Chapter 8
Circulating Tumor Cells in Mesenchymal Tumors

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8.1 Mesenchymal Stem Cells

Many authors have previously isolated CTCs from carcinomas and demonstrated their prognostic value in different tumors. However, the majority of methods used for isolating these CTCs are based on epithelial antigen-targeted antibodies, and thus they neither allow the isolation of the CTCs undergoing epithelial-mesenchymal transition (EMT) nor the detection of CTCs from sarcomas [35, 60].

The ability to differentiate along different lineages and the ability to self-renew are characteristic of stem cells [49]. Embryonic stem cells (ESCs) and adult stem cells compose two large groups and the first are associated with tumorigenesis [2, 4]. Based on this observation at the turn of the 1960s and 1970s, Friedenstein was one of the pioneers of the theory that bone marrow is a reservoir of stem cells of mesenchymal tissues in adult organisms. In his study, Friedenstein noted in vitro cultivation that ectopic transplantation of bone marrow (BM) into the kidney capsule resulted in the formation of bone, not only in the proliferation of bone marrow cells [24]. According to McCulloch, cells from the BM can give rise to multilineage descendants while retaining the ability to self-renew [45, 59, 61].

Proposed by Caplan in 1991, the term "mesenchymal stem cells" (MSCs) was used due their ability to differentiate into more than one type of cells capable to form connective tissue in many organs [10]. The MSCs are multipotent cells that are present in several adult tissues, such as the umbilical cord, adipose, peripheral blood, liver, and bone marrow [21, 29].

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The mesenchymal stem cell phenotype is characterized by the presence of CD73, CD90, CD105 surface antigens and the absence of protein expression CD45, CD34, CD14, CD11b, CD79a, or CD19 or class II histocompatibility complex antigens (HLA II, human leukocyte antigens class II). Furthermore, these cells must have the ability to differentiate osteoblasts, adipocytes, and chondroblasts [8, 18, 31] (Fig. 8.1).

8.2 Detection of Circulating Tumor Cells of Sarcomas

Sarcomas are a heterogeneous group of soft tissue and bone neoplasms that arise out of mesenchymal tissues and consequently may arise from mesenchymal stem cells [42, 62]. In patients with localized disease, distant metastases develop in 50% of cases, with lungs being the most common metastatic site [42]. Detection of circulating tumor cells (CTCs), as a measure of metastatic potential, could provide a way to diagnose and monitor patients. However, the clinical significance of CTCs, as a prognostic or predictive marker in sarcoma, is poorly explored (Table 8.1).

The detection of CTCs in sarcomas are relatively recent due to the limited number of patients, the absence of specific markers expressed by sarcoma tumor cells, and their high diversity/heterogeneity.

Considering that most CTCs are frequently larger than that of normal circulating cells in blood, cell size represents a potential criterion for isolating sarcoma CTCs. Chinen et al. [16] and Braun et al. [9], were the first to describe the isolation by size method to isolate sarcoma CTCs, but other studies, with other techniques, have been

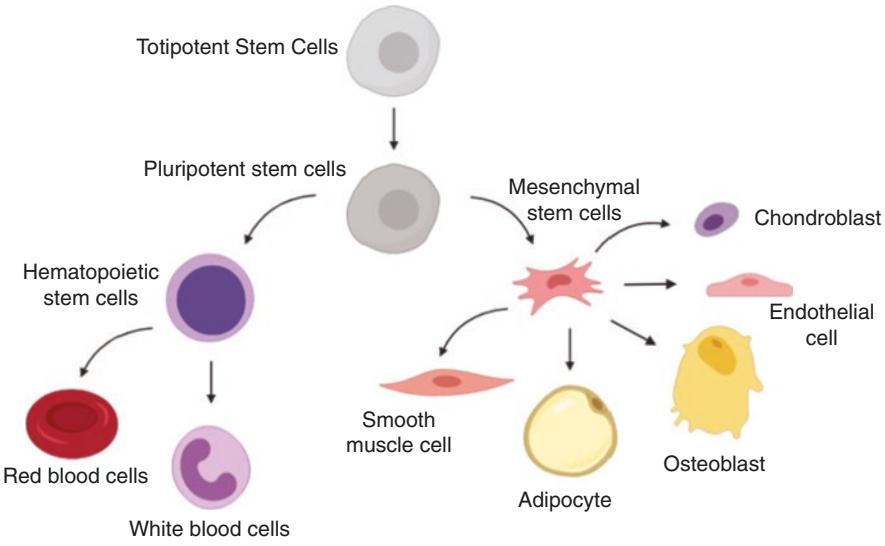

Fig. 8.1 The ability of mesenchymal stem cells (MSCs) to differentiate in other cells

Table 8.1 Studies with CTCs and their main findings in diverse types of sarcomas

Author	Year of publication	Patients (*n*)	Histology	Method of detection	Source	Finding
Peter et al.	1995	36	Ewing's sarcoma	RT-PCR	PB and BM	Detection of EWS-FLI1 fusion transcript
Pfleiderer et al.	1995	16	Ewing's sarcoma	RT-PCR	PB and BM	Detection of EWS-FLI1 fusion transcript
Kelly et al.	1996	11	Rhabdomyosarcoma	RT-PCR	BM	Detection of PAX3-FKHR fusion transcript
West et al.	1997	16	Ewing's sarcoma/PNET	RT-PCR	PB and BM	Detection of EWS-FLI1 fusion transcript
Fagnou et al.	1998	62	Ewing's sarcoma	RT-PCR	BM	CTC detection in BM but not PB associated with reduced survival
Zoubek et al.	1998	35	Ewing's sarcoma	RT-PCR	BM	EWS/ets-oncogene fusion transcripts
de Alava et al.	1998	28	Ewing's sarcoma	RT-PCR	PB	EWS-FLI-1, EWS-ERG
Thomson et al.	1999	12	Alveolar rhabdomyosarcoma, Ewing's sarcoma	RT-PCR	PB and BM	PAX3-FKHR, EWS-FLI1
Wong et al.	2000	11	Osteosarcoma	RT-PCR	PB	Quantification of COLL mRNA
Burchill et al.	2001	49	Neuroblastoma	RT-PCR	PB	Tyrosine hydroxylase mRNA
Schleiermacher et al.	2003	172	Ewing's sarcoma	RT-PCR	PB and BM	CTC detection in PB or BM associated with poor outcome among patients with localized disease
Avigad et al.	2004	26	Ewing's sarcoma	RT-PCR	PB and BM	Detection of EWS-FLI1 fusion transcript
Gallego et al.	2006	16	Rhabdomyosarcoma	RT-PCR	PB	Isolation of PAX3-FKHR correlated with prognosis
Hoshino et al.	2009	1	Alveolar sarcoma	RT-PCR	PB	Isolation of ASPSCR1-TFE3-positive cells
Dubois et al.	2010	1	Ewing's sarcoma	Flow cytometry	PB and BM	Isolation of CD99+CD45− cells

