REVIEW

Circulating tumor cells: basic concepts and some clinical applications

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Abstract

The spread of cancer requires the presence of circulating tumor cells (CTCs), which are rare cells surrounded by billions of normal hematopoietic cells in the bloodstream. It is believed that CTCs tend to metastasize to certain organs, thus, their presence may determine invasive tumor behavior. Generally, these cells are undetectable by conventional histopathological analysis and imaging exams with high resolution. Therefore, more sensitive immunohistochemical and molecular assays have been developed that have allowed the specific detection of metastatic tumor cells in regional lymph nodes, peripheral blood and bone marrow. This article reviews the literature regarding CTCs and tumors of the breast, colorectal, pancreas and lung as it pertains to forms of detection and clinicopathological correlations, in addition to future outlooks.

Keywords: circulating; immunohistochemistry; neoplastic cells; review literature as topic.

INTRODUCTION

Concept and Overview of Circulating Tumor Cells

Initial tumors grow as non-vascularized masses that can proliferate through pre-existing vasculature in a microenvironment. After reaching a size of approximately 2-3 mm, tumors require their own vasculature[1]. Accordingly, the induction of angiogenesis and tumor vasculature are regarded as pathognomonic factors of the malignant process and are necessary for tumor progression[2].

Cancer cells can spread, invading neighboring blood vessels or using capillaries formed within the tumor. In both instances there is induction of epithelial-to-mesenchymal transition (EMT), which constitutes a change in the expression of adhesion molecules (e.g., integrins, laminins) and in the activation of proteases (e.g., matrix metalloproteinases), which eventually allows tumor cells to enter in circulation[3].

The spread of cancer requires the presence of circulating tumor cells (CTCs), which are rare cells surrounded by billions of hematopoietic cells in the bloodstream[4]. The presence of CTCs in peripheral blood was first reported by Thomas Ashworth[5], an Austrian physician, when he performed the autopsy of a patient with metastatic subcutaneous tumors located in the anterior wall of the chest and abdomen. He noted circulating cells (obtained from the saphenous vein of the right leg) identical to those from tumors and postulated that these cells were derived from an existing tumor structure that must have traversed much of the circulatory system to reach the inside of the saphenous vein of the right leg.

Human blood is composed of white blood cells (5-10 x 10^6/ml), red blood cells (5-9 x 10^9/ml) and platelets (2.5-4 x 10^8/ml). Depending on the type of primary tumor, few CTCs can be found in patients with known metastatic disease: about less than one CTC per ml of blood[6].

It is believed that what are called CTCs tend to metastasize to certain organs that are selective to certain tumors, conforming to the historical concept of "seed and soil", proposed by Stephen Paget reviewed by Lurje et al.[7]. Currently, metastatic animal models have suggested that about 1 x 10^6 tumor cells/g of tumor enter the bloodstream daily. However, CTCs have low levels of survival in circulation and 85% of them disappear in 5 minutes. Those animal models have shown that 2.5% of CTCs cause micrometastases and that only 0.01% of them proliferate and form macroscopic metastases[8]. CTCs that survive will probably develop survival mechanisms in the highly oxygenated environment that is the peripheral blood (seen leaving the tumor, a site of hypoxia) and escape from immune response. This escape is performed through the ability to form groups of microtumores, or microemboli, which affect distant sites. It has been shown that an aggregate of 5-10 occult CTCs escaping the immune system promotes the recruitment of proangiogenic factors from the local microenvironment and expression of new surface markers[9].

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The spontaneous circulation of tumor cells and/or microemboli determines the invasive tumor behavior[10]. Generally, they are undetectable by conventional histopathological analysis and imaging exams with high resolution. Therefore, more sensitive immunohistochemical and molecular assays have been developed that have allowed the specific detection of metastatic tumor cells in regional lymph nodes, peripheral blood and bone marrow[11].

The primary detection of CTCs may help identify patients in need of further systemic therapy after surgical resection of the primary tumor. Despite all the therapies being developed to prevent metastatic relapse, patient selection is based on the statistical risk of developing recurrence, without actually knowing if the patient has any CTCs. This leads to the overtreatment of these patients with toxic agents with serious side effects[12]. Thus, including the detection or counting of CTCs in the analysis of patients would make treatment more targeted and perhaps even less intolerable because it would be with those who really needed the treatment, avoiding overexposure.

