Chapter 4
Circulating Tumor Cells in Colorectal Cancer

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4.1 Introduction

Colorectal cancer (CCR) is the second most common cancer diagnosed in women and third most in men, accounting for approximately 10% of all annually diagnosed cancers and cancer-related deaths worldwide [8]. These rates also vary geographically, with the highest rates seen in the most developed countries. It is a prevalent disease in older patients, but the incidence is rising in younger ones, especially rectal cancer and left-sided colon cancer [19].

CCR is largely an asymptomatic disease until it reaches an advanced stage; in these cases, symptoms such as rectal bleeding, change in bowel habits, anemia, or abdominal pain should alert patients to look for a doctor. In asymptomatic patients, screening methods are important. Colonoscopy, occult blood in feces, and sigmoidoscopy are the most common used methods, but each one has its own limitations [12]. Thus, new and less invasive methods need to be investigated.

For metastatic CCR, systemic therapy typically includes chemotherapy backbone paired with a biological treatment. Fluoropyrimidines combined with oxaliplatin (FOLFOX) and irinotecan (FOLFIRI) chemotherapies are the most commonly used regimens [12]. In terms of response rate and survival, the addiction of a biologic (anti-VEGF or anti-EGFR) antibody in the chemotherapy regimen, depending on the tumor-specific factor, must be considered.

Figures separated by Ludmilla T.D. Chinen and revised by Mauro Saieg (Cytopathologist from AC Camargo Cancer Center, São Paulo, SP – Brasil)

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It is known that genetic intratumor heterogeneity contributes to treatment failure and drug resistance [14]. Several studies comparing mutational profiles of primary tumors and associated metastatic lesions [13, 36] and local recurrences [29] have provided evidence of intratumor heterogeneity.

Early during the formation and growth of a primary tumor, cells are shed from the primary tumor and then circulate through the bloodstream. These circulating tumor cells (CTCs) can be enriched and detected by different technologies, which take advantage of their physical and biological properties. CTC analysis is considered a real-time "liquid biopsy" for patients with cancer [3].

Compared with conventional biopsy, the "liquid biopsy" has some advantages: requires only a small amount of blood [23], is minimally invasive [24], allows early detection of cancer [17] and-real time monitoring for treatment responses and resistance, by repeated analysis [6]. Some disadvantages are the lack of standardization techniques [9] and insufficient clinical and technical validation [4].

In CCR, CTCs can be used for screening (early detection of invasive cancers), in localized cancer (risk stratification), prognosis and monitoring after treatment, and metastatic cancer (selection of therapy, monitoring of response, and resistance mechanisms).

4.2 CTCs for Colorectal Cancer Screening

Although the prognostic value of CTCs in the early stages of CCR has already been evaluated in several clinical studies, its role in screening and early detection remains controversial, but it is a very promising topic [22, 30].

The main study on CTCs with the screening approach was recently presented at ASCO 2018 with 620 participants (182 healthy controls, 111 participants with precancerous lesions, and 327 patients with stage I-IV CRC). The results were compared to a standard clinical protocol, including colonoscopy and biopsy results, revealing an overall accuracy of 88% for all stages of the disease, including precancerous lesions. It is the first study to show high sensitivity in the detection of precancerous colorectal lesions [33].

The simple collection of blood for liquid biopsy can be easily integrated into the routine physical examination of the patient, increasing adherence to the test and, thus, allowing an increase in early diagnosis without the need for invasive tests; however, we still need more studies to support this tracking strategy in colorectal cancer.

4.3 CTCs for Evaluation of Minimal Residual Disease in metastatic CCR

Treatment for patients with localized CRC consists of surgery, and in some cases, stages II and III, adjuvant treatment with chemotherapy in addition to surgery is indicated. Identifying patients at high risk of recurrence and treating them with adjuvant

therapy remains an important clinical issue. In current practice, we used tumor markers such as carcinoembryonic antigen and clinical-pathological factors to define the risk of recurrence and prognosis, with limitations in identifying minimal residual disease (MRD). Therefore, the monitoring of CTCs during post-surgical follow-up evaluations may allow the patient to better stratify in relation to the risk of recurrence.

In a study with 141 patients (stages II and III), the presence of CTCs after curative surgery was associated with worse progression-free survival and overall survival. In this study, recurrence occurred in 72.5% of patients with positive CTCs after surgery, on the other hand, recurrence occurred in only 12.2% of patients with negative CTCs [20].

A research with 138 patients showed that postoperative patients with positive CTC and negative CTC before surgery is an independent indicator of poor prognosis for CRC patients treated with curative resection [38].

A study with 130 patients with stage II-III CRC demonstrated that the postoperative CTC counts were earlier than the preoperative CTCs in predicting tumor recurrence survival in patients with non-metastatic CRC undergoing surgery. In addition, the authors developed CTC-based prognostic models to predict tumor recurrence in stage II-III CRC, which can be used to identify patients at high risk for recurrence and guide aggressive treatment to improve the clinical outcomes of these patients [35]. Please see some pictures of CTCs isolated from localized colon cancer by ISET in Figs. 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 4.10, 4.11, 4.12, 4.13, 4.14, 4.15, 4.16, 4.17, 4.18, 4.19, and 4.20.