(continued)

Table 8.1 (continued)

Author	Year of publication	Patients (*n*)	Histology	Method of detection	Source	Finding
Chinen et al.	2013	11	Soft-tissue sarcomas	ISET	PB	Isolation by size of tumor cells (antivimentin and CD45)
Przybyl et al.	2014	38	Ewing's sarcoma	RT-PCR	PB and BM	EWS-FLI-1, EWS-ERG
Satelli et al.	2014	28	Ewing's sarcoma/osteosarcoma/ leiomyosarcoma/Angiosarcoma	Flow cytometry	PB	Detection by a novel monoclonal antibody (cell-surface vimentin)
Braun et al.	2018	18	Soft-tissue Sarcomas	ISET	PB	Isolation by size of tumor cells and EGFR expression detected
Mihály et al.	2018	15	Synovial sarcoma	ddPCR	PB	Detection of SS18-SSX2 fusion transcript
Przybyl et al.	2019	38	Synovial sarcoma	RT-PCR	PB	Detection of SS18-SSX fusion transcript

performed involving these tumors. Filtration methods are relatively rapid, sensitive, and easy technique. After isolation, CTCs are characterized by immunocytochemistry. Chinen et al. [16] investigated the feasibility of using isolation by size of tumor cells (ISETs) for isolation, identification, and characterization of CTCs derived from patients with high-grade and metastatic sarcomas. The researchers studied 11 patients and blood samples (8 ml) were collected from patients with advanced soft tissue sarcomas (STSs). In these studies, all patients showed CTCs, with numbers ranging from 2 to 48 per 8 mL of blood.

Braun et al. [9] quantified CTCs and identified CTM as well as the EGF receptor (EGFR) protein expression in these cells and correlated with clinical outcome in metastatic STS. Blood was prospectively collected from patients with different types of high-grade STS, before the beginning of chemotherapy. The samples were processed and filtered by ISET (Rarecells, France) for the isolation and quantification of CTCs and CTMs. EGFR expression was analyzed by immunocytochemistry (ICC) on CTCs/ CTMs (Fig. 8.2).

The authors analyzed 18 patients with median age of 49 years (18–77 y). The positivity for EGFR protein expression in CTCs was observed in 93.75% of the patients. The authors were the first to demonstrate the expression of EGFR protein in CTCs from sarcoma patients. These results may open an area for future investigations.

Another strategy for CTC detection in sarcomas is the use of common mesenchymal cell markers such as vimentin. Satelli et al. [57] used a new marker on

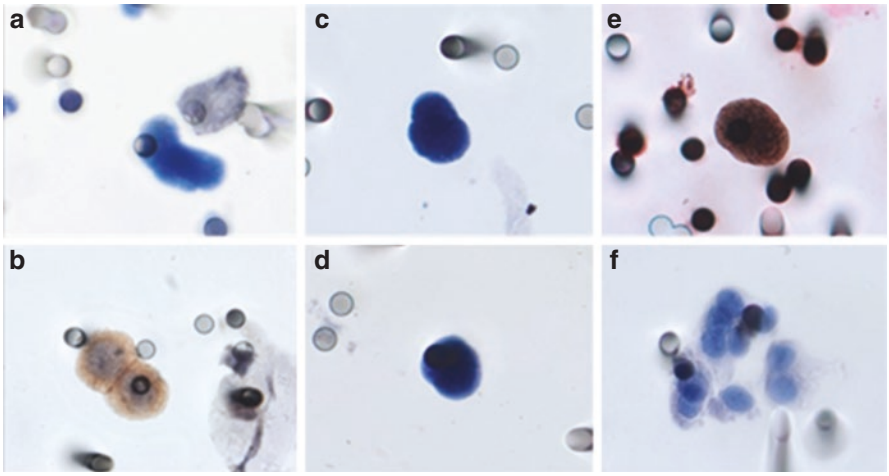

Fig. 8.2 (**a**) Negative control, A-549 cell line "spiked" in healthy blood and negative for EGFR. (**b**) Positive control, FaDu cell line "spiked" in healthy blood and stained for EGFR. (**c**, **d**) Examples of an isolated CTC of sarcoma patient with cytomorphological features (negative staining for CD45, nucleus size ≥12 μm, hyperchromatic and irregular nucleus, visible presence of cytoplasm, and a high nucleus–cytoplasm ratio (Krebs et al. [43]) 15. (**e**) Immunocytochemistry of CTC with anti-EGFR antibody and counterstaining with DAB. (**f**) One CTM from STS patient observed in the blood filtered using the ISET. (Ref. Braun et al. [9])

sarcoma CTC regardless of the tissue origin of the sarcoma as detected by a novel monoclonal antibody. In this study, the authors reported cell-surface vimentin (CSV) as an exclusive marker on sarcoma CTC. Using flow cytometry and FISH, they suggested that this new marker established the first universal and specific CTC marker described for enumerating CTCs from different types of sarcoma, thereby providing a key prognosis tool to monitor cancer metastasis and relapse.

Gallego et al. [25] used detection of muscle markers for CTC detection in rhabdomyosarcoma patients. They performed the analysis combining the detection of a fusion gene product and muscle-specific markers, including MyoD1 and myogenin. In this study, patients with positivity in peripheral samples at the end of treatment showed a poorer prognosis than patients with negative samples.

