Relationship of Circulating Tumor Cells’ Detection to Serologic and Imaging Response in Germ Cell Tumors: a pilot study. Is it a new prognostic factor in a new Era?

Cristina Ligia Cebotaru¹, Rareș Buiga², Alina Alexandra Lazăr⁴, Adriana Pălciintar⁵, Nicolae Ghilezan¹,³

¹“Ion Chiricuță” Institute of Oncology, Dept Radiotherapy I Chemotherapy, Cluj-Napoca, Romania; ² “Ion Chiricuță” Institute of Oncology, Head Dept Anatomopathology, Cluj-Napoca, Romania; ³“Iuliu Hațieganu” UMPh Cluj-Napoca, Romania; ⁴“Niculae Stănciu” Heart Institute, Dept of Cardiology, Cluj-Napoca, Romania; ⁵“Ion Chiricuță” Institute of Oncology, Day Hospital Dept, Cluj-Napoca, Romania

**Purpose:** As treatment options are currently standardized for germ-cell tumors, and poor-prognosis patients still have a 50% chance to die of the disease, a blood marker with a prognostic and a predictive role could guide treatment. We tested the hypothesis that circulating tumor cells (CTCs) could predict clinical and serologic outcome in patients with germ-cell tumors with poor-prognosis features.

**Patients and Methods:** We prospectively enumerated CTCs in the peripheral blood in two patients with poor-prognosis germ-cell tumors at baseline, before starting chemotherapy, and after one cycle and at the end of chemotherapy, with correlation with serum markers decrease and radiological response. CTCs were measured using a density gradient centrifugation separation technique and an immucytochemistry technique.

**Results:** Enumeration of CTCs in the peripheral blood at baseline and after treatment correlated with serum markers decrease and radiologic response.

**Conclusions:** The number of CTCs before, during and at the end of the treatment correlates with serum markers decrease and radiologic response in poor-prognosis germ cell tumor patients. CTCs may provide prognostic information in addition to former prognostic scores in order to adjust the therapy.

**Key words:** Circulating tumor cells, Germ cell tumors, Poor prognosis group, Prognostic factors.

**Introduction**

Testicular cancer is one of the leading tumors among young men. Peak incidence rates occur in men age 25 to 39 years. With modern treatment 5-year survival rates exceeds 90%. The role of the histologic type and extent of disease in identifying groups for risk-adapted treatment decisions is well established. In a cancer that is so curable, it is important to identify any influence that confers an increased risk of specific mortality. Additional research is needed, enabling the development of additional prognostic factors. (1)

Studies have reported attempts to isolate, identify, and classify circulating tumor cells (CTC) from patients diagnosed with various urologic cancers. The detection method of circulating tumor cells has mainly been based on gene expression and phenotypic characteristics common to neoplastic epithelial cells. Recent studies used nested PCR techniques to detect circulating malignant cells from peripheral blood isolated from patients with germ cell testicular tumors of various stages and treatment regimens. (2)

Circulating tumor cells are rare malignant cells found in the peripheral blood that originate from the primary tumor or metastatic sites. New techniques have been developed to isolate and characterize these cells. CTC enumeration has been incorporated into different fields of oncology as a prognostic marker, a tool to monitor therapy response, and a method to understand basic tumor characteristics. Solid tumors diagnosed in an early stage are typically treated by local resection, with or without additional chemotherapy aimed at eliminating microscopic metastatic disease. These metastases are initiated by the invasion of tumor cells into the systemic circulation. Detection and characterization of metastatic or CTC may provide important prognostic and predictive information to guide and monitor the treatment. (3)
Concomitant with the rapidly growing number of treatment options for metastatic carcinomas is the need for biomarkers to guide and monitor their use. Tumor cells shed into the blood during metastasis may help meet this need by serving as generic biomarkers for a variety of carcinomas. Assessment of circulating tumor cells (CTC) has already been shown to provide prognostic information regarding progression free and overall survival in metastatic breast cancer (MBC), colorectal (MCRC), and prostate cancer (MPC). In addition to predicting outcomes and monitoring treatment, CTC has also been used to detect the presence of treatment targets. The use of CTC can provide real-time information assisting the selection of specific therapies or families of therapies, making CTC an invaluable tool in the practice of evidence-based personalized medicine. (4)

CTCs can be detected in blood from patients with metastatic and primary carcinomas: breast cancer, colorectal, prostate, lung. (5, 6, 7, 8, 9) The development of immunomagnetic techniques has permitted accurate enumerations of CTC at extremely low frequencies. (9)

