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Review

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Assessment of circulating tumor cells in peripheral blood using flow cytometry in patients with surgery for colorectal cancer – review

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Abstract

***Introduction**: Colorectal cancer (CRC) is the third most common neoplasia in the world. Circulating tumor cells (CTC) have a prognostic value and can be useful in monitoring solid neoplasia. Only one method for CTC identification has received the approval and this is the CellSearch® system based on the immunomagnetic separation. Multiple markers are used in CTC identification, as epithelial markers and cytokeratines. CTC identification in peripheral blood is associated with a worse prognostic and reduced free survival in CRC. **Material and methods**: We performed a systematic search in PubMed database for articles that reports the circulating tumor cells in CRC until July 2019. We selected studies in English and French and the main words used for search were 'circulating tumor cells', 'colorectal cancer', 'colon cancer', 'rectal cancer', 'flow cytometry', 'peripheral blood'. We included studies with more than 10 patients, where samples were collected from the blood in relation with surgery and flow cytometry was used as analyzing technique. **Results**: We included 7 studies in final analysis, that showed in flow cytometry analysis a cut-off value of CTC that can vary from 2-4 CTC/ 7.5 ml peripheral blood with a sensitivity of 50.8% and specificity of 95%. Patients with positive CTC were associated with higher T stage and positive lymph nodes, with a worse overall survival (OS) and disease free survival (DFS) comparing with negative patients.* ***Conclusion:** CTC are considered to be a prognostic factor who needs more validation studies in order to be included in the clinical practice.*

***Keywords:** colorectal cancer, flow cytometry, circulating tumor cells*

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Introduction

Colorectal cancer (CRC) is the third most common neoplasia in the world (1) and the risk of developing a colorectal carcinoma during one's lifetime is > 5% in general population due to lack of complete curative results of current therapies (2). Prognostic in colorectal cancer is associated with circulating tumor cells (CTC) that are proved to be a marker for metastasis and penetration available for solid tumor (3). Liquid biopsy refers to the use of free circulating tumor DNA and cells, and is consider useful in monitoring multiple types of neoplasia (4,5). Detection of free circulating cancer cells in the blood of patients with CRC can be used as a prognostic tool and a mode for colorectal cancer staging (6), the presence of CTC being associated with a poor prognosis (7). Identification of CTC in blood of patients with CRC is significantly associated with aggressive disease free progression ($p<0.001$) and the persistence of CTC reduces disease survival (8), thus being considered an independent prognostic factor (9). Related to surgical resection, the presence of CTC in the peripheral blood for at least 24 hours postoperatively, is considered to be an independent prognostic marker of recurrence especially in stage III CRC (10,11). No-touch surgical resection technique based on primary ligation of the lympho-vascular pedicle applied in CRC is used in order to reduce blood spreading of CTC (12) while the laparoscopic approach is consider not to have any influence on CTC number (13). Identification of CTC as a prognostic marker has proven useful for other multiple neoplasia such as breast, prostate, gastric and lung cancer (14–16). Multiple techniques such as reverse transcription polymerase chain reaction (RT-PCR) and immunocytochemistry (17) are used for CTC identification with a variable specificity and sensitivity. The first standardized system approved by The Food and Drug Administration (FDA) in the USA for CTC detection is the CellSearch® system based on a semiautomatic immunomagnetic method for identification. In a meta-analysis CellSearch® system proved to have a prognostic utility with a significant value of CTC detected in metastatic CRC patients compared with non-metastatic ones ($p<0.01$) (18). There are two-types of markers that are used in order to identify CTC in CRC: epithelial specific markers such as epithelial cells adhesion molecule (EpCAM) and cytokeratines 18, 19, 20 and specific markers such as carcinoembryonic antigen (CEA) (1). Flow-cytometry is another technique used to identify CTC (19) and a protocol for detection and enumeration of circulating endothelial and progenitor cells in human blood was published (20) and it presented a technique that offers the advantage of morphologically examining targeted cells. CTC are a prognostic marker for CRC patients treated with chemotherapy and can be considered to be a predictive marker for the response to oncologic treatment (21). In rectal cancer, CTC can predict response to neoadjuvant radio-chemotherapy, with a significant reduction of CTC number after treatment in responders compared to non-responders (22). Due to the diversity of methods in identifying CTC, in order for it to become a powerful prognostic tool, a standardization in the detecting and reporting of the number of CTC is necessary and it should come about through large-scale multicenter validation studies (23).