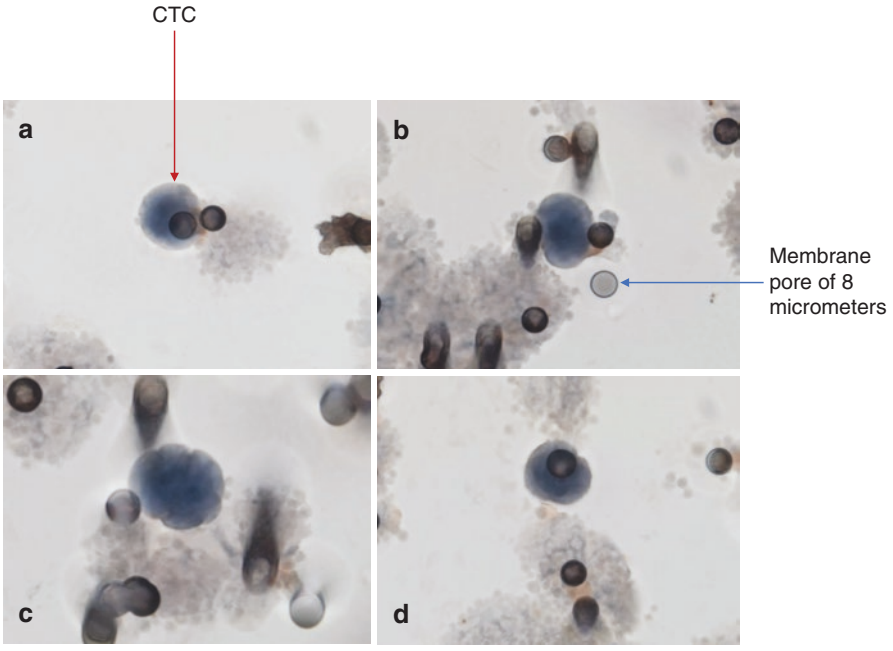

Fig. 4.1 Patient with 58 years old, male, with stage IIIC (1st collection, at diagnosis). CTC count was 4.60 CTCs/mL. The CTC count was 0.33 CTCs/mL after surgery and 4.33 CTCs/mL after adjuvancy. On letter C, we can better visualize nuclear irregularity and lobular nuclei. In boxes (**a–d**) we can oberve CTCs with different shapes

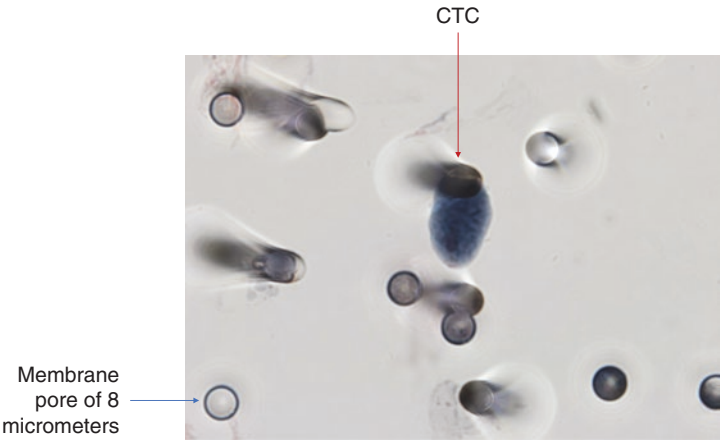

Fig. 4.2 Patient with 61 years old, male, stage IIC. Here, we can observe irregular nuclei. The CTC count was 3.80 CTCs/mL in baseline (blood collection at diagnosis)

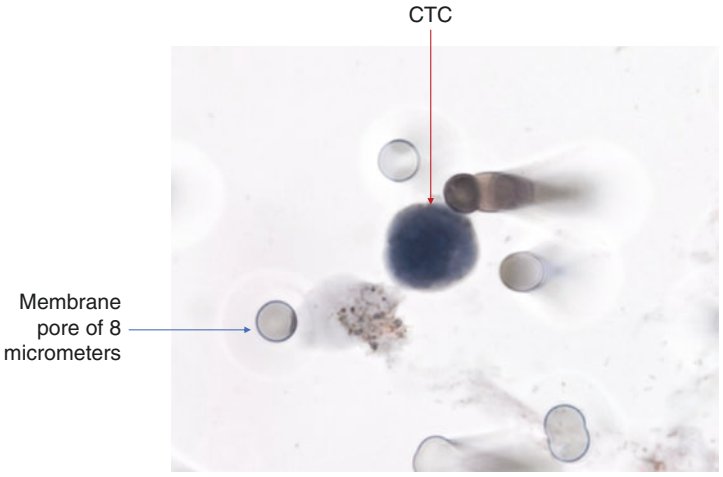

Fig. 4.3 Patient with 51 years old, male, stage IIA. The CTC count was 6.0 CTCs/mL in the 1st collection (cell with irregular nuclei and abundant cytoplasm). After surgery (second blood collection), it was 5.33 CTCs/mL