CLINICAL APPLICATION

CTCs and breast cancer

Slade et al.[13] conducted a study to compare a detection method of CTCs (CellSearch™ system) with the monitoring system of bone marrow micrometastases (ICC - immunocytochemistry and RT-PCR) in follow-up patients after the diagnosis of breast cancer without evidence of metastases. The results showed that some patients with an initial diagnosis of breast cancer had evidence of micrometastases during follow-up, despite the absence of the clinical evidence of metastases. Seven out of 18 (39%) patients classified as T1N0 had CTCs when compared to those that were node positive (23 of 33, corresponding to 70%, $p = 0.042$). Despite the limited number of cases, these authors found that the detection of CTCs and disseminated tumor cells (DTCs) in bone marrow was greater in patients with higher risk of recurrence ($p = 0.042$ and < 0.001, respectively) and that the number of CTCs were higher in patients with worse prognosis. In this study, both DTCs and CTCs led to the same conclusions, thus being equivalent in terms of clinical significance.

Nakagawa et al.[14] demonstrated a direct correlation between the presence of CTCs in blood and lymph node status by RT-PCR in real time. CTCs were found in 36/90 (43%) patients in clinical stages I-III of breast cancer studied. These cells were not found in the healthy volunteers. By *in situ* hybridization, Fehm et al.[15] showed that the vast majority of CTCs in the peripheral blood of patients with breast cancer derived from the primary tumor.

Progress has occurred with the development of systems of enrichment and immunohistochemical detection of CTCs. The best known of these is the CellSearch™ system. Using this system, Cristofanilli et al.[16], in a study with 177 patients with metastatic breast cancer, counted CTCs before and after the start of treatment for metastatic disease. Patients with levels of CTCs equal or superior to 5 per 7.5 ml of blood, when compared with those who had less than 5 CTCs per 7.5 ml, had lower progression-free survival (PFS) (2.7 months *vs.* 7 months $p < 0.001$) and lower overall survival (OS) (10.1 months *vs.* 18 months, $p < 0.001$). After the first follow-up following the start of treatment, this difference between the groups persisted (with respect to survival and the number of CTCs). On multivariate analysis, the levels of CTCs before and after the start of treatment proved to be significant predictors of OS and PFS. Yet, approximately 70% of patients were observed to have metastatic disease above 1 CTC per7.5 ml of peripheral blood.

Using the same CTC detection system, an Italian group[17] conducted a study with 80 patients with metastatic breast cancer who were evaluated before the start of treatment, 4 to 8 weeks after the first clinical evaluation and every two months thereafter. Before the start of treatment, 49 patients had ≥ 5 CTCs. In multivariate analysis, the levels of CTCs before treatment were significantly associated with PFS (relative risk (RR) of 2.5 times, CI 95%). Patients with persistent levels of ≥ 5 CTCs had a greater risk of progression than those with < 5 CTCs (RR 6.4, CI 95%). Those studies indicate the probable use of CTCs as a measure of metastatic tumor response.

De Giorgi et al.[18] evaluated the relationship between detection and CTC prognostic significance with sites of metastases detected by 2-fluorine-18-fluoro-2-deoxy-D glucose positron emission tomography/computed tomography (18F-FDG PET/CT) in patients with metastatic breast cancer. In all, 195 patients participated and greater numbers of CTCs were observed in patients with bone metastases (detected by PET/CT) compared to those patients without metastases (mean of 65.7 *vs.* 3.3, $p = 0.012$), as well as for patients with multiple metastases in relation to one or two bone lesions (mean 77.7 *vs.* 2.6, $p < 0.001$). CTCs were predictors of OS in 108 patients with multiple metastases, including bone ($p \leq 0.0001$), but not in 58 without bone metastasis ($p = 0.411$) and in 29 with bone involvement only ($p = 0.3552$). In multivariate analysis, CTCs, but not bone metastases, remained significant predictors of OS.

The SUCCESS study evaluated the levels of CTCs at the time of primary diagnosis and during adjuvant therapy in 1767 patients with lymph node-positive breast cancer. The CTC values were obtained in 852 follow-up patients. Before the start of adjuvant therapy, 10% of patients had more than one CTC. The presence of CTC was not correlated with tumor size, histological grade, hormonal status or HER-2 in the primary tumor. However, CTCs correlated significantly with lymph node metastasis ($p = 0.02$). None of the 24 healthy controls showed more than one CTC. Among the 852 patients evaluated, 11% were positive for

CTCs before adjuvant therapy and 7% had more than one CTC after completing treatment. Among those initially positive for CTCs, 10% remained positive and 90% became negative after the end of chemotherapy. The presence of CTCs before therapy showed no prognostic significance for PFS (p = 0.89) or OS (p = 0.71). However, the persistence of CTCs after chemotherapy was a significant predictor for the reduction of both (p = 0.04 and p = 0.03, respectively). These results indicate a potential role of CTCs in monitoring treatment of early breast cancer[19].