Although the traditional staging in CRC has a prognostic value in most of the cases, there are many patients in whom tumor behavior does not overlap with classical staging methods, this data suggesting that other prognostic factors, such as tumor microenvironment, intestinal microbiota, immune infiltrate and CTC, should be evaluated. The purpose of our study is to evaluate the feasibility of detection of CTC in patients with surgery for CRC by using the flow cytometry

method and to show the necessity of a standardized protocol for clinical implementation.

Material and methods

Search strategy

We performed a systematic search for relevant studies in PUBMED database with no time restriction up until July 2019. We were searching for articles that report the circulating tumor cells in CRC. We only selected studies written in English and French and the main words used for the search were 'circulating tumor cells', 'colorectal cancer', 'colon cancer', 'rectal cancer', 'flow cytometry', 'peripheral blood' linked with Boolean operators AND. Two independent researchers (AMM and IH) performed the systematic search. We used the recommendations from the preferred reporting items for systematic reviews and metaanalyses (PRISMA) guidelines (24) that helped us in study selection in order to evaluate the quality of items from the included articles.

Inclusion criteria

In order to be included in our review, all the eligible studies had to fulfill the following criteria: 1) studies with more than 10 patients included in the series; 2) the samples had to be collected from the peripheral blood of patients; 3) flow cytometry had to be the used as the analyzing technique; 4) samples had to be collected in relation to surgical stages.

Exclusion criteria

Studies were excluded from the review if: 1) the samples were used from other sources than peripheral blood (peritoneal cavity, bone marrow, lymph nodes, solid tumor); 2) the analyzing technique was different than flow cytometry; 3) the studies analyzed less than 10 patients; 4) the sample collection had no association to the surgery undergone by patients; 5) there were multiples types of cancer included in the same article. All data represented by abstract meetings were excluded due to the lack of sufficient information they were presenting.

Quality assessment of the articles

The quality of the studies was assessed by evaluating the methodological quality of each article, based on the Cochrane Handbook for Systemic Review of intervention and an assessment list. The two researchers before mentioned independently assessed the evaluation of each article. All studies were evaluated in order to analyze: the number of cases, consecutive series and prospective studies, flow cytometry technique and antibodies description, blood sampling and collection time, the collecting of samples' relation to surgery, tumor stage and complete results reported. Each study received a score of 1 to 9 (Table 1, Figure 1). The performance of each study was measured based on the detection rate, the sensibility and accuracy of the method used for CTC identification and the estimated survival rates with a 95% confidence interval. We decided to exclude the case series and studies with less than 10 patients included because we considered this limit necessary in achieving the learning curve in the implementation of the flow cytometry protocol. Since the number of selected studies is limited, we decided to consider the 10 patients limit the cut-off.

Data extraction

All data were extracted only from the original studies published, all the review and metaanalysis being excluded. We reviewed all the included studies and extracted the interest information on a preformed database. We extracted the first author and year of publication, country, patient number, sampling time in relation to surgery, situs collection of the blood and blood volume, cancer type and staging according to Dukes or Union for International Cancer Control (UICC),

Table 1. Quality assessment of the studies evaluated by the authors

Author	Patients > 10	Consecutive Series	Prospective Studies	Blood samples	Collection time	Flow-cytometry technique details	Markers used reported	Surgery related	Complete results
Fang et al. (24)	+	+	+	+	+	+	+	+	+
Galizzia et al. (25)	+	+	+	+	+	+	+	+	+
Tralhao et al. (26)	+	+	+	+	+	+	+	+	-
Cohen et al. (27)	+	+	+	+	+	+	+	+	+
Tseng et al. (28)	+	+	+	+	+	+	+	+	+
Tsavellas et al. (29)	+	+	-	+	+	+	+	+	+
Bahnassy et al. (30)	+	+	+	+	+	+	+	+	+