Circulating tumor cells of sarcoma subtypes associated with specific chromosomal translocations leading to the expression of a unique fusion product are more easily identified, and most studies were performed on Ewing's sarcoma by RT-PCR analysis for the research of the fusion gene product associated with the disease: EWS-FLI-1 and EWS-ERG markers [46]. Results from clinical studies of patients with Ewing's sarcoma suggest that the detection of CTCs at diagnosis may be associated with worse clinical outcomes and that CTCs may be an early marker of recurrent disease.

West et al. [63] studied 16 patients with nonmetastatic disease, three of 16 were RT-PCR positive for EWS/HumFLI1 RNA in BM and three of 10 were positive in PB. In this study, they showed that it is possible to amplify the EWS/HumFLI1 RNA by RT-PCR from the BM and PB of a subset of patients with both nonmetastatic and metastatic ES or PNET, which implies that occult tumor cells are present at these sites.

In the study of Schleiermacher et al. [58], the researchers studied 172 patients with Ewing tumor. RT-PCR targeting EWS-FLI-1 or EWS-ERG transcripts was used to search for occult tumor cells in peripheral blood and bone marrow at diagnosis. The presence of circulating tumor cells (CTCs) was more frequently observed in patients with large tumors ($P = .006$), and CTCs were associated with a poor outcome among patients with clinically localized disease ($P = .045$). The study's conclusion was that patients with localized Ewing tumor and BM micrometastasis or CTC are comparable to patients with metastases in terms of the localization of the primary tumor and relapse pattern.

Avigad et al. [1] reported the prognostic potential of the positive chimeric transcript (EWS/FLI1) in bone marrow (BM) and/or peripheral blood (PBL) in 26 patients with EFTs (Ewing family tumors), during a long follow-up period (median, 61 months), and the results suggested that occult tumor cells in BM and/or PBL samples during long follow-up are strong predictors of recurrent disease in patients with nonmetastatic EFTs.

Semi-quantitative RT-PCR was described by Wong et al. [64]. The researchers correlated mRNA levels of "osteoblast-related genes like" in CTCs from peripheral blood of osteosarcoma patients and found that type I collagen levels were significantly higher in osteosarcoma patients than in healthy subjects.

Hatano et al. [30] developed a similar methodology. They used a system with a polymerase chain reaction assay based on an enzyme-linked immunosorbent assay (PCR-ELISA) to detect circulating osteosarcoma cells in a mouse metastatic model. Osf2/Cbfa1, hereafter called Osf2, a member of the runt family of transcription factors, was used as a target gene, and the amount of the splicing variant of Osf2 mRNA was significantly higher in the blood of mice with metastasis than in the blood of the control group. The researchers demonstrated that PCR-ELISA using Osf2 mRNA was a potential method to detect circulating osteosarcoma cells in peripheral blood.

Multiple studies use flow cytometry to detect CTCs. To isolate these cells, pre-enrichment steps are required in combination with specific antigen recognition for discriminating CTCs from circulating hematopoietic cells (anti-CD45 marker) and epithelial cells (pan-cytokeratin-related marker) [17]. Dubois et al. [19] studied Ewing sarcoma cell line A673, peripheral blood mononuclear cells (PBMCs), and bone marrow mononuclear cells (BMMCs). In this study, the cells were stained for CD99 and CD45 in order to detect CD99+CD45− cells by flow cytometry. Known quantities of A673 Ewing sarcoma cells were spiked into control PBMCs to test the accuracy of this method, and control PBMCs were evaluated to access the level of background staining. The authors suggested that multicolor flow cytometry for CD99+CD45− cells provides a new strategy for detecting circulating Ewing sarcoma cells.

8.3 Epithelial-Mesenchymal Transition (EMT)

To initiate metastasis, tumor cells (CTs) need to leave the primary site to colonize distant tissues. Within the cascade of events that would allow migration, the so-called epithelial-mesenchymal transition (EMT) is presented, a process present during embryogenesis, when epithelial tissue healing is performed. Carcinoma cells can also pass through this process, by loss of epithelial properties and acquisition of partially or totally mesenchymal ones [33, 34].

Carcinoma cells are of epithelial origin and so, undergo to cell-to-cell interaction through adhesion molecules such as cadherins, claudins, or plakoglobin [27, 41].

EMT is a transformation that, apart from being highly dynamic, can be reversible, and in the case of tumor cells, it is characterized by stimulating the invasiveness toward other tissues, by a series of events such as the detriment of cell-cell adhesion proteins within the tumor, in addition to the loss of cellular-atomic-basal polarity [33, 44].

It is documented that EMT is probably triggered by paracrine signaling of the transforming growth factor beta (TGF-β), the Wnt signaling pathway, platelet-derived growth factors, interleukin-6 (IL-6), and some different agents such as nicotine, alcohol, and ultraviolet light. These activators would stimulate transcription factors, such as the basic helix-loop-helix factor (TWIST) and zinc-finger

E-box-binding homeobox (ZEB), which help to maintain the mesenchymal phenotype by autocrine signaling. Due to the breakage of tight and adherent junctions, together with the cytoskeleton variations, epithelial markers such as EpCAM and E-cadherin are negatively regulated, and at the same time the expression of keratins is altered, together with a positive regulation of mesenchymal markers, such as vimentin [32].

To invade the extracellular matrix, tumor cells enter and exit the bloodstream using different cell forms and alternating between the rounded (or amoebic) and the elongated (or mesenchymal) shape, directed by Rho GTPases (RHO) – RHO-associated protein kinase (ROCK) RHO-ROCK [39, 40, 52]. The mesenchymal mode demands the Rac small GTPase (Rac). Cells with amoebic motility exhibit rounded or ellipsoid morphology. These cells also present weak interaction with surrounding matrix, induced by elevated RHO levels, that stimulate membrane blebbing by ROCK-dependent myosin II phosphorylation and consequent actin-myosin contractility [53, 56]. The balance of activated RAC and RHO may determine the mesenchymal or amoebic mode, and the mutual antagonism contributes to maintain different modalities of cell motility [28, 65]. However, the activation mechanism is still confusing [36, 53, 55].