Finally, a study with 438 patients, with the objective to evaluate the presence of CTCs in the pre- and postoperative scenario in patients with colorectal cancer in stages I-III undergoing curative resection and, thus, identifying a subgroup of patients at high risk of relapse, suggested that the persistent presence of CTCs in the postoperative period can be a crucial prognostic factor, in addition to conventional tumor markers in patients with CRC undergoing curative resection. The identification of these high-risk patients with persistent positive CTCs is important and, therefore, can help to define patients for adjuvant therapy with this tumor entity [34].

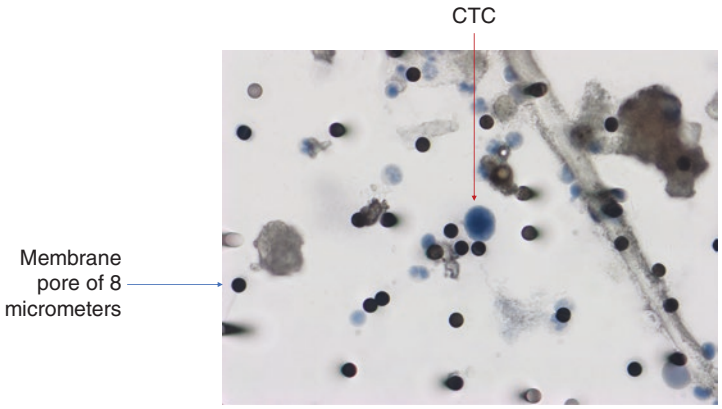

Fig. 4.4 CTCs from the same patient of Fig. 4.3. Isolated CTC of 3rd collection (after adjuvancy). The CTC count was 5.66 CTCs/mL

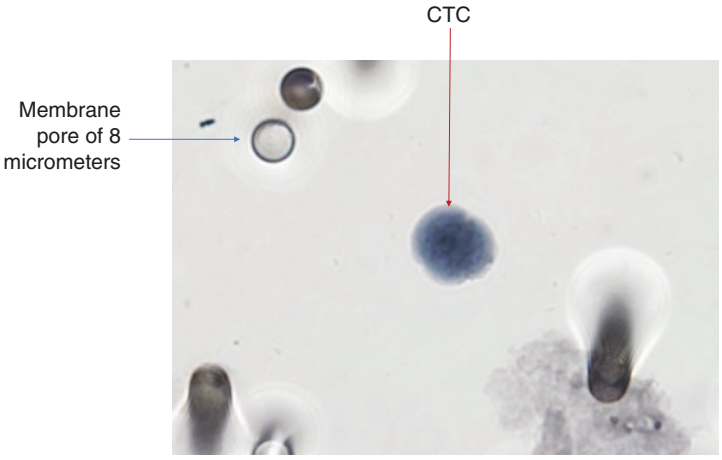

Fig. 4.5 Patient with 37 years old, woman, stage IIIB. The CTC count was 4.80 CTCs/mL in the 1st collection

4.4 CTCs for Prognostic Evaluation in Metastatic Disease

The role of CTCs in the prognostic stratification of patients with metastatic CRC has been demonstrated in several studies emphasizing that the presence of CTCs can predict future metastasis (disease progression) and unfavorable outcome as demonstrated in Table 4.1.

In a previous publication of our group, with 54 mCRC patients, we demonstrated that in addition to the initial CTC count, kinetics was also important for prognostic definition [27]. Evaluating CTC kinetics, when we compared the baseline (pretreatment) CTC level (CTC1) with the level at first follow-up (CTC2), we observed that

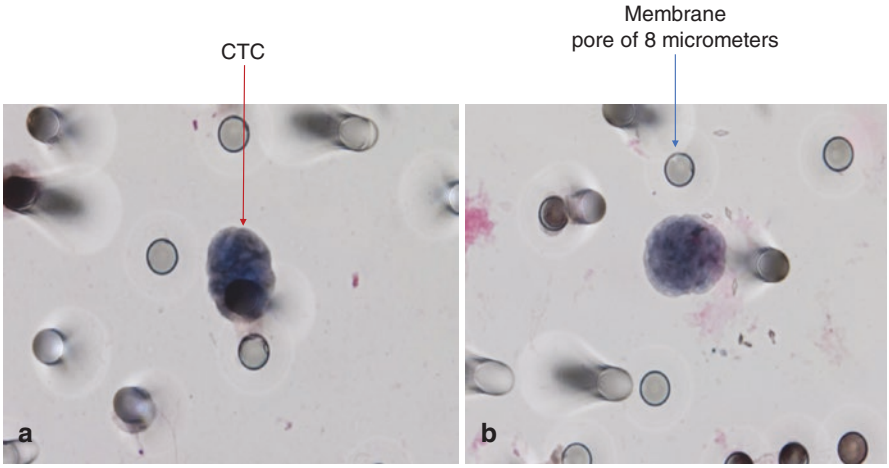

Fig. 4.6 Patient with 70 years old, male, stage I disease. The CTC count was 1.0 CTCs/mL in the 1st collection. This figure is of 2nd collection (on letter (**a**): cytoplasm staining with ERCC1). In letters (**a**, **b**), we can observe chromatin irregularity. The CTC count was 4.67 CTCs/mL