- absence of the criteria, + presence of the criteria

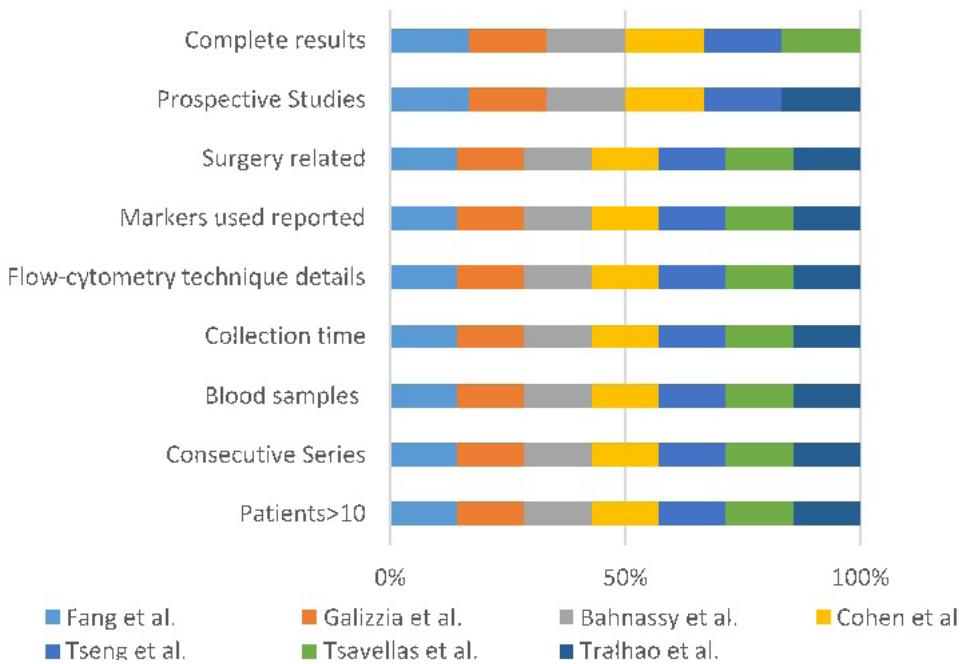

Fig.1. Quality of the studies performed by the authors

type of surgery, tumor localization, patient age, identified markers, detection rate, number of CTC and cut-off for CTC, follow up median, diagnostic specificity and outcomes.

Results

In PubMed database, we recorded a number of 1833 items. Based on the title and abstracts we excluded 1328 articles. We took 505 articles into evaluation out of which we selected 32 articles. Out of those we excluded another 25 articles either because the sampling was not in relation to the surgical moment or the analysis was performed for a precursor of circulating tumor cells. In the end we evaluated seven articles to be included in this study.

Study characteristics (Table 2)

A total number of 562 patients were evaluated in our included articles (25–31) and associated 62 case control patients (25–27). Publication time was from 2006 to July 31-st 2019 with no more than one study published each year. Most of the articles evaluated a small series of patients (25–27,29–31) and only one article evaluated more than 100 patients (28). In two of the articles a group control was selected in order to compare the analysis (25,27). Median age of the patients included was 64 with a range between 21-91 years of age. In only two of the studies (25,29) was the histopathologic type mentioned (adenocarcinoma=115, mucinous=14). The samples were collected from peripheral blood and in only one study is the situs puncture referred to as the antecubital vein (30). Four of the studies used only one blood sample that was collected in relation to the moment of surgery (25,28,30,31): before surgery (25,30), during surgery (31) and after surgery (28). In three studies the samples were collected thusly: two blood samples, before surgery and one month after surgical resection (29), three blood samples, at surgical incision, after tumor resection and at the end of the surgical intervention (26) and three samples during surgery at every one minute interval (27). The median sample blood size was 12.5 ml with a range between 7.5- 84 ml (25–30) and in one study the size is not mentioned (31). Tumor localization was mentioned in five out of the seven studies (25,26,28,29,31) and was represented by rectum (n=168, 29.89%) and colon (n=139, 24.73%), out of the latter left colon (n=49, 35.25%) and right colon (n=42, 30.21%). In two of the studies the tumor site was not specified so the patients were considered with colorectal localization (n=193, 34.34%) associated with liver metastasis (n=16, 2.84%) (27,30). Surgical treatment was applied for all cases included in our studies but in only two of the articles (26,28) are the surgical procedures described (Table 2). Tumor stage was reported in all cases using TNM or Dukes classification and was as follows: stage I (n=25), stage II (n=62), stage III (n=158), stage IV (n=124) (Table 3).