Recent advances in technology now permit reproducible detection of circulating tumor cells from a blood test. The clinical utility of CTCs was standardized and reviewed in breast, colorectal and prostate cancer, using studies evaluating the correlation of CTC number with radiological outcome and survival. The exploitation of CTCs in cancer management is now extending beyond prognostication. Current studies observe the potential of CTCs as pharmacodynamics and predictive biomarkers and potentially their use in revealing drug resistance in real time. The opportunity to learn more about the biology of metastasis through CTC analysis is now being realized with the horizon of CTC-guided development of novel anticancer therapies. (10)

Patients and method

Study Design

This is a pilot study conducted prospectively in our institution in two germ cell high-risk patients to evaluate the feasibility of CTC isolation and enumeration and the potential role in correlating response in term of radiologic and serologic tumor markers decrease with CTC profile.

The analyses described in this article focus only on the two germ cell poor-prognosis male patients with newly diagnosed, measurable disease who were about to start their first line of systemic therapy. Both had Eastern Cooperative Oncology Group (ECOG) performance status 0-1 (1 for the first patient and 0 for patient number 2). The institutional review board approved the study protocol and the patients provided written informed consent.

Before the initiation of therapy, patients had imaging evaluation (including computed tomography scans of chest, abdomen and pelvis, chest X-ray), serum tumor markers and a baseline blood draw for isolation and enumeration of CTC. Blood specimens were collected every three weeks for reassessment of tumor markers (i.e. before every other cycle of chemotherapy) and for CTC assessments, at the beginning and at the end of four cycles of chemotherapy for the first patient, and before treatment and after one cycle of chemotherapy (i.e. three weeks) for the second one. Reassessment of disease status by the same modalities used at baseline, were conducted at 12 weeks (CT scan of thorax, abdomen and pelvis and serum tumor markers) and an additional cardiac ultrasound was performed at the end of the four cycles of chemotherapy for the first patient. Serum tumor markers levels (alpha-fetoprotein = AFP, betahuman choriogonadotrophin = b-HCG and lactic dehydrogenase = LDH) were measured at baseline and every three weeks (i.e. before every other cycle of chemotherapy).

Isolation and Enumeration of CTCs

Blood samples (from peripheral blood) were drawn into 7.5 mL EDTA Vacutainer tubes, to which a cell preservative was subsequently added. Erythrocyte separation was performed by osmotic lysis. Samples were first refrigerated, but the second samples were maintained at room temperature and were processed in the first six hours.

Protocol of red cells lysis

The protocol used for red blood cells lysis, altogether with preserving viable lymphocytes and circulating tumor cells is the following: We pelleted the blood cells by centrifuge at 300xg for 10 minutes and re-suspended it in 5 mL of ice with 0.75% ammonium chloride, on ice, for five minutes. We pelleted the cells, discarded the red supernatant, and re-suspended the cell pellet in normal medium. Virtually, all the red blood cells were gone and the lymphocytes and the circulating tumor cells were still viable. We repeated the process if red blood cells were still evident in the pellet and then washed the pellet.

For the enumeration of blood nucleate cells, we used the Burger-Turk camera and a buffer (PBS). The formula for nucleate cells calculator is the following:

Total number of Nucleate cell = N/n x 250 x 20, where: N = number of nucleate cells in at least three big quadrants; N = number of small quadrants where enumeration was made; 250 = correction factor for reposing leucocytes/mm3; 20 = correction factor for dilution (i.e. dilution 1:20)
**Enumeration of blood nucleate cells**

The definition of “Total number of blood nucleate cells” is currently the sum between the white blood cells number (leukocytes, i.e. normal elements of blood), the circulating tumor cells number and the number of epidermal cells which could often contaminate the blood sample while puncturing the skin. The epidermal epithelial cells and the CTCs may be easily seen, due to their large diameter, and making possible the differentiation between those large cells and the white blood cells.

The whole quantity of cells obtained in this suspension was gently centrifuged and stained by the immunocytochemistry technique. The CTCs expressed the cytokeratin AE1/AE3, allowing the diagnosis. Contaminating epidermal normal cells are described differently due to their cell and nucleus morphology.

**Isolation of Circulating Tumor Cells**

Blood samples were drawn into 7.5-mL EDTA Vacutainer tubes, to which a cell preservative was twice subsequently added, and then samples were centrifuged at the room temperature. Samples were maintained at the room temperature and processed within 72 hours after collection. After CTCs were identified by immunocytochemistry technique, these were enumerated and related to the sample volume (per 1 mL or per 7.5 mL).