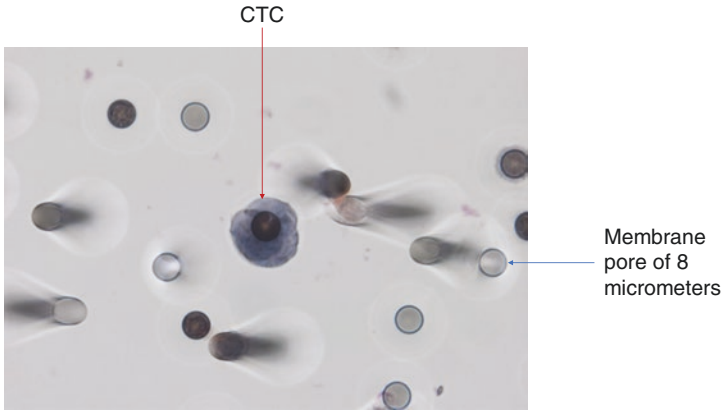

Fig. 4.7 Patient with 85 years old, woman, with stage IIA. The CTC count was 2.25 CTCs/mL at baseline. This figure is of 2nd collection (after surgery), the count was 1.33 CTCs/ml

CTC1-positive patients (CTCs above the median), who became negative (CTCs below the median) had a favorable evolution ($n = 14$), with a median progression-free survival (PFS) of 14.7 months. This was higher than that for patients with an unfavorable evolution ($CTC1^-$ that became $CTC2^+$; $n = 13$, 6.9 months; $p = 0.06$). Patients with WT KRAS with favorable kinetics had higher PFS (14.7 months) in comparison to those with WT KRAS with unfavorable kinetics (9.4 months; $p = 0.02$). Moreover, patients whose imaging studies showed radiological progression had an increased quantification of CTCs at CTC2 compared to those without progression ($p = 0.04$). This study made possible the presentation of ISET as a

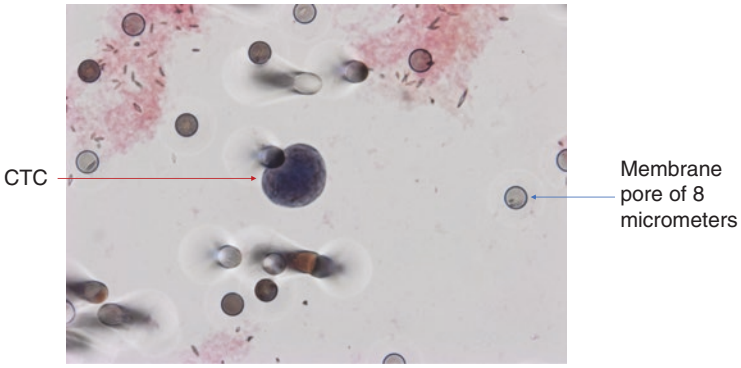

Fig. 4.8 Patient with 56 years old, woman, with stage I. The CTC count was 6 CTCs/mL at baseline. This figure is of 2nd collection (after surgery), the count was 5 CTCs/ml (cytoplasm staining for ERCC1). We can observe a classical CTC and an ISET pore

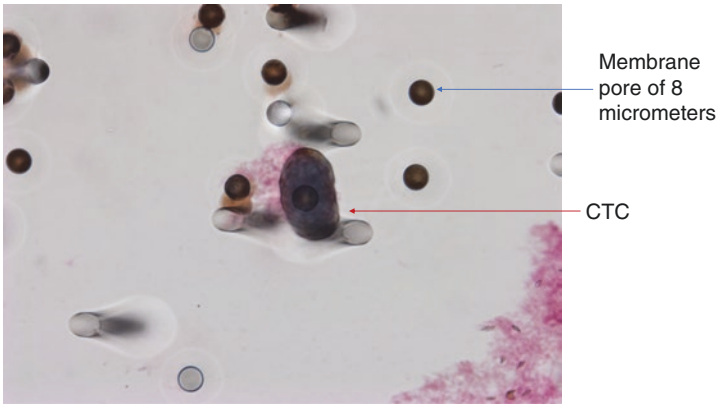

Fig. 4.9 Patient with 59 years old, woman, with stage IIIC. The CTC count was 2.50 CTCs/mL at baseline (cytoplasm staining with TIMP1)