Markers used in CTC identification using flow cytometry (Table 3)

All studies (25–31) used CD45 as a marker in flow cytometry analysis. Two of the studies (25,27) used CD45, EpCAM and cytokeratines (CK) markers in order to identify CTC, and positive CTC were considered to be cells CK+ and CD45-. One study (31) used epidermal growth factor marker (EGFR) and nucleic acid stain in association with CK and CD45, positive CTC being considered CD45-/CK+/ nucleic acid dye+/EGFR+. Tseng et al. (30) defined the positive CTC as ESA+ (epithelium specific antigen)/ CD45-. One study (28) used multiple markers in analysis and considered as positive CTC the CD133+, CD54+, CD44+, CD45- cells. Galizia et al. (29) associates to the EpCAM and CD45 analysis, CD326, and considered CTC positive as EpCAM/CD326+ and CD45-.

Table 2. Characteristics of the studies evaluating CTC in colorectal cancer

Author	Year	Country	Patient number	Median age	Histological type	Blood sampling	Situs collection	Blood volume	Collection time
Fang et al. (24)	2017	China	152	61.59 ± 12.08	no specification	peripheral blood	No specification	No specification	T1- after surgery
Galizia et al. (25)	2013	Italy	66	66	ADK	peripheral blood	No specification	7.5 mlx 2	T1- before surgery T2- 1 month after surgery
Tralhao et al. (26)	2010	Portugal	40 6 control	64±10 (41-90)	no specification	peripheral blood	peripheral blood	12 mlx 3	T0- surgical incision; T1- after tumor resection; T2- end of the surgical intervention;
Cohen et al. (27)	2006	USA	49	61 (24-80)	no specification	peripheral blood	No specification	7.5 ml	T1- surgery moment
Tseng et al. (28)	2014	Taiwan	172	67 (30-91)	no specification	peripheral blood	antecubital vein mesenteric vein	10 ml	T1- before surgery
Tsavellas et al. (29)	2015	China	20 CCR 16 control	69	no specification	peripheral blood	No specification	3X 28 ml at every one minute interval	T1- after anesthesia before surgery
Bahnassy et al. (30)	2019	Egypt	63 CRC 40 control	44.8 ± 12.7 (21-72)	ADK= 49 Mucinous= 14	peripheral blood	No specification	7.5 ml	T1-before surgery

ADK- adenocarcinoma

Table 3. Markers used and detection procedure used in CTC identification and CTC value in relation with surgery moment in the included studies

Author	Identified markers	CTC positive	Flow cytometry antibodies used/ Source	Type of flow cytometer	Number CTC identified Nr. Patients	Cu-off CTC	p value
Fang et al.	CD133-APC, CD44-FITC, CD54-PerCP-Cy5.5, CD54-PE, CD45-BV 510,	CD133+, CD54+, CD44+, CD45-	CD133-APC, CD44-FITC, CD54-PerCP-Cy5.5, CD54-PE, CD45-BV 510 (BD Biosciences, San Diego, CA, USA).	FACS	21.20% (CD45), 27.10% (CD54-PE) 26.70% (CD44) 0.91% (CD133-APC)	no specification	no specification
Galizia et al.	CD326/ EpCAM PerCP-Cy5.5 CD45-APCCy7 CD326/Ep-CAM magnetic beads	EpCAM/ CD326+, CD45-	CD326/EpCAM-PerCP-Cy5.5 CD45-APCCy7 (Becton Dickinson, San Jose, CA, USA) CD326/EpCAM magnetic beads (Miltenyi Biotech, Auburn, CA, USA)	BD FacsARIA III cell sorter (Becton Dickinson).	0-130 CTC **Preoperative:** 0 CTC-14 pts; 1 CTC- 3 pts; 2 CTC- 5 pts; >3 CTC- 54 pts **Postoperative:** mean CTC= 1.92±3.06, 95 % CI 1.22–2.62, (range 0 to 12) median CTC= 0	≥3 CTC/ 7.5 ml	**Preoperative:** CTC+ (n=54) CTC- (n=22) p=0.05 **Postoperative** CTC- (n=53) CTC+ (n=16) median CTC= 0 (p<0.001 vs. preoperative)