A German group Muller et al.[20], in a study of neoadjuvant therapy for breast cancer, conducted tests for the determination of CTCs before and after neoadjuvant chemotherapy. The level of CTC positivity before treatment was around 22%, dropping to 14% post-treatment. Another German group, in a study with 14 centers (GeparQuattro Neoadjuvant Trial) also trying to correlate the levels of CTCs before and after neoadjuvant chemotherapy (NT) in peripheral blood of patients with breast cancer, detected ≥ 1 CTC per 7.5 ml in 46 (21.6%) of 213 patients before NT and in 22 (10.6%) of 207 patients after NT (p = 0.002). Twenty patients (15%) initially positive for CTCs became negative after NT, while 11 cases (8.3%) were positive, although none had CTCs detected before NT. This group also noted that the detection of CTC did not correlate with the characteristics of the primary tumor. Contrary to expectations, there was no correlation between tumor response to NT and CTC detection[21]. Additionally, this study showed that NT with trastuzumab in HER-2-positive patients had a limited effect on the number of CTCs in HER-2 overexpression. However, Reuben et al.[22] demonstrated that trastuzumab was able to reduce the level of CTCs expressing HER-2 even in patients whose tumors showed normal levels of this protein. These results suggest not only a therapeutic potential for trastuzumab, but that CTCs may genotypically and phenotypically differ from the original tumors as well.

Gradilone et al.[23] searched for CTCs in the blood of 42 patients with metastatic breast cancer and established its presence with ALDH1, predictive and prognostic values and proteins related to drug resistance. The authors emphasized that PFS was lower in patients whose CTCs expressed two or more proteins related with drug resistance. No relationship between tumor characteristics and ALDH1 was found in that study. The correlation between the number of proteins related to drug resistance and ALDH1 was statistically significant.

Georgoulias et al.[24] divided 75 patients with HER2-negative breast cancer and CK19 mRNA-positive CTCs before and after NT in two groups: 36 patients were treated with trastuzumab and 39 patients were only observed. The CK19 mRNA-positive CTCs were detected by RT-PCR and double stained CK-positive/HER-2-positive cells by immunofluorescence. The authors noted that 89% of the patients analyzed had HER-2 expressing CTCs. After treatment with trastuzumab, 75% of patients became CK19 mRNA negative, whereas this profile was only found in 17.9% of the observation-only patients. These results suggest that trastuzumab may eliminate the resistance to therapy of CTCs expressing CK19 mRNA positive, reducing the risk of disease recurrence and prolonging disease-free survival.

The monitoring of CTCs was used in a study of Pachmann et al.[25] using therapy with trastuzumab as a control in HER 2-positive breast cancer patients. Seventy-nine patients were selected with this phenotype, 35 were treated without trastuzumab and 36 treated with this drug for one year. The CTCs were separated by FITC-anti-EpCAM and analyzed during chemotherapy and between 2 and 10 times during one year of maintenance treatment and observation. The patients treated with trastuzumab had better recurrence-free survival. The increase in the number of CTCs was also accompanied by an increased number of cells containing a high number of HER2/*neu* gene copies. These results suggest that an analysis of the behavior of CTCs may contribute to the development of the effectiveness in therapies used, as well as sparing patients unnecessary treatments and reduce costs.

Aktas et al.[26] analyzed the blood of 193 patients with metastatic breast cancer at diagnosis of metastasis. Immunomagnetic enrichment was performed using the AdnaTest BreastCancerSelect™ (AdnaGen, Germany) followed by RNA isolation and gene expression analysis by Multiplex-PCR in tumor cells previously separated. The CTCs were analyzed by three breast cancer-associated markers: EpCAM, Muc-1 and Her-2. Estrogen receptor expression and progesterone was observed by RT-PCR. The authors observed that 45% (87/193) of patients presented CTCs. Through analysis, 71% expressing EpCAM (62/87) 73% Muc-1 (64/87) 48% Her-2 (42/87) 19% estrogen receptor (17/87) and 10% progesterone receptor (9/87) were found. It was also possible to observe that in 77% (48/62) of patients, the tumor expressed estrogen receptor but not CTCs. The same occurred with 43% of patients when analyzing the progesterone receptor. Tumors and CTCs expressed estrogen receptors and progesterone concurrently in 41% (p = 0.260) and 45% (p = 0.274) of patients, respectively.

Banys et al.[27] investigated the influence of the removal of the primary tumor in the incidence and phenotype of CTCs in patients with primary breast cancer. The study included 209 patients with this pathology. Peripheral blood was collected pre-and postoperatively and analyzed by the AdnaTest™ method. There were three tumor markers (GA733-2, Muc-1 and Her-2) and two hormone receptors (ER and PR) observed. Bone marrow was also collected at surgery. The authors noted that 21% (43/209) of patients had CTCs at pre- and/or postoperative.