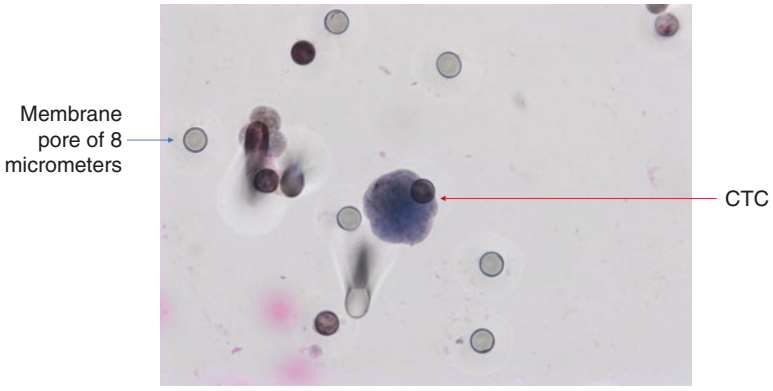

Fig. 4.10 CTCs from the same patient of Fig. 4.9. This picture is of the 2nd collection. The count was 3 CTCs/ml (cytoplasm staining for ERCC1)

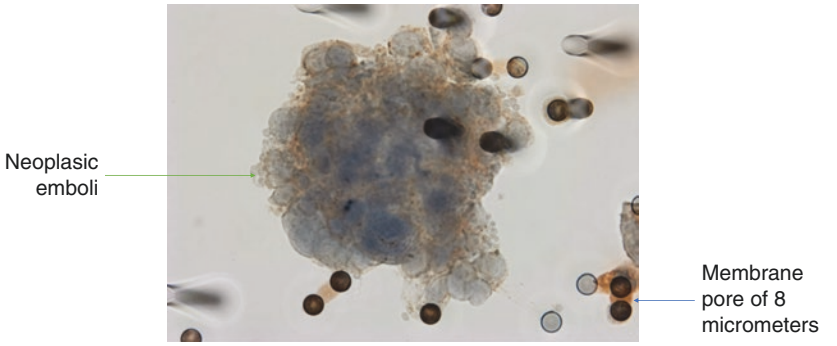

Fig. 4.11 Patient with 71 years old, woman, with stage IIIB. The CTC count was 7 CTCs/mL at baseline (cytoplasm staining with TYMS). Here, we can observe a neoplastic emboli with three-dimensional arrangement of epithelial cells

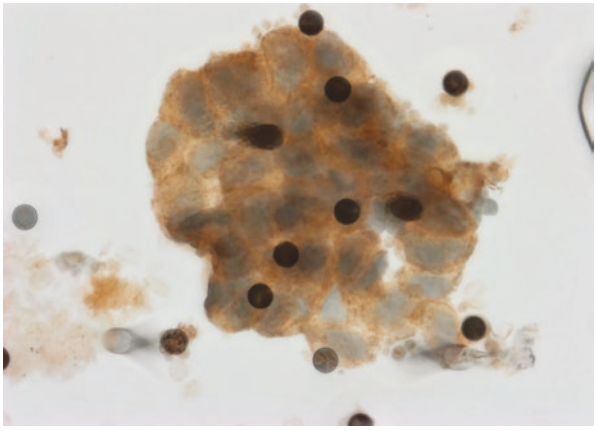

Fig. 4.12 Patient with 69 years old, man, with stage IIA. The CTC count was 3.6 CTCs/mL at baseline (microemboli staining for TYMS)

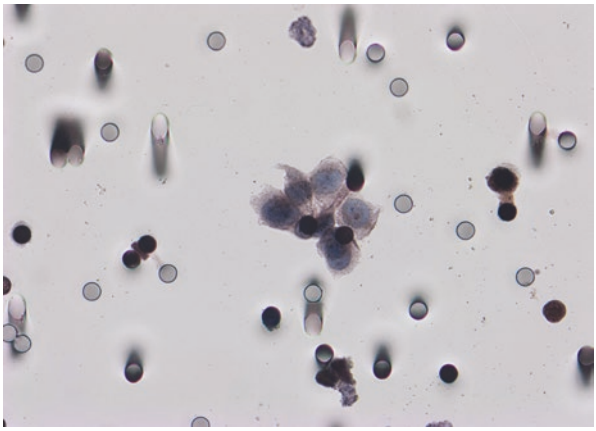

Fig. 4.13 Patient with 63 years old, man, with stage IIIB. The CTC count was 7.0 CTCs/mL at baseline (microemboli staining for β-GAL)

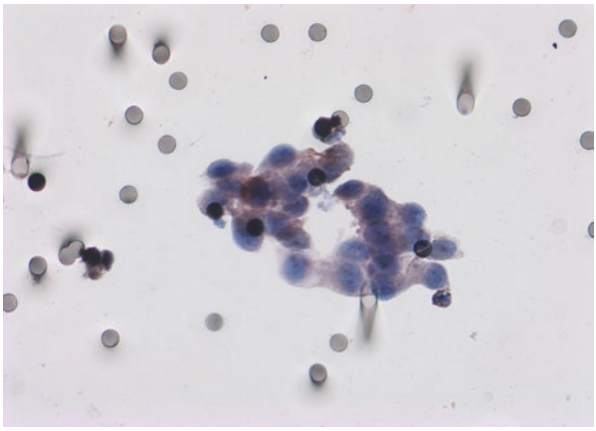

Fig. 4.14 Same patient of picture Fig. 4.13. Here, we can observe a proliferation of epithelial cells with three-dimensional arrangements and columnar-looking cells. Staining for TGF-βRI