Tralhao et al.	CD45 -PerCP Cy5.5 CK	CD45-/CK+	CD45-PerCP-Cy5.5 (clone 30- F11, BDB, San Jose, CA, USA) Anti-CK with FITC (clone MNF116, DakoCytomation, Glostrup, Denmark).	Flow cytometer (BDB, San Jose, CA, USA); CellQuest software (BDB, San Jose, CA, USA). Paint-a-Gate Pro software (BDB, San Jose, CA, USA).	**CRC cancer or liver metastasis (34 pts):** 7±7 (0-26) before incision; 4±4 (0-13) after specimen removal; 4±6 (0-26) end of the surgery; **Colorectal cancer (18 pts)**: 6±8 (0-26) before incision; 2±2 (0-6) after specimen removal; 3±4 (0-10) end of the surgery; **Liver metastases (16 pts):** 9±4 (0-14) before incision; 7±4 (0-13) after specimen removal; 6±9 (0-26) end of the surgery;	no specification	p<0.501 p<0.141 p<0.088
Cohen et al.	CK nucleic acid stain EGFR CD45	CD45-/CK+, nucleic acid dye+, EGFR+	CK nucleic acid stain anti-EGFR CD45	no specification	Median 2 CTC/ 7.5 ml > 1CTC/ 7.5 ml= 77% pts; 1-5 CTC/ 7.5 ml= 54%; >5 CTC/7.5 ml= 25% pts	2 CTC/ 7.5 ml	no specification
Tseng et al.	CD45- PE-Cy7 CD326- FITC ESA	ESA+, CD45-	FITC-conjugated anti-CD31, PE-conjugated anti-CD4, APC-conjugated anti-CD8a PE-conjugated anti-CD49f (Dx5) (BD Biosciences) PE-conjugated anti-IL-17RA (eBiosciences) 7-AAD exclusion (BD Biosciences).	FACS, Kaluza software (Beckman Coulter).	0-0.815%	no specification	p=0.0218
Tsavellas et al.	EpCAM CD45 CK	CK+/CD45-	IgG monoclonal antibody specific EpCAM. IgG anti-CK with FITC (Dako, UK). IgG anti human CD45 with PE (Sigma, UK)	FACS CaliburTM flow cytometer (Becton Dickinson, Oxford, UK); CellquestTM (Becton Dickinson, Oxford, UK)	2 CTC (0.75-4) (0-4.5) control group	median 2 CTC	p=0.07
Bahnassy et al.	EpCAM CD45 CK	CD45-/CK+	EpCAM enrichment anti CK-FITC anti-CD45-TRITC (Thermo Scientific)	no specification	98 CTC (2-198 CTC/7.5 ml) in non-metastatic CRC;	4 CTC	no specification

ESA- Epithelium-specific antigen, EpCAM- epithelial cells adhesion molecule, CTC- circulating tumor cells, EGFR- epidermal growth factor marker , CK-cytokeratines, PE-Cy7- phycoerythrin-cyanine7, FITC- fluorescein isothiocyanate (FITC), PerCP Cy5.5- peridinin chlorophyll protein cyanine 5.5, APC- Allophycocyanin, PE- phycoerythrineconjugated

Diagnostic procedure for CTC identification and CTC value (Table 3)

All studies (25–31) used flow-cytometry as the identification technique, with different types of flow cytometers (26–30). In one study Kaluza software was used for analysis (30), in another two studies, CellQuest (26,27) and Paint-a-Gate Pro software (26) while in another four studies (25,27–29,31) the analysis software was not specified. The number of CTC identified are reported in all studies and this value varies in relation to the surgical resection (26,29) (Table 3). The cut-off value for CTC is reported in four studies, with a value of 2 CTC/7.5 ml blood (27,31), ≥ 3 CTC/7.5 ml blood (29) and 4 CTC (25). In three studies (26,28,30) the cut-off value is not specified. Bahnassy et al. (25) reports a sensitivity of 50.8% of flow cytometry in detecting CTC (≥ 4 CTC/7.5 ml blood) with a 96.3% specificity and 76.2% accuracy associated with a positive detection rate (CTC CK+/CD45-) of 50.8%. One study (31) reports a sensitivity of 50% for the same method and another three studies (27–29) report a 95% specificity.