According Li et al. [38] and Caramel et al. [11], mesenchymal tumors are characterized by early metastasis, frequent relapse, and unfavorable clinical outcomes; thus, sarcomas exhibit an aggressive clinical phenotype [26]. EMT has been observed mainly in carcinomas; however, EMT-like processes have also been reported in non-epithelial cancers. Based on that, some studies indicate that sarcomas can undergo phenotypic changes reminiscent to the EMT/MET (mesenchymal-epithelial transition) [15, 20, 22] (Fig. 8.3).

Studies with melanoma have shown that cells spread in a mesenchymal state throughout the body during embryogenesis and settle in the skin. These studies suggested that a subpopulation of melanoma cells transiently acquires a mesenchymal-like state [13, 38].

In Ewing sarcoma, several research groups have shown that individual tumor cells can switch back and forth between more epithelial and more mesenchymal phenotypes.

Chaturvedi et al. [14], using an orthotopic xenograft model, showed that EWS/FLI-induced repression of α5-integrin and zyxin expression promotes tumor progression by supporting anchorage-independent cell growth. This selective advantage was paired with a trade-off in which metastatic lung colonization is compromised, demonstrating that phenotypes can change.

Franzetti et al. [23] demonstrated in their study that cell-to-cell heterogeneity of EWSR1-FLI1 activity determines proliferation/migration choices in Ewing sarcoma cells, using proteomic analysis.

These data together suggest that certain sarcomas can undergo to an EMT- and MET-related process through pathways classically involved in the EMT/MET in carcinomas. The activation of one or another pathway appears to be crucial for the

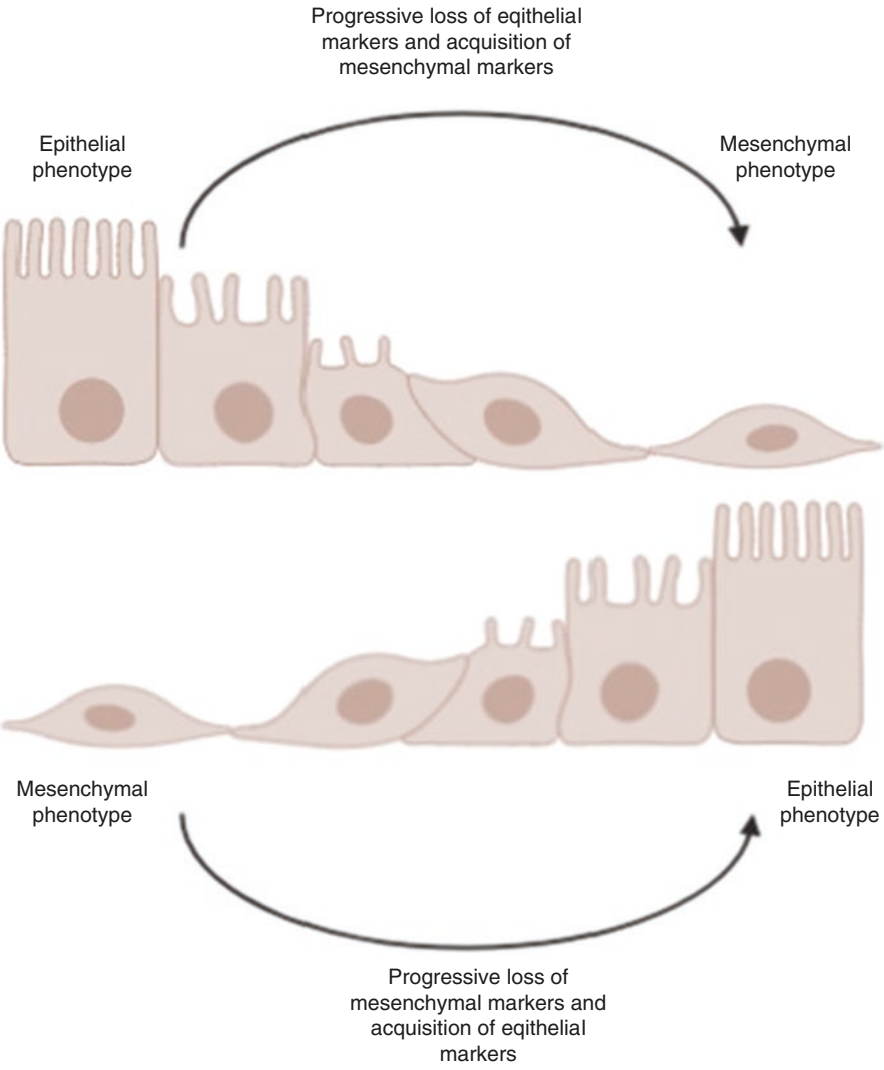

Fig. 8.3 Illustration of the EMT/MET

phenotypic switching of sarcomas toward either a more epithelial or mesenchymal phenotype.

Sannino et al. [54] proposed in a review that certain sarcoma subtypes reside in a peculiar metastable state that enables individual tumor cells to undergo EMT/MET-related processes due to specific cues, combining both epithelial and mesenchymal biological features in a single tumor, which makes metastable sarcomas highly aggressive.

8.4 Plasticity of Circulating Tumor Cells

As previously described, EMT is a complex process that occurs in a broad range of tissue types and developmental stages. EMT involved various mechanisms of the dissemination of cancer including the release of CTCs [6, 7, 47].

Most of the assays for detecting CTCs use cell surface proteins, which pose a challenge to any detection system. In addition, not all steps of EMT are required for carcinoma cells to become invasive and enter the circulation [3, 5, 51].

In 2009, Aktas et al. analyzed blood samples of 39 patients suffering from metastatic breast cancer using the AdnaTest Breast Cancer and observed that 97% of 30 healthy donor samples investigated were negative for EMT and 95% for ALDH1 transcripts. CTCs were detected in 69/226 (31%) cancer samples. In the positive CTC group, 62% were positive for at least one of the EMT markers and 69% for ALDH1. In the negative CTC group, the percentages were 7% and 14%, respectively. In non-responders, EMT and ALDH1 expression were found in 62% and 44% of patients, in responders the rates were 10% and 5%, respectively.