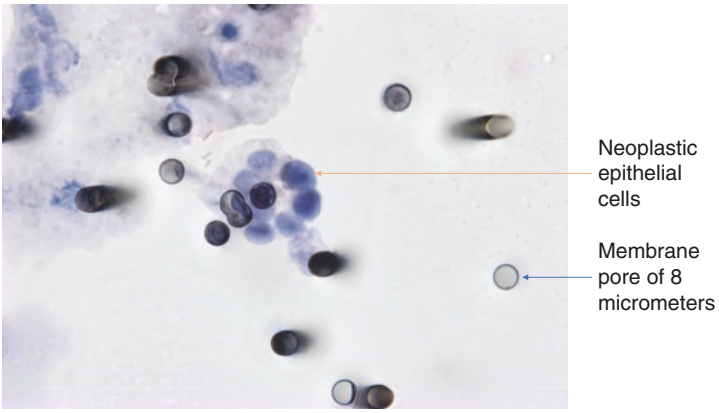

Fig. 4.15 Patient with 71 years old, woman, with stage IIIC. The CTC count was 7.0 CTCs/mL at baseline. We can see neoplastic epithelial cells sketching acinar arrangement

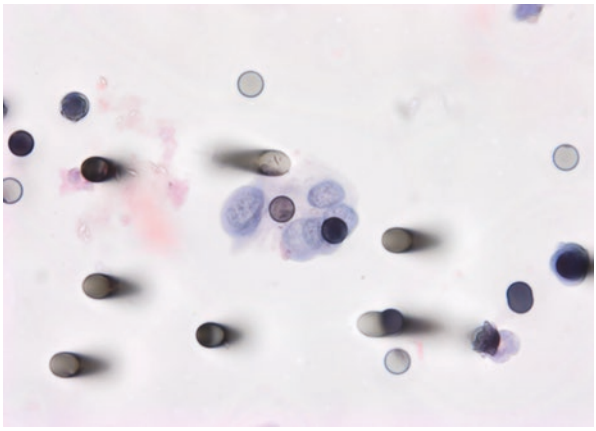

Fig. 4.16 Patient with 57 years old, man, with stage IIIB. The CTC count was 2.5 CTCs/mL at baseline (at diagnosis)

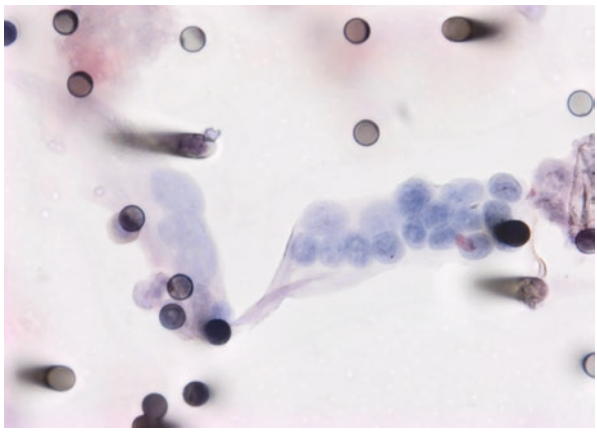

Fig. 4.17 Same patient of Fig. 4.16

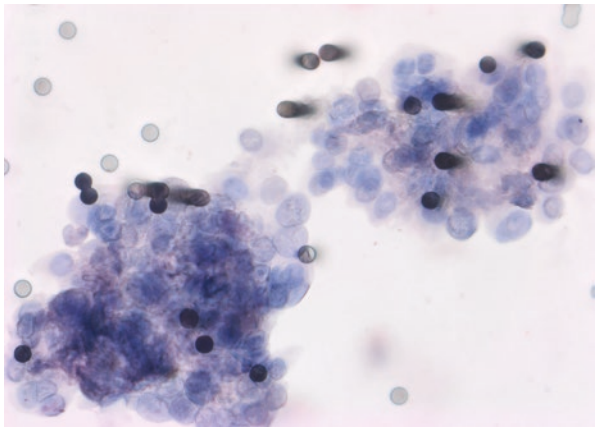

Fig. 4.18 Same patient of Fig. 4.16. Three-dimensional cluster of neoplastic epithelial cells