Positivity after surgery was higher, but not significantly different (p = 0.264). DTCs were found in 15% of the cases (32/209). Patients with DTCs in the bone marrow had significantly more CTCs in peripheral blood, either pre-or postoperatively, compared to those patients without DTCs. The most common phenotype of CTCs (24 patients) was triple negative, followed by HER2+/ER-/PR- (10 patients) and ER and/or PR positive (9 patients). It was also seen that 95% (41/43) of primary tumors were ER and PR positive.

Seeking to develop a new profile of an independent prognostic variable through CTC status, Molloy et al.[28] developed a study that analyzed the gene expression of a group of 72 patients with breast cancer, whose CTC status had been determined in a previous study. The profile generated was validated in two different databases of 49 and 123 patients, and confirmed to be predictive of CTCs and an independent prognostic factor. Thus, this study developed a signature that can predict the status of CTCs in patients with breast cancer through gene expression of the primary tumor.

Aboagye et al.[29] compared the changes in the level of CTCs with changes in tumor proliferation using metabolic imaging by [18F] 3-deoxy-3'-fluorothymidine positron emission tomography (FLT-PET) in women with advanced breast cancer before and during therapy with docetaxel. In those patients that CTCs had been detected, a decrease could be correlated with a decrease in FLT-PET signal within two weeks. The authors concluded that, when combined, these two technologies may provide an excellent tool for assessing therapeutic response.

CTCs and colorectal cancer

With respect to colorectal cancer (CRC), it is known that the first strategy for the treatment of this disease is complete resection of the lesion, which sometimes includes resection of surrounding organs and extensive lymph node dissection procedures that affect the quality of life of patients. However, despite the radical resection, some patients experience a recurrence which is believed to be due to sites of residual micrometastases. A study by Katsumata et al.[30] used RT-PCR reaction to detect CTCs through the use of cytokeratin and CEA (carcinoembryonic antigen) genes. The authors analyzed 57 patients with CRC who underwent surgery and also evaluated the presence of CK20 in peripheral blood. CK20 mRNA was found in 42.1% of patients and correlated with lymph node metastasis (p = 0.037). The 5-year survival rate for CK-20-positive patients was 62.5%, while for CK-20-negative patients was 87.5% (p = 0.048). Thus, the authors defend the idea of searching for CTCs as the best predictor of disease recurrence. However, it is known that hematopoietic cells can "illegitimately" express tumor-associated antigens (TAA) or tumor-specific antigens (TSA) to epithelial cells, while pseudogenes can lead to PCR products identical to marked genes, which leads to a considerable number of false-positive results by RT-PCR[31].

Hiraiwa et al.[32], in a study using the CellSearch™ system designed to evaluate CTCs in 130 patients (44 gastric, 48 colorectal, 38 esophageal) with gastrointestinal tumors, correlated clinicopathological characteristics with CTCs. The CTC count was significantly higher in metastatic gastric cancer (n = 27) than in non-metastatic (n = 14) and healthy controls (n = 41). A number equal to or greater than two CTCs per 7.5 ml of blood correlated with peritoneal dissemination of gastric or colorectal cancer. The survival of patients with metastatic cancer with ≥ 2 CTCs was significantly lower than those with less than 2 CTCs. Despite a small number of patients, the results were important and consistent with prior studies.

Cohen et al.[33] conducted a pilot study demonstrating that CTCs can be isolated in patients with metastatic CRC (mCRC). In 2008, this same group published a prospective study with 55 clinical centers in the US, the lower European Union countries and England with the purpose of evaluating interobserver agreement between the numbers of CTCs with the response of imaging and the ability of these numbers to predict PFS and OS in patients with mCRC. The principal inclusion criteria were: patients with mCRC starting any first - or second line systemic therapy or third line systemic therapy associated with epidermal growth factor receptor (EGFR) inhibitor. CT or MRI of the chest, abdomen and pelvis were performed at diagnosis and every 6 to 12 weeks after the start of treatment. Peripheral blood for analysis of CTCs through the CellSearch™ system was collected before the start of chemotherapy and at four different times: every 1-2, 3-5, 6-12 and 13-20 weeks after the start of treatment. In this study, the group established by interim analysis a threshold (≥ 3) for CTCs in CCR. They included 430 patients between 2004 and 2006, of which 26% had ≥ 3 CTCs per 7.5 ml of blood. Patients with liver metastasis and poor performance status had worse CTC levels at diagnosis. By comparing the results of CTCs with imaging exams (CT or MR) between 3-5 weeks of treatment, CTCs had a sensitivity of 27% (95% CI, 17-39%), specificity of 93% (95% CI, 89-96%), positive predictive value of 53% (95% CI, 36-69%), negative predictive value of 81% (CI 95%, 76-85%) and an accuracy of 78% (CI 95%, 73-82%). Patients with unfavorable CTC levels compared to favorable at diagnosis had lower average PFS (4.5 months *vs.* 7.9 months, p = 0.002) and lower OS (9.4 months *vs.* 18.5 months, p < 0.001). These differences persisted after four different collection periods. The authors concluded that the presence of at least 3 CTCs at diagnosis and at follow-up is a strong independent predictor for worse PFS and OS, and when used in conjunction with imaging, may provide additional prognostic information.