**CTC definition**

Circulating tumor cells are those cells present in the blood that possess antigenic and/or genetic characteristics of a specific tumor type (11). Many different methodologies have been used for the detection of CTC with variable sensitivity and specificity. To qualify as a CTC, an object must be round or oval, have nucleus (as determined by positive 4`, 6-diamidino-2-phenylindole staining) contained within the cytoplasm (as determined by positive cytokeratin 8, 18, 19-phycoeritin staining), and lack expression of CD 45 (as determined by negative CD45-allophycocyanin staining). Results are always expressed as the number of cells per 7.5 mL of whole blood. Cell size ranges from 4 um to more than 30 um, and large heterogeneity in morphology is observed. (12)

**Results**

**Patient Characteristics**

The first patient, 28 years old, Caucasian, male, was confirmed by mediastinal biopsy to have a poor-risk prognosis, primary Yolk sac mediastinal tumor, stage IIIC, T4 (mediastinum, involving big vessels and the right atrium), N3 (mediastinal), S3 (AFP), M1b (bone metastases). A standard treatment of four cycles of BEP chemotherapy was started, with imaging, serum markers and initial CTC counts, followed by serum markers dosing every 21 days, before every other cycle. After four cycles of BEP, imaging, serum markers and CTC counts was reassessed.

The second patient, 22 years old, Caucasian, male, diagnosed on 02-FEB-2010 after a right inguinal orchidectomy with testicular choriocarcinoma, was staged as IIIC, poor-prognosis group, with pT2N0M1a (lung metastases) S3 (HCG). He was scheduled to receive four cycles of standard chemotherapy of BEP, with imaging (CT-scans and chest X-ray), serum markers and CTC counts before the first cycle of chemotherapy, than after 21 days, before the second BEP, serum markers, chest X-ray and CTC counts were reassessed, in order to demonstrate a correlation between CTC, imaging and serum markers.

**Relationship of CTC to Radiographic Imaging**

Patients were assessed for radiographic response before initiation of first cycle of chemotherapy, during the chemotherapy courses and at the end of the four standard first-line cycles of BEP, as per international guidelines for poor-prognosis germ cell patients.

For the first patient, initial thorax, abdominal and pelvic computed tomography (CT) scan, described on 12-OCT-2010, a large superior and middle mediastinal tumor of 13 cm x 9 cm, involving big vessels (right subclavicular artery, azygos vein, ascendant aorta, right pulmonary artery, right mammary artery, the sternum and anterior ribs), right lateral tracheal lymph nodes and right atrium involvement, with right parietal collateral circulation. There were multiple bone lesions involving the right iliac bone, left sacro-iliac joint and left acetabullum (Fig. 1-4).

The initial CTC number was 175,000/7.5 mL or 43,890/1 mL (Fig. 5-10, 27).

After four cycles of BEP, on 01-FEB-2011, the CT scan showed shrinkage of the mediastinal tumor to 7 cm x 6.4 cm with the involvement of the right atrium and superior cava vein (Fig. 11-14).

The heart ultrasound and the trans-esophageal ultrasound described a right atrium tumor of 2.8 cm x 2.2 cm, with the surface of 6.3 cm2 (Fig. 15-17).

After four cycles of BEP, on 03-FEB-2011, no CTC were found in the peripheral blood (Fig. 18, 27).

For the second patient, initial imaging made on 08-FEB-2011, described multiples disseminated lung metastases of 1-5 cm, without other lesions (Fig. 19-22).

The number of CTC was 16,500/7.5 mL, or 2,200/1mL (Fig. 23-25, 27).
After 21 days (one cycle of BEP), we performed a chest X-ray with stable lesions in the lungs, but no more circulating tumor cells (Fig. 26, 27).

**Relationship of CTC to Serologic Response**
As per initial staging procedures, patients were assessed for serum tumor markers and, additionally, for CTC.

The first patient, with a Yolk sac mediastinal primary tumor stage IIIC, had on 18-OCT-2010, the following serum tumor markers: AFP = 24,703 mg/mL, HCG=30.8 mU/mL, LDH=649 U/L, before initiation of chemotherapy. The level of CTC was 175,000/7.5 mL (or 43,890/1 mL).

The serum marker levels decreased after each 21-days cycle of chemotherapy, as follows: after the first cycle, on 19-NOV-2010, AFP was 7531 mg/mL, HCG=0.4 mU/mL, and LDH=497 U/mL; after cycle 2, on 10-DEC-2010, AFP= 672.9 mg/mL, HCG=5.2 mU/mL, and LDH= 440 U/mL; after the third cycle, on 13-JAN-2011, AFP level was 63.34 mg/mL, HCG<0.1 and LDH=340; and after four cycles of BEP, on 03-FEB-2011, AFP was 32.81, HCG<0.1 and LDH 524, probably due to G-CSF (Fig. 28, 30).