Correlation of CTC and TNM stage (Table 4)

Only three studies (25,29,30) showed a correlation of CTC and TNM stage.

Galizia et al. (29) report that CTC positive (≥3 CTC) patients were associated with advanced T stage (p=0.001), M stage (p=0.005) and Dukes stage (p=0.001). Patients in early Dukes stages associated with normal postoperative CTC levels have a significant lower risk of tumor relapse compared to advanced Dukes stages and high postoperative CTC levels patients (p<0.001).

Bahnassy et al. (25) show that patients with positive CTC are associated with a higher T stage (30 CTC positive patients out of 53 patients, p=0.034), stage III TNM (21 CTC positive patients out of 30 patients, p=0.004) and positive lymph nodes stage (20 CTC positive patients out of 28 patients, p=0.005).

Tseng et al. report that a higher number of CTC in mesenteric blood was identified in stage II CRC compared to stage I (p=0.52), III (p=0.023) and IV (p=0.035) (30).

Results of each studies and impact of CTC in patient's survival

Fang et al. (28) report that patients who received both primary tumor and liver metastasis surgical resection had a better survival than those who received only primary tumor resection (3-year OS, 70.8%, 43.8%, and 4.5%, respectively; p<0.001). Patients with a high expression of CD133+CD54+ (p<0.001), CD133−CD54+ (p=0.004) and CD133+CD44+CD54+ (p=0.003) CTC had a worse overall survival compared to patients with low expression. In multivariate analysis, CD133+CD44+CD54+ subpopulations of CTC (HR=6.459; 95% CI=1.461– 28.558; p=0.014) were considered an independent prognostic factor for patients with CRC. High value of CD133+CD44+CD54+ subpopulation was associated with a worse survival in patients without surgical resection of liver metastasis than in patients with low expression (3 year OS, 9.1% vs. 57.1%; p<0.001) (28).

When analyzing the number of CTC in patients with colorectal cancer associated with liver metastasis, CRC or liver metastasis group alone, the number of CTC varies in relation to the surgical moment (before incision, specimen removal or end of surgery) but without statistical signification (p=0.501, p=0.141, respectively p=0.088). These results give rise to the question of the role of the ''no touch'' technique in patients with colorectal cancer and liver metastasis (26).

At a CTC cut-off value ≥ 3 Galizia et al. (29) report differences between the preoperative CTC positive and negative patients (54 versus 22 patients) with a higher progression free survival in patients with zero to two CTC than in those with ≥ 3 CTC identified (p=0.06). Preoperative ≥ 3 CTC were significantly associated with T

Table 4. Staging of CRC and type of surgery reported in included studies

Author	Year	Country	Patient number	Cancer type	Tumor localization Nr. Patients	Stage	Type of Surgery
Fang et al. (24)	2017	China	152	colorectal	rectum=106 left hemicolon = 17 right hemicolon = 29	I= 15 II=31 III=28 IV=78	Surgical treatment for primary tumor= 32; Surgical treatment for primary and metastatic tumor= 24 (liver metastasis resection, radiofrequency ablation, transcatheter arterial chemoembolization)
Galizia et al. (25)	2013	Italy	66	colorectal	right colon= 13 left colon= 33 rectum= 30	III=69 IV= 13	No specification
Tralhao et al. (26)	2010	Portugal	40 (6 control)	colorectal	rectal= 9 colon= 8 colorectal= 1 only liver metastasis= 16 group control=6	III=18 IV=16	R0 resection; right hepatectomy= 4; extended left hepatectomy= 1, left hepatectomy= 1; central hepatectomy= 1, left lobectomy= 4; bisegmentectomy= 3; segmentectomy= 2; subsegmentectomy= 4; total colproctectomy with ileostomy= 2, total colectomy= 2, subtotal colectomy=1, right hemicolectomy= 1, left hemicolectomy=3; anterior resection= 6; anterior resection with ileostomy= 1; abdominal perineal resection= 2
Cohen et al. (27)	2006	USA	49	colorectal	colon= 35 rectum= 14	I= 2 II=6 III=14 IV= 27	No specification
Tseng et al. (28)	2014	Taiwan	172	colorectal	no specification	I 31 II 63 III 48 IV 30	No specification
Tsavellas et al. (29)	2015	China	20 CCR (16 control)	colorectal	no specification	I-III-17 IV-3	No specification
Bahnassy et al. (30)	2019	Egypt	63 CRC (40 control)	colorectal	Colon=40 Rectum=23	IA=2 IB=6 IIA=16 IIB=9 IIIA=3 IIIB=13 IIIC=14	No specification