In a study from Madrid, Sastre et al.[34] found a positive correlation between the number of CTCs and clinical stage in 97 patients with the following characteristics: Newly diagnosed nonmetastatic CRC or rectal cancer without

neoadjuvant chemo - or radiotherapy; newly diagnosed mCRC, CRC recurrence at follow-up. Thirty healthy patients were used as control. The threshold of ≥ 2 CTCs per 7.5 ml was chosen to define a test as positive. No relationship was observed between any CTCs and primary tumor location, increased levels of CEA or lactate dehydrogenase, or differentiation grade.

Tol et al.[35] using samples of patients with mCRC who participated in a multicenter phase III study (CAIRO2) attempted to observe a correlation between the levels of CTCs in patients treated with chemotherapy and targeted therapy with the results obtained by imaging (CT). In all, 467 patients were included, of which 129 showed high levels of CTCs before the start of treatment, and correlated with poor PFS (HR = 1.5) and OS (HR = 2.2). When compared with imaging exams, the sensitivity and specificity of CTCs before the start of treatment for predicting disease progression were 16.7 and 70.1% respectively and after the start of treatment were 20 and 95.1% respectively. The values after treatment are similar to those obtained by Cohen et al.[36], which shows that the association of these two techniques can improve the accuracy of diagnosis.

A meta-analysis of 36 studies was published in 2010 which included 3094 patients. The authors concluded that the detection of CTCs in peripheral blood is an indicator of poor prognosis in patients with primary CRC[37].

Lu et al.[38] conducted a study in China with 141 patients with stage II and III CRC in order to determine whether the persistence of CTCs postsurgery could predict a high risk of early relapse. Four markers were used for mRNA (human telomerase reverse transcriptase, CK 19, CK 20 and CEA) to detect CTCs in the peripheral blood of patients before surgery with curative intent. Of the evaluated patients, 48 (34%) had early relapse and 93 (66%) did not. Early relapse correlated with lymph node metastasis (p = 0.025), vascular invasion (p = 0.002), perineural invasion (p = 0.001), laparoscopic surgery (p = 0.019), high postoperative serum CEA levels (p = 0.001), and persistent postoperative presence of CTCs (p < 0.001). On multivariate analysis, perineural invasion (p = 0.034, HR = 1.974, CI 95%: 1.290 - 3.861), high postoperative serum CEA levels (p = 0.020, HR = 2.377, CI 95%: 1.273-4.255) and the persistent postoperative presence of CTCs (p < 0.001, HR = 11.035, CI 95%: 4.396-32.190) proved to be independent predictors of postoperative early relapse. Additionally, persistent postoperative presence of CTCs correlated with poor overall and disease-free survival (both p < 0.001).

CTCs and pancreatic cancer

From the standpoint of gastrointestinal tumors, pancreatic cancer is a highly aggressive disease. It presents a high mortality rate since the disease is difficult to diagnose. Curative resection appears to be crucial for increased survival, but even with resection, most patients have local relapse or hematogenous spread[39]. Due to the poor prognosis of patients with pancreatic cancer, few studies have been conducted in relation to the disease with CTCs. There are some case reports and rare studies with series of patients.

Kurihara et al.[40] assessed the levels of CTCs in 26 patients with pancreatic cancer in stages II to IVb over a period of two years. Healthy individuals were used to confirm the specificity of the test and it was noted that none of them had any CTCs in circulation. Eleven patients had CTCs, all in stage IV. There was a significant difference between the levels of CA 19-9 found in CTC-positive and -negative patients (p < 0.05). The median survival of CTC-negative patients was greater than positive patients, showing that the positivity of CTC may affect survival.