After four cycles of BEP, on 03-FEB-2011, the number of CTC was zero (Fig. 18, 27).

The first patient was considered as a positive marker, partial responder after the four standard BEP chemotherapy cycles.

For the second patient, we managed to measure CTC, imaging and serum markers levels were assessed initially, after the orchidectomy, before the first cycle of BEP, and, after 21 days, i.e., the first cycle of treatment. Before chemotherapy, on 08-FEB-2011, the level of CTC was 16,500/7.5 mL or 2,200/1mL, and the serum markers level was: before orchidectomy, on 02-FEB-2011: HCG= 190, 586 mU/mL, AFP= 1527 mg/mL, and LDH= 342 U/L. After orchidectomy, and before the first BEP, on 08-FEB-2011, HCG level was 116,931, AFP=350 and LDH=268 (Fig. 27-30)
Fig. 5: Patient 1: Group of CTCs before treatment, HE 40x: Group of sac yolk CTCs (primary mediastinal tumor) in the central 60 d left quadrant. We can see an abundant cytoplasm, amphophile, and with thin granules, and moderate pleomorphic nuclei, with variable shapes and dimensions. The large up-side cell is multinucleate and is fagocytising a neutrophil cell (emperipolesis). Along with the CTC, we can observe a lot of thrombocytes (with small dimensions), and neutrophils. We also can see in the right lower quadrant, one small lymphocite with very small amount of cytoplasm. Celulele tumorale sunt insotite de numeroase trombocite de mici dimensiuni si de PMN neutrofile.

Fig. 6: Patient 1: Small group of CTCs before treatment; HE 40x: In the left quadrant we can see two large cells, with a big amount of cytoplasm, amphophile, with granules. The up-side cell is trinucleated. The tumoral nuclei have various shapes and dimensions. In the right quadrant we can see two normal neutrophils and some quiescent thrombocytes.

Fig. 7: Patient 1: Two CTCs before treatment; col. Papanicolaou 40x: In the right quadrant we can see two large cells with large nuclei, with irregular shape and a much pale color than those from surrounding neutrophiles.

Fig. 8: Patient 1: One large isolated CTC before treatment; HE 40x: A large CTC with a large nucleus, with ovalar shape and nucleoli, pale cytoplasm surrounded by numerous plasmocytes, neutrophiles and some few lymphocytes.

Fig. 9: Patient 1: A false-positive normal epidermal epithelial cell, contaminating the blood sample due to the needle puncture; HE 40x: Although this normal squamous cell has large dimensions, we must see the small round nucleus and the perinucleated drops of keratohialin, making the difference with a CTC.

Fig. 10: Patient 1: Apoptotic megakaryocyte (before treatment); HE 40x: A physiological aspect, occasionally seen in the peripheral blood, and must not be confounded with a CTC. These normal cell has a large hyperchromatic nucleus.
Fig. 11: *Patient 1:* Imaging after four cycles of BEP (PR)

Fig. 12: *Patient 1:* Imaging after 4xBEP

Fig. 13: *Patient 1:* Imaging after 4xBEP

Fig. 14: *Patient 1:* Imaging assessment after 4xBEP

Fig. 15: *Patient 1: Cardiac ultrasound after chemotherapy*

Fig. 16: *Patient 1: Cardiac ultrasound assessment after chemotherapy*

Fig. 17: *Patient 1: Cardiac ultrasound assessment for right atrial involvement*

Fig. 18: *Patient 1: No more CTCs.* Thrombocytes and normal neutrophils in the peripheral blood after treatment. HE 40x
Fig. 19: Patient 2: Chest X-ray before first cycle of BEP

Fig. 20: Patient 2: Thorax CT scan before treatment

Fig. 21: Patient 2: Thorax CT scan before chemotherapy

Fig. 22: Patient 2: CT scan assessment before treatment

Fig. 23: A small isolated CTC, immunostained for citokeratin, surrounded by normal thrombocytes and neutrophils from the blood. AE1/AE3 staining, 40x. (Patient 2, before initiation of chemotherapy)

Fig. 24: False – positive cell. (artifact)
After the first cycle of BEP, on 28-FEB-2011, HCG was 1,419 mUI/mL, AFP= 16.12 and LDH=337. (Fig. 28-30).

After just one cycle of chemotherapy, no more circulating tumor cells could be detected in the peripheral blood (