stage (p<0.001), M stage (p<0.005), Dukes stage (p<0.001) and CEA serum level (p<0.02) with lower progression free survival in univariate analysis in CTC positive patients with potentially curative surgery (p=0.05, 47 positive patients vs. 22 negative patients). Postoperative median CTC is 0 with no cell identified, with significant differences (p<0.001) in progression free survival (PFS) compared to preoperative levels when analyzing the patients (53 negative vs 16 positive patients). The progression free survival was 89.6% at one year, 76.1% at two years and 68.3% at tree years, with a CTC accuracy of 88.4% of predicting tumor recurrence. High postoperative CTC levels were correlated with tumor relapse and in multivariate analysis it was the only independent factor correlated with increased tumor recurrence rate (p<0.001) (29).

Tsavellas et al. (27) report a median of 2 CTC positive (IQR (interquartile range)=0.75-4) in patients with colorectal cancer, but without significant differences compared to no cancer patients (p=0.07, IQR=0-4.5) (27).

Cohen et al. (31) report that during treatment no significant differences in CTC number was observed in progression compared to no progression patients after the flow cytometry analysis (+0.6 cells vs. -1.9 cells, p=0.48) and time to progression was not predicted by the CTC number (6.6 months for < 2 CTC, 6 months ≥ 2 CTC, p=0.89) (31).

Patients with ≥ 4 CTC detected by flow cytometry had a statistically reduced 5 year DFS (27.5% vs 100%, p=0.001) and overall survival (17.2% vs 91.7%, p= 0.001) compared to patients CTC negative (≤ 4 cells) (25). In univariate analysis, the CTC number detected by flow cytometry was an independent prognostic factor for both PFS (p<0.001) and OS (p=0.01) but only for PFS in multivariate analysis (p<0.001)(25).

The number of CTC identified in mesenteric vein was shown to be higher than the number identified in forearm vein (p=0.0218) with a positive correlation of those two (p=0.0017). Patients with high number of CTC in forearm vein were significantly associated with a high number of CTC in mesenteric vein (p=0.001). Patients with a high number of CTC in mesenteric vein had a significant reduced PFS compared to patients with low CTC number (p=0.0016) (30).

Discussion

Circulating tumor cells are cancer stem cells that can derive from both primary and metastatic tumor and their presence is associated with progression and metastasis. The CTC presents a immunophenotype signature by expression of CD45-/EpCAM+ and an epithelial origin (32). The CTC identification in patients with CRC is possible using different methods: physic analysis that use centrifugation and filtration methods or phenotypic analysis that evaluate the surface markers expression and specific antibodies (by flow cytometry). Morphologic identification uses cytological analysis or markers' identification through immunohistochemistry or immunofluorescence. Molecular biology techniques like in situ hybridization (FISH) perform the ARN analysis and, in association with RT-PCR, can identify specific mutations (33). Immunohistochemistry using monoclonal antibodies in CTC identification has low sensitivity and requires prior tumor cells' enrichment (34).

Flow cytometry presents some advantages when compared to other CTC detection methods. Flow cytometry allows for a higher CTC yield when compared to fluorescent microscopy and ensures an accurate quantification and an imunophenotypic assessment of each cell in the sample.

CTC characterization through flow cytometry associated with gene identification is a noninvasive predictor factor for clinical response to treatment and could guide new therapies and drug development (31). Flow cytometry also allows single-cell analysis and allows inclusion

and exclusion of different cell populations when their origin is uncertain at any time after sample acquisition (29). The disadvantages of using flow cytometry are represented by the higher costs, the necessity for high technical expertise, the time consuming protocol for sample acquisition and the evaluation of a quantitative expression of receptors that are not well documented in the literature (26). The development of high-speed flow cytometry ensures the acquisition of 20.000 events per second with a reduced time for the study protocol and thus the increasing interest in using this technique for rare events identification such as CTC in CRC (26). Still, the lack of a standardized protocol validated in multicenter prognostic studies for CTC identification in CRC cancer is a limitation on the use of this technique.