Khoja et al.[41] prospectively compared the use of two methods for enumeration and characterization of CTCs in patients with pancreatic cancer. As a result, peripheral blood was collected from 54 patients and analyzed by CellSearch™ and ISET (isolation by size of epithelial tumor cells). CellSearch™ explores immunomagnetic capture of CTC-expressing epithelial markers, while ISET is an independent marker, an apparatus for blood filtration. The authors observed that ISET detected CTCs in 93% of patients, while CellSearch™ detected CTCs in 40% of patients. Furthermore, the number of CTCs detected by ISET (0-240) was greater than the number detected by CellSearch™ (0-140). Thus, the authors observed that ISET detects more CTCs than CellSearch™ and offers flexibility in the characterization of these cells, offering a vast opportunity to study the biology of this cell type, looking for new markers of pancreatic cancer.

CTCs and lung cancer

Finally, another type of tumor that has been studied in relation to the detection of CTCs is the lung. Patients with this tumor have a poor prognosis and most that are in stages III and IV die in two years[42]. One study[43] conducted with 88 patients with non-small-cell lung cancer (NSCLC) showed that CTC levels decreased significantly after treatment with standard chemotherapy in relation to the values obtained before the same (p < 0.005) suggesting that these cells be used as prognostic factors for NSCLC.

Marrinucci et al.[44] reported a case of a non-smoker diagnosed with stage III NSCLC. The patient had recurrence two years after treatment. After two years of therapy for recurrence, 67 CTCs were identified in peripheral blood by an immunofluorescence protocol and stained using Wright-Giemsa stain. By comparing the cytomorphology of CTCs with the cells isolated from the primary tumor (archived slides), it was possible to note that CTCs had morphology similar to the latter. This demonstrates that

cells of similar appearance to those of the primary tumor mass can be found in blood circulation at a relatively late stage of the development of metastatic disease.

Tanaka et al.[45], with the aim to investigate the relevance of CTCs in discriminating between primary lung tumors and non-malignant disease, as well as the role of CTCs in predicting metastasis, conducted a study with 150 patients, 25 patients with nonmalignant disease and 125 with primary lung cancer, of which 31 patients had distant metastases and 94 did not. The count of CTCs (by CellSearch™) was observed higher in patients with tumors than in those with non-malignant disease. Among the cancer patients, CTC levels increased throughout tumor progression, especially in those who developed metastases.

Hou et al.[46] analyzed the peripheral blood of 97 small cell lung cancer (SCLC) patients who received chemotherapy. Analysis was performed using immunomagnetic detection based on EpCAM and a filtration-based technique. Apoptosis and Ki67 were examined, as well as the number of CTCs and circulating tumor microemboli (CTM), which were associated with prognostic factors. CTCs were present in 85% (77/97) of patients. CTM and apoptotic CTCs were detected in 32% and 57% of patients, respectively. The number of CTCs found changed after one cycle of chemotherapy. Overall survival was 5.4 months for patients with 50 or more CTCs in each 7.5 ml of blood, and 11.5 months ($p < 0.0001$) for patients with less than 50 CTCs in the same amount of blood, prior to chemotherapy. The authors were then able to conclude that both the baseline number of CTCs and the change in the number of CTCs after one cycle of chemotherapy are independent prognostic factors for SCLC.

Franco et al.[47] collected the peripheral blood of 45 patients with NSCLC immediately after surgical resection. The authors analyzed the expression of chemokine receptors CXCL12 and CXCR4 in CTCs. The primary tumor was used to obtain microvascular density (MVD) and blood vessel invasion to investigate the receptor expression of CXCL12, CXCR4 and CXCR7. In 11 cases (23.9%) cytokeratin-positive cells were detected in the blood. CK-positive cells also expressed CXCR4 in 8 of 11 cases. Tumor tissue with high CXCR4 expression was significantly associated with high MVD expression ($p = 0.046$), high expression of CXCR7 ($p = 0.001$) and the presence of CTCs in the pulmonary veins ($p = 0.001$). Moreover, the invasion of blood vessels in relation to high MVD was found. Through that study the authors were able to observe the ability of CXCR4 receptors to determine vessel formation and migration of tumor cells by promoting the development of metastases.

A study conducted in England[48] with 101 patients with NSCLC without prior treatment, stage III or IV before and after the administration of one cycle of standard chemotherapy, investigated the ability of CTCs to indicate response to therapy. CTCs were evaluated by the CellSearch™ system and their number was higher in patients with stage IV (n = 60) compared to those with stage IIIB (n = 27) and IIIA (n = 14), where no CTCs were detected (n = 14). PFS was 6.8 months *vs.* 2.4 months ($p < 0.001$) and OS was 8.1 months *vs.* 4.3 months ($p < 0.001$) for patients with up to 5 CTCs compared with those with 5 or more CTCs prior to chemotherapy. In multivariate analysis, the number of CTCs was the strongest predictive factor for OS (HR = 7.92, 95% CI 2.85-22.01, $p < 0.001$) and the estimated HR increased with the second CTC sample collected after the first chemotherapy cycle (HR = 15.65, 95% CI 3.63-67.53, $p < 0.001$).