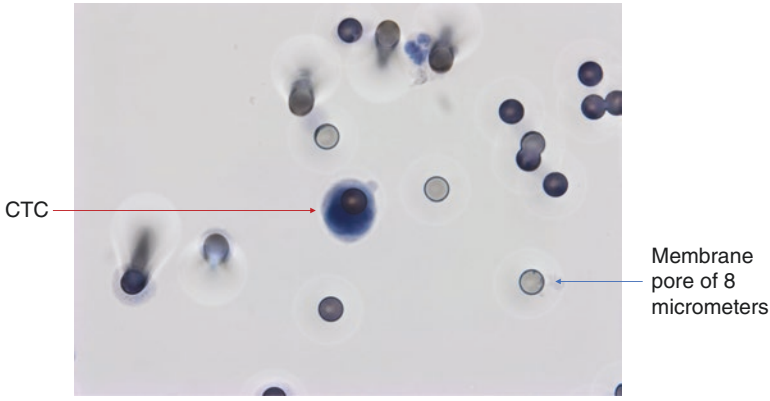

Fig. 4.19 Patient with 69 years old, male, with stage IIA. The CTC count was 7 CTCs/mL at baseline. This figure is of 3rd collection, made after adjuvancy (3.33 CTCs/mL)

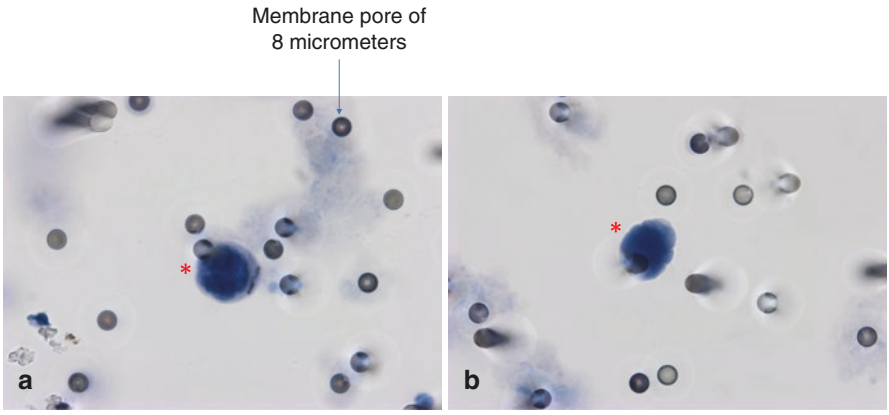

Fig. 4.20 Patient with 59 years old, woman, with stage IIIC. The CTC count was 2.80 CTCs/mL at diagnosis. This figure is of 3rd collection (after adjuvancy) and the count was 5.33 CTCs/mL. The asterisk represents CTCs stained with hematoxylin

feasible tool for evaluating CTC kinetics in patients with mCRC, which can be promising in their clinical evaluation.

These data are reinforced by the meta-analysis with 13 studies that showed that the rate of disease control was significantly higher in patients with CRC with low CTC compared to high CTC (RR = 1354, 95% CI [1002–1830], $p = 0.048$). CRC patients in the CTC-high group were significantly associated with poor progression-free survival (PFS; HR = 2500, 95% CI [1746–3580], $p < 0.001$) and poor overall survival (OS; HR = 2856, 95% CI [1959-4164], $p < 0.001$). Patients who converted from low CTC to high CTC or who were persistently high CTC had a worse disease progression (OR = 27.088, 95% CI [4960–147,919], $p < 0,001$), PFS (HR = 2095, 95% CI [1105–3969], $p = 0.023$) and OS (HR = 3604, 95% CI [2096–6197], $p < 0,001$) than patients who converted from high CTC to low CTC. Thus, it concludes that CTCs can be used as a new marker capable of predicting the response to chemotherapy in patients with metastatic CRC [15].

Another more recent meta-analysis with 15 published studies containing 3129 patients reinforces that the presence of CTCs was significantly associated with poor mortality (overall survival: HR = 2.36, 95% CI: 1.87–2.97; $P = 0.006$) along with aggressive disease progression (progression-free survival: HR = 1.83, 95% CI: 1.42–2.36; $P < 0.00001$) (Yi Tan et al. 2017).

Another study by our group in the metastatic setting evaluated the expression of TYMS in CTCs, in 34 samples and was TYMS considered positive in 9 (26.5%). Six of these patients had tumor progression after treatment with 5-FU. An association was found between CTC TYMS staining and disease progression (PD), although without statistical significance ($p = 0.07$). Patients who had a CTC count above the median (2 CTCs / mL) had higher TYMS expression ($p = 0.02$) correlating with a worse prognosis. These results suggest that TYMS analysis may be a useful tool as a biomarker predictor of 5-FU resistance if analyzed in CTCs of

Table 4.1 Studies showing that the presence of CTCs can predict future metastasis (disease progression) and unfavorable outcome