CTCs were detected in 69/226 (31%) cancer samples. Those results indicate that a major proportion of CTC of metastatic breast cancer patients shows EMT and tumor stem cell characteristics.

Lecharpentier et al. [37] found the presence of hybrid epithelial-mesenchymal CTCs in six NSCLC patients that was reported in a pilot study. They observed the presence of clusters of dual CTCs strongly co-expressed vimentin and keratin in all patients (range 5–88/5 ml) and showed for the first time the existence of hybrid CTCs with an epithelial/mesenchymal phenotype in patients with NSCLC.

Alix-Panabières et al. [12] in a review exposed that CTCs with mesenchymal features in patients with various tumor entities can be attributed to higher disease stages, presence of metastases, and in some studies even to therapy response and worse outcome.

Future studies should focus more on the detection and characterization of CTCs with mixed epithelial mesenchymal features.

8.5 Perspectives

The use of CTCs detection in sarcoma patients might be an important diagnostic tool for the earlier detection of metastatic disease for monitoring therapeutic response and for identifying the time point during treatment at which an adjustment in therapy is indicated. CTCs, CTM, and EMT/MET in these cells can be used as tools to measure the effectiveness of treatment and better select patients for clinical intervention. Studies with a larger cohort of patients, with well-defined treatment and follow-up are necessary to confirm data.

Advances may help clarify the extent to which EMT is involved in the various disease states and point to avenues through which our current understanding of the

EMT pathway and transitional events can be exploited to target tumors and/or make them more susceptible to treatment regimes.

8.6 Pictures from Patients

Here, we show some pictures (Figs. 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 8.10, 8.11, 8.12, 8.13, 8.14, 8.15, 8.16, 8.17, 8.18, 8.19, 8.20, 8.21, and 8.22) of CTCs and CTM from patients with diverse types of sarcomas, treated and followed-up at ACCamargo Cancer Center, São Paulo, Brazil. All pictures were selected by Dr. Ludmilla T.D. Chinen and reviewed by Dr. Mauro Saieg (cytopathologist).

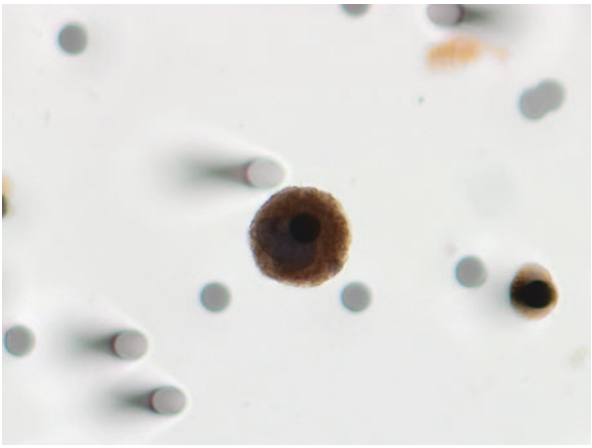

Fig. 8.4 Macrophage isolated from blood, by ISET. Woman, 57 years old, with pleomorphic sarcoma. At the time of blood collection, before first-line treatment with epirrubicin and ifosfamide, she showed 4.5 CTCs/ml. In brown: DAB (anti-β-galactosidase). Microscope magnification: 60×

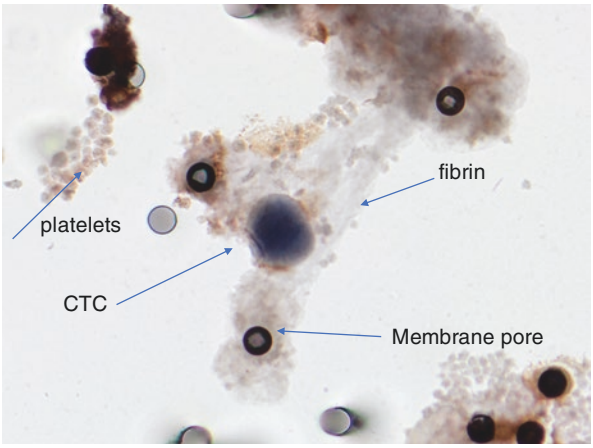

Fig. 8.5 CTC isolated from blood, by ISET. Woman, 59 years old, with leiomyosarcoma. At the time of blood collection, before first-line treatment with gemcitabine, she showed 9.6 CTCs/ml. In brown: DAB (anti-β-galactosidase). Microscope magnification: 40×. Membrane pore diameter of 8μm and CTCs nucleus size ≥ 12 μm

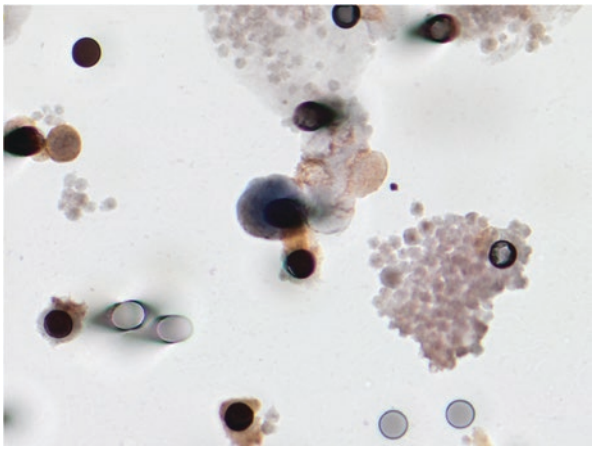

Fig. 8.6 CTC from the same patient Fig. 8.5. In brown: DAB (anti-βgalactosidase). Blue: hematoxylin