Besides CRC, the analysis of CTC proves to be a prognostic marker in breast cancer where patients with > 5 CTC/7.5 ml blood have a worse progression free and overall survival ($p<0.0001$) than patients with < 5 CTC (4). Identification of cytokeratin 19 using flow cytometry ensures a CTC detection rate of 86% in stage IV breast cancer (35). The presence of CTC is considered the first step to metastasis and for patients with curable surgery and > 5 CTC, the risk of presenting distant metastasis in the first year is 8 times higher (36). In stage III and IV CRC, the mean number of CTC identified using flow cytometry was 5 ± 1 cells/ml for CD133+/CD45- and respectively 29 ± 3 cells/ml for CK20+/CD45- that consider CTC identification as a useful biomarker for personalized metastatic risk evaluation (37). Almost 95% of the colorectal cancers and metastasis with this origin are CK20 positive, which makes cytokeratin a suitable marker in CTC with CRC origin identification (38). In order to predict survival in colorectal cancer, new robust prognostic biomarkers are necessary and clinical trials with specific protocols for CTC identification are initiated (39). When a cut-off ≤ 30/ml CTC is established, the OS and PFS are 37.1 and respectively 13.3 months while at > 30 CTC/ml median OS is 14.9 months and PFS is 5.1 months, with significant differences ($p<0.001$) in metastatic colorectal cancer (40). For patients undergoing liver resection for metastatic colorectal cancer, CTC identification in central venous blood is associated with patient's prognosis compared to CTC identification in peripheral blood (41). CTC identification in mesenteric blood predicts clinical survival compared to CTC identification in central blood (42). Preoperative search of CTC in non-metastatic CRC is associated with a low number of CTC identified (43). CTC identification can predict progression and distant metastasis even for patients with nonmetastatic colorectal cancer, patients with ≥5 CTC having an 8 times higher risk of developing liver metastasis during the first year post- surgery (44). Identification of > 2 CTC using immunostaning fluorescence in situ hybridization at three months after surgery in CRC is associated with worse PFS than in patients with < 2 CTC (45). When detected, perioperative CTC are an independent prognostic factor (46) and when they are associated with lymph node status they allow for patients' classification into high and low-group with impact in treatment management (47).

When comparing open surgery to laparoscopic approach for CRC, the CellSearch® system shows significant differences between these two techniques when both peripheral and portal venous blood was analyzed ($p=0.002$), with a lower number of CTC being identified during the minimally invasive approach probably because of the medial to lateral approach resection (48). A number of > 2CTC can appear in 30% of patients with CRC and in 57% of patients with prostate cancer (49) with a detection rate that varies from 4% to 57% as reported in a systematic review (17).

Ongoing trial is evaluating the role of liver resection in colorectal cancer liver metastasis in order to research the role of surgical approach in haematogenous cancer cells dissemination and prognostic value (50). In a metaanalysis it is shown that the CTC positive patients have a significantly increased liver metastatic rate of up to 21% (22 of 203 patients) compared to 8% (16 of 207 patients) in negative patients (OR=6.38, CI 2.67-15.25) (6) and CTC detection is associated with disease progression and poor survival (51). In **conclusion**, CTC identification is useful and it was proven to have a role in predicting clinical evolution in patients with CRC, a high number of CTC preoperatively or postoperatively being associated with a worse prognostic in terms of overall survival and disease free survival. Studies show that CTC can be considered as an important prognostic marker but in order for it to be included in clinical practice new trials for clinical validation are necessary. Flow cytometry is a feasible method used for CTC identification but needs standardized protocol and trained technician in order to be widely used.

Abbreviations

CRC- colorectal cancer
CTC- circulating tumor cells
OS- overall survival
DFS- disease free survival
RT-PCR- reverse transcription polymerase chain reaction
EpCAM- epithelial cells adhesion molecule

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Conflict of interest

The authors declare no conflict of interest.

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