CTC Detection systems

As to methods for detecting CTCs, many tests have been developed, but the need to enrich the cells after its withdrawal from the patient's blood still remains. Briefly, the methods used until now have been: immunohistochemistry combined with fluorescence, immunomagnetic separation techniques, fluorescence-based assays and RT-PCR. The RNA markers usually include cytokeratins 19 and 20. Although highly sensitive, the techniques of RT-PCR are not as specific, leading to false positive results due to contamination or choice of target gene expression found in non-malignant cells[49]. The AdnaTest™ (AdnaGen AG, Langenhagen, Germany), using RT-PCR, is the CTC separation medium in most widespread use, however, it does not permit any further analysis of the cells[50].

The CellSearch™ system (Veridex, Huntingdon Valley, PA) is the only method approved by the US Food and Drug Administration-FDA[51] for use in breast, prostate and metastatic colorectal cancer (www.accessdata.fda.gov/cdrh_docs/reviews/K071729). This system allows CTCs to be viewed and numerically identified. It combines fluorescence data with the data of cell morphology simultaneously in the observation of the same cell. Cell separation is made by magnetic beads using anti-EpCAM to separate CTCs and identify them by means of anti-cytokeratin antibodies (8, 18 and 19), making the selection negative with anti-CD45 (leukocyte separation). To be considered a CTC, a cell must have an oval nucleus under microscopy, be positive for cytokeratin, negative for CD45 and positive for DAPI staining (4'-6-diamidino-2-phenylindole) to prove the presence of nucleus and exclude the possibility to be only a cell fragment. The analysis requires only 7.5 ml of peripheral blood and the test has a minimum sensitivity that allows the detection of 1-2 cells per sample. The results showed to be reproducible between samples in duplicate and multiple operators. No more than one CT was found in patients without cancer, suggesting high levels of specificity[50,52,53].

Deng et al.[54] developed a slide visualization method that combines the use of three fluorescent antibodies with the information obtained in cell morphology observed in

optical microscopy to identify CTCs. This method was applied by Ariol®, an automated collection and imaging analysis system which combines the data of fluorescence with cell morphology simultaneously in the observation of the same cell. By comparing this system with the CellSearch™ system, these authors showed that Ariol® was more sensitive, more accurate and more able to reproduce the results. Additionally, the Ariol® system was able to detect grouped CTCs, also known as microemboli, unlike the CellSearch™ system, which only identifies cells in isolation. As seen earlier, the importance of grouped CTCs has recently been highlighted. It is believed that these clusters of epithelial tumor cells have advantages in survival, proliferation and the establishment of micrometastatic lesions in distant organs[10]. Moreover, Al-Mehdi et al.[55] demonstrated that these CTC clusters can metastasize without extravasation, only by adherence to the vessel wall of arterioles and capillaries, proceeding proliferation within the vasculature, capillary wall rupture and formation of micro- and macrometastases. Thus, it has been postulated that the presence of CTC clusters in the blood may be indicative of a high metastatic potential. According Pantel et al.[56], analysis using the Ariol® system also requires the same amount of blood (7.5 ml) and makes positive selection using anti-cytokeratin antibodies (8, 18 and 19) and negative selection with anti-CD45.

Briefly, a cell adhesion molecule (EpCAM) is a type of adhesion molecule that is normally present in the basolateral surface of simple, pseudostratified and transition epithelial. Its *in vivo* expression is related to increased epithelial proliferation and negatively correlates with cell differentiation[57]. EpCAM is overexpressed in many solid tumors, including lung, colon, breast and squamous cell carcinoma[58].

Cytokeratins form parallel arrays of intracellular filaments that participate in maintaining the structural integrity of cells. Each epithelial cell type can be characterized by its content of cytokeratin polypeptides, whereas the expression pattern varies according to the type of epithelium. During the transformation process of normal epithelial cells into malignant cells, the pattern of cytokeratin expression is usually maintained. This property has allowed the use of these proteins as histological tumor markers, especially for tumors not easily classified[59].

Another method for detecting CTCs that has been used is ISET, which constitutes a direct technique of epithelial cell enrichment by filtration. It is based on the observation that the majority of peripheral blood leukocytes (lymphocytes and neutrophils) are the smallest cells of the body, with a size ranging from 8 to 11 µm. Thus, these cells can be eliminated by blood filtration through a polycarbonate membrane with 8 µm-calibrated pores. The simplicity of the method avoids the loss of rare cells in multi-step cell isolation. Once isolated, the CTCs can be evaluated by Giemsa, hematoxylin-eosin, or characterized by immunocytochemistry, FISH, TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) or microdissected for molecular analysis[10,56].