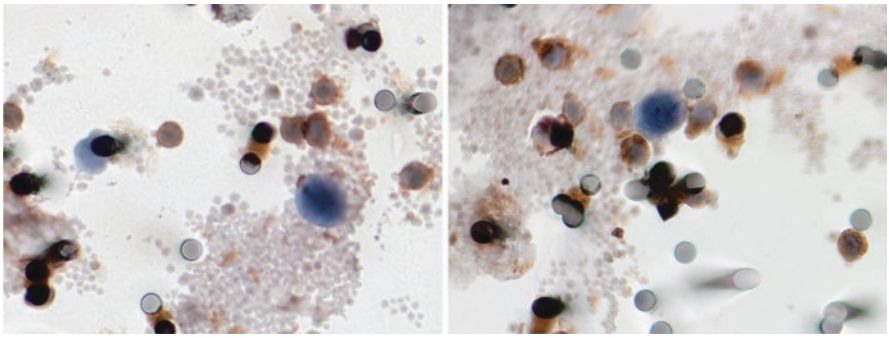

Fig. 8.7 CTC from the same patient Fig. 8.5. CTCs with evident nuclei

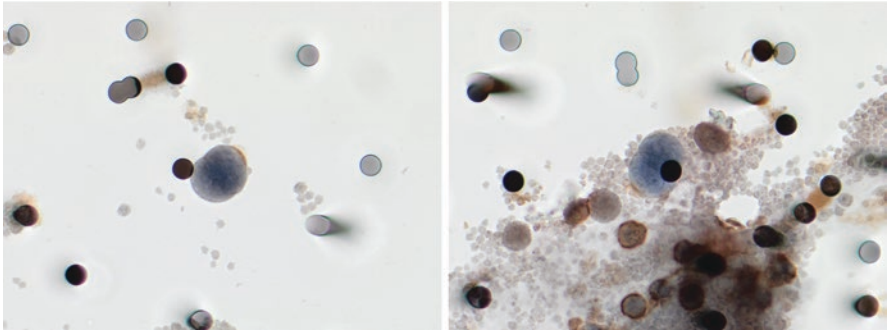

Fig. 8.8 CTC from the same patient Fig. 8.5. In brown, cell on left side, with evident nuclei and anti-EGFR staining with DAB. Cell on right side: CTC in the middle of the field showing changes in the N/C ratio and binucleation, with irregular chromatin and high nuclear/cytoplasmatic ratio

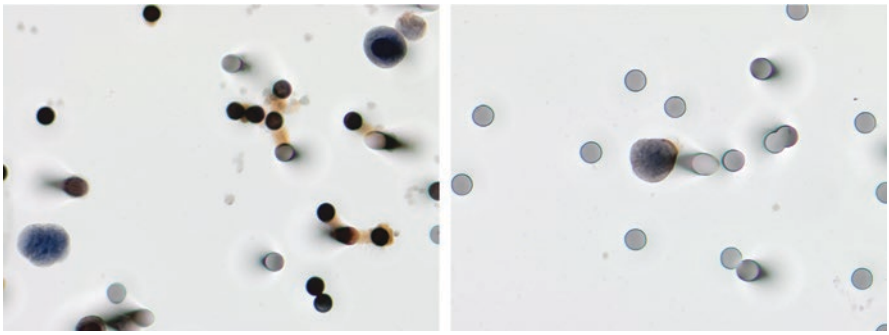

Fig. 8.9 CTC from the same patient Fig. 8.5. In brown: DAB (anti-EGFR). In blue: hematoxylin

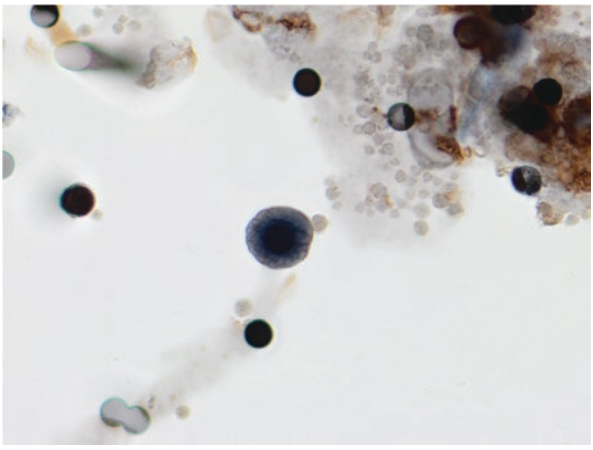

Fig. 8.10 CTC from the same patient Fig. 8.5. In brown: DAB (anti-βgalactosidase); in blue: hematoxylin. CTC in the middle of the field showing changes in the N/C ratio and binucleation, with irregular chromatin and high N/C ratio

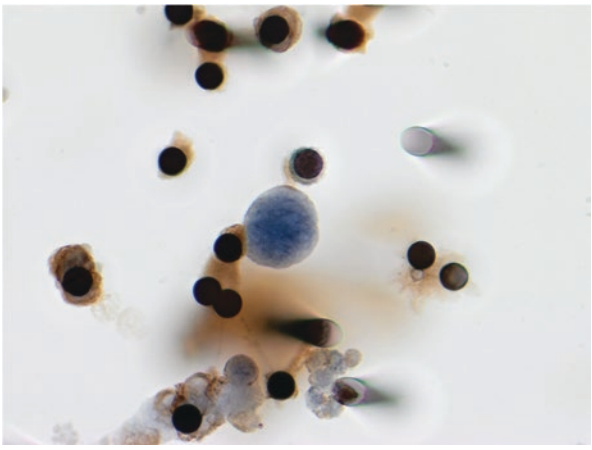

Fig. 8.11 CTC from the same patient Fig. 8.5. In brown: DAB (anti-EGFR), in blue: hematoxylin. CTC in the middle of the field showing chromatin irregularity and alteration of the nuclear/cytoplasmatic ratio

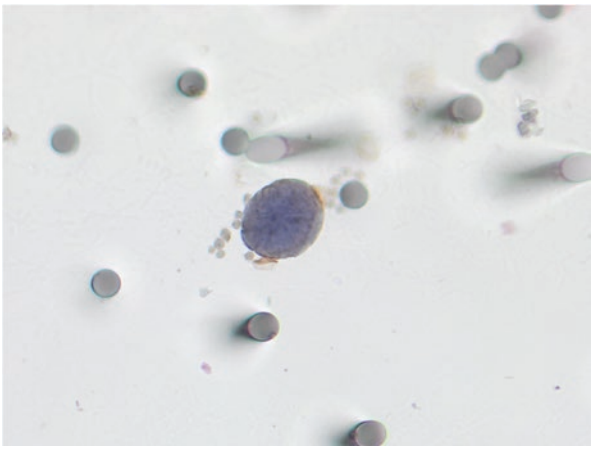

Fig. 8.12 CTC from the same patient Fig. 8.5. In brown: DAB (anti-TGF-β-RI). In blue: hematoxylin. CTC showing high N/C ratio, nuclear and chromatin irregularity