Author, year	Number of patients	Population	CTC's evaluation	Treatment	Main results
Sastre et al. (2012) [16]	1202	mCCR	CellSearch System	Chemotherapy + Mab	bCTC presented in 41% of patients; association with worse ECOG, stage IV, >3 metastatic sites and CEA levels
Bidard et al. (2019) [7]	131	mCCR	CellSearch System	Chemotherapy + surgery (metastasectomy)	bCTC was associated with OS; no association of CTC and metastatic hepatic resection
Tan et al. (2018) [18]	9	mCCR	Size-exclusion method	Chemotherapy +/− Mab	CTC kinetics during chemotherapy was associated with disease progression and trends in CEA levels
Yang et al. (2017) [37]	2363 (metanalysis)	Non-metastatic CCR	RT-PCR	Adjuvant chemotherapy for III and part of II	CTC positive was associated with shorter OS (HR = 3.07, $P < 0.001$) and disease-free survival (HR = 2.58, $P < 0.001$)
Chen et al. (2017) [10]	90 (and 151 healthy donors)	CCR and healthy donors	RT-PCR in marker genes in RNA extracted of CTCs	–	The expression of ECT2 in the CTC could serve as an alternative measurement in the diagnosis and monitoring of colorectal cancer patients

Table 4.1 (continued)

Author, year	Number of patients	Population	CTC's evaluation	Treatment	Main results
Souza e Silva et al. (2016) [26]	54	mCCR	Isolation by size of epithelial tumor (ISET) cells	Chemotherapy +/− Mab	ISET was proved a feasible tool for evaluating CTC kinetics, that, together with CTC levels were associated with prognosis
Abdallah et al. (2015) [1]	54	mCCR	Isolation by size of epithelial tumor (ISET) cells	Chemotherapy +/− Mab surgery +/− metastasectomy	Thymidylate synthase (TYMS) expression in CTC was a predictor biomarker of 5-FU resistance
Barbazan et al. (2014) [5]	50	mCCR	Multimarker CTC detection panel	Chemotherapy +/− Mab	A multimarker model based on expression levels of a six-gene panel of tissue-specific and EMT-related markers in CTC was associated with of OS and PFS
Sastre et al. (2012) [25]	108	mCCR	CellSearch System	Chemotherapy + bevacizumab	CTC count is a strong prognostic factor for PFS and OS
De Albuquerque et al. (2012) [11]	60	mCCR	Immunomagnetic enrichment with BM7 and VU1D9 Ab	Chemotherapy +/− Mab	CTC positivity was prognostic factor and associated with radiographic disease progression

(continued)

Table 4.1 (continued)

Author, year	Number of patients	Population	CTC's evaluation	Treatment	Main results
Matsusaka et al. (2011) [21]	64	mCCR	CellSearch System	Chemotherapy +/− bevacizumab	CTC number before and during treatment was associated with PFS and OS in oriental population
Tol et al. (2010) [31]	477	mCCR	CellSearch System	Chemotherapy +/− Mab	CTC count before and during treatment was associated with PFS and OS and provides additional information to CT imaging
Cohen et al. (2008) [28]	430	mCCR	CellSearch System	Chemotherapy +/− Mab	CTC number before and during treatment was associated with PFS and OS in occidental population

Abbreviations: *bCTC* baseline CTCs, *Mab* monoclonal antibody, *ECT2* epithelial cell transforming sequence 2, *BM7* antibody which target mucin 1, *EMT* epithelial-mesenchymal transition, *mCCR* metastatic colorectal cancer, *OS* overall survival, *PFS* progression-free survival, *VU1D9* antibody which target EpCAM

patients with mCRC [1]. In addition, in another study developed by our group, we analyzed the immunocytochemical expression of MRP1 and ERCC1 in patients with metastatic CRC who had previously detectable CTCs. Among patients treated with irinotecan-based chemotherapy, 4 out of 19 cases with MRP1-positive CTCs showed a worse progression-free survival (PFS) compared to those with negative MRP1 CTCs (2.1 months vs. 9.1 months; $p = 0.003$). These results show MRP1 as a potential biomarker of resistance to treatment with irinotecan when found in CTCs of patients with mCRC [2].

4.5 CTCs as a Predictive Factor in the Treatment of Locally Advanced Rectal Cancer

Neoadjuvant chemoradiation (NCRT) followed by total mesorectal excision (TME) is the standard treatment for locally advanced rectal cancer (LARC). Our group developed a study aiming to explore the role of CTCs in patients undergoing NCRT followed by surgery for treatment of LARC. In addition, we evaluated the predictive values of TYMS and RAD23B expression in CTC before and after NCRT. The initial analysis of 30 patients was published and demonstrated that the complete pathological response (*p*CR; $p = 0.02$) or the partial response ($p = 0.01$) could correlate with CTC counts. Regarding protein expression, TYMS was absent in 100% of CTCs from patients with pCR ($p = 0.001$) yet was expressed in 83% of non-responders at S2 ($p < 0.001$). Meanwhile, RAD23B was expressed in CTCs from 75% of non-responders at S1 ($p = 0.01$) and in 100% of non-responders at S2 ($p = 0.001$); 100% of non-responders expressed TYMS mRNA at both timepoints ($p = 0.001$). In addition, TYMS/RAD23B was not detected in the CTCs of patients exhibiting pCR ($p = 0.001$). Thus, TYMS mRNA and/or TYMS/RAD23B expression in CTCs, as well as CTC kinetics, have the potential to predict non-response to NCRT and avoid unnecessary radical surgery for LARC patients with pCR [32].

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