As cited by Bednarz-Knoll et al.[50], there are also other techniques such as CTC-chip, not yet validated, which makes the positive and negative selection with the same antibodies as the other techniques mentioned earlier. The MagSweeper™ is a device that is able to analyze a large volume of blood and performs the detection of the cells by the visualization of their morphology. The separation of cells is done by means of "magnetic beads". Using laser scanning cytometry, Maintrac® selects cells that are EpCAM positive and CD45 negative. The Ikoniscope® robotic fluorescence microscope imaging system makes the positive selection of cells with anti-cytokeratin antibodies (7 and 8), EpCAM and PSA in cases of patients with prostate cancer. Through this technique, it is possible to conduct FISH analysis. The EPithelial ImmunoSPOT (EPISPOT) technique selects cells by proteins CK19, MUC1, Cath-D (in breast cancer), PSA (prostate cancer) and TG (in thyroid cancer). The collagen adhesion matrix (CAM) method makes the positive selection of cells with anti-EpCAM antibodies, ESA, and cytokeratins 4, 5, 6, 8, 10, 13 and 18. The negative selection is made with anti-CD45 antibody. The techniques using FAST (fiber-optic array scanning technology) and versatile label free biochip (microfluidic device) make the positive selection of cells with anti-CK antibodies and the negative selection with anti-CD45 antibodies. The selected cells possess a DAPI-positive nucleus and are also analyzed morphologically. Table 1 contains a brief summary of the CTC detection systems discussed.

CONCLUSION

From what has been described earlier, the counting of CTCs at diagnosis and during chemotherapy can anticipate clinical outcome and ultimately change the direction of treatment. However, despite the counting of CTCs being able to predict the evolution of a disease to a metastatic state, these data still need greater solidification. It is known that CTCs may undergo the process of epithelial-to-mesenchymal transition during metastasis, changing the composition of their membrane markers, which constitutes one of the limiting factors for detection of CTCs by the methods described earlier. Moreover, CTCs may die after the removal of the tumor of origin, as death can be caused by several factors such as blood oxygenation and immune response (e.g., NK cell activity, factors of the complement system).

It is also important to emphasize that CTCs may or may not have resemblance to the original tumor. There is speculation that these cells are different from the

Table 1. CTC detection systems and their associated techniques and antibodies.

Detection Systems	Techniques and Antibodies Used
AdnaTest™	RT-PCR
	Positive markers: MUC1, HER2 and EpCAM
CellSearch™ system	Immunocytochemistry
	Positive markers: CK8, 18, 19
	Negative markers: CD45
	DAPI-positive nucleus
Ariol	Immunocytochemistry
	Positive markers: CK8, 18, 19
	Negative markers: CD45
	DAPI-positive nucleus
ISET (Isolation by Size of Epithelial Tumor Cells)	After filtration, CTCs can be stained by hematoxylin and nucleus seen.
	Molecular techniques can be performed after isolation of CTCs
CTC-chip	Immunocytochemistry
	Positive markers: CK8, 18, 19
	Negative markers: CD45
	DAPI-positive nucleus
MagSweeper™	Viewed by microscope: morphology
Maintrac® (laser scanning cytometry)	Immunocytochemistry
	Positive markers: EpCAM
	Negative markers: CD45
Ikoniscope imaging	Immunocytochemistry
	Positive markers: EpCAM, CK 7/8, PSA (only for prostate)
	FISH
	DAPI-positive nucleus
EPISPOT	Proteins secretions: CK19, MUC1, Cath-D (breast), PSA (prostate), TG (thyroid)
Collagen Adhesion Matrix (CAM)	Immunocytochemistry
	Positive markers: EpCAM, ESA, pan-CK 4, 5, 6, 8, 10, 13 and 18
	Negative markers: CD45
FAST	Immunofluorescence
	Morphology observation
	Positive markers: CK
	DAPI-positive nucleus
Versatile label free biochip	Immunofluorescence
	Morphology observation
	Positive markers: CK
	DAPI-positive nucleus
	Negative markers: CD45

primary tumors in that they have undergone mutations that have given them greater resistance and survivability and therefore are resistant to standard therapies. Many questions still need to be answered, including whether the metastatic site is populated by CTCs or tumor stem cells that migrate clustered. If the latter, what has been postulated for mRNAs? If a tumor is completely removed by surgical resection, can CTCs be found in circulation? If the answer is yes, what has been observed in animal models in regard to where these cells come from? Do they have the capacity for dormancy? For now, we have more questions than answers, which makes the study of CTCs so challenging and compelling.

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