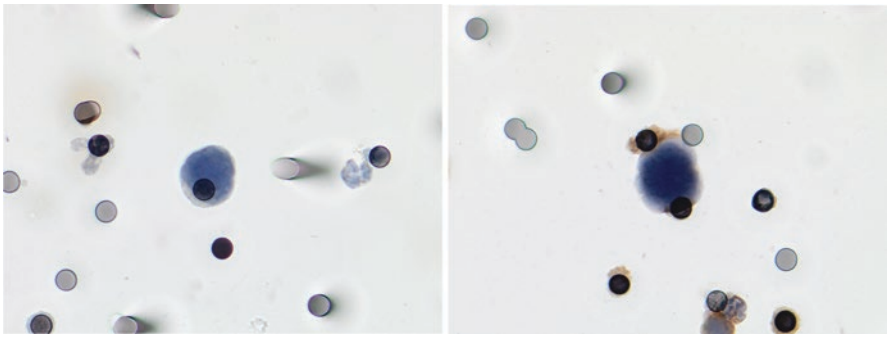

Fig. 8.13 CTC isolated from blood, by ISET. Woman, 46 years old, with leiomyosarcoma. At the time of blood collection, before surgical rescue, she showed 0.75 CTCs/ml. Microscope magnification: 40×

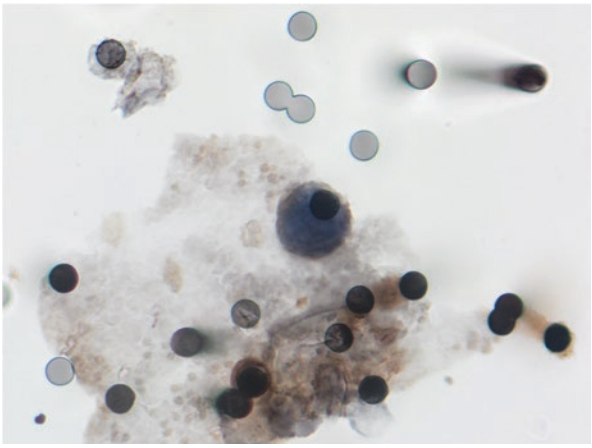

Fig. 8.14 CTC isolated from the same patient Fig. 8.13. In brown: DAB (anti-EGFR). Microscope magnification: 40x

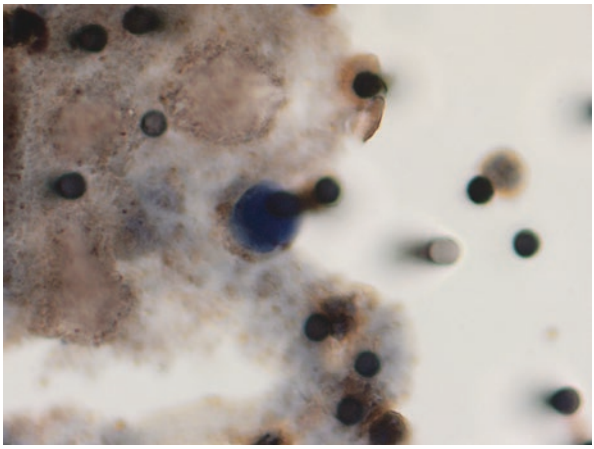

Fig. 8.15 CTC isolated from blood, by ISET. Man, 69 years old, with liposarcoma. At the time of blood collection, before treatment with doxorubicin, he showed 11.25 CTCs/ml. Microscope magnification: 40×

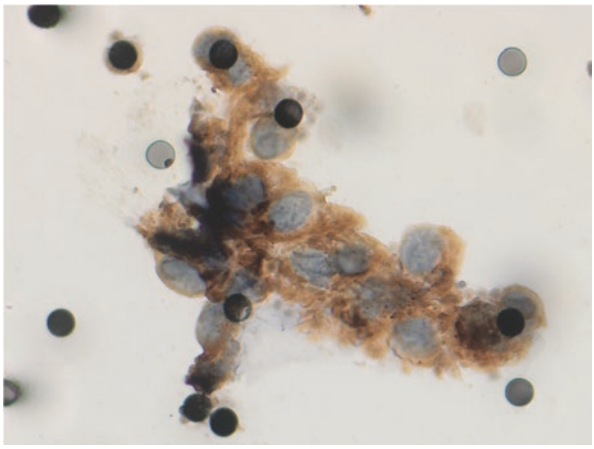

Fig. 8.16 CTC isolated from the same patient of Fig. 8.15. Neoplastic cell block, sometimes spindle shaped, with cytoplasmic marking for vimentin (DAB). Microscope magnification: 40×

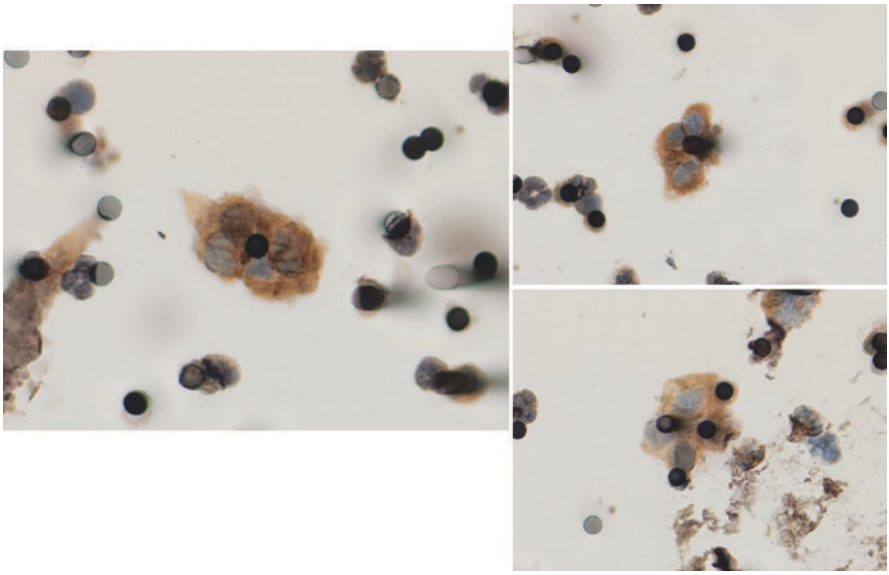

Fig. 8.17 CTM isolated from the same patient of Fig. 8.15. Neoplastic cell blocks, with cytoplasmic marking for vimentin (DAB). Microscope magnification: 40×

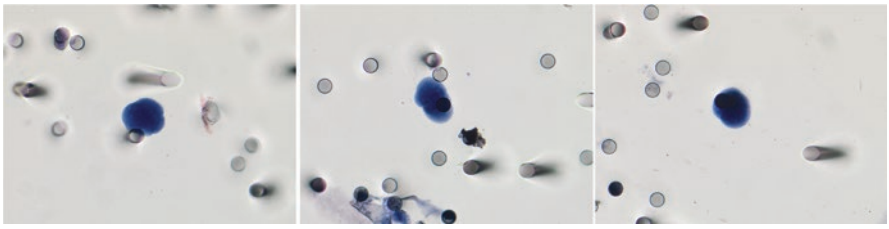

Fig. 8.18 CTC isolated from blood, by ISET. Man, 76 years old, with pleomorphic sarcoma. At the time of blood collection, before treatment with gemcitabine and docetaxel, he showed 5.62 CTCs/ml. Microscope magnification: 40×

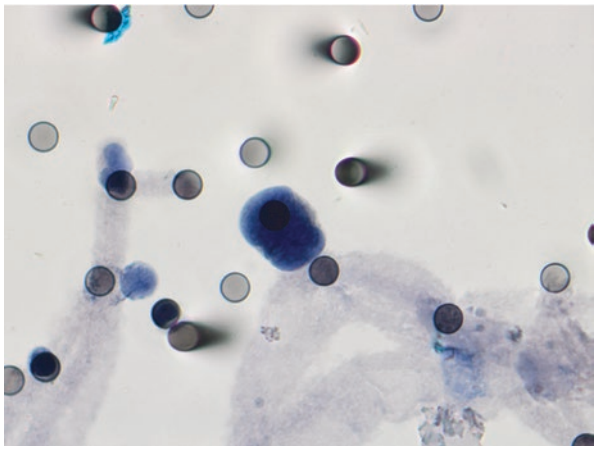

Fig. 8.19 CTC from the same patient of Fig. 8.18. We can observe intense atypia, with multilobulated nucleus, irregular chromatin, and hyperchromic nucleus

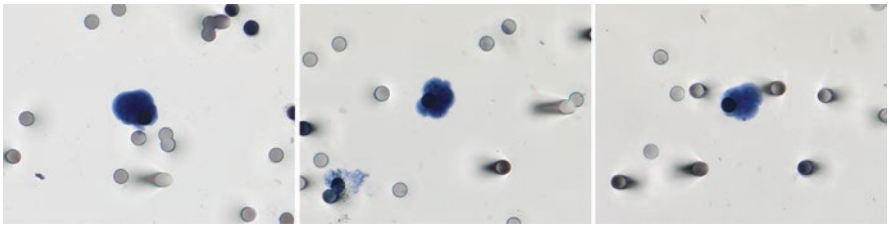

Fig. 8.20 CTCs from the same patient of Fig. 8.18

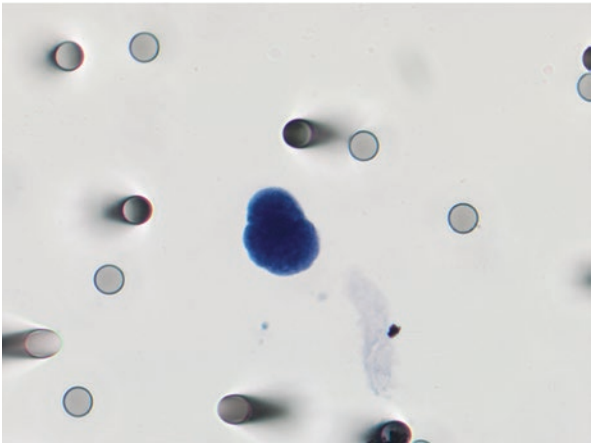

Fig. 8.21 CTCs from the same patient of Fig. 8.18. We can observe an irregular, multilobulated nucleus, with hyperchromasia and nuclear irregularity

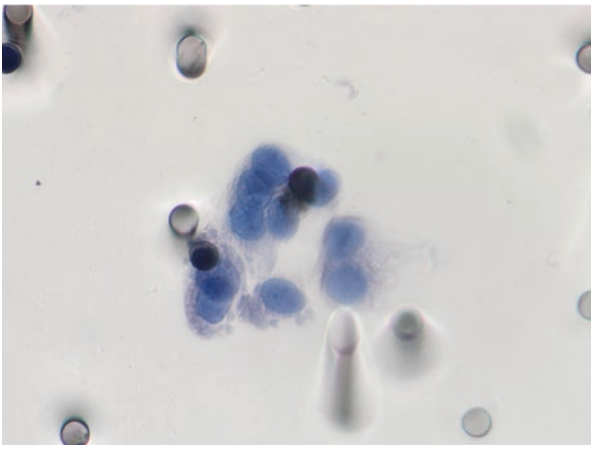

Fig. 8.22 CTC isolated from blood, by ISET. Woman, 27 years old, with synovial sarcoma. At the time of blood collection, before treatment with epirrubicin and ifosfamide, she showed 1.0 CTC/ml. We can observe a group of neoplastic cells showing nuclear irregularity and three dimensionality. Microscope magnification: 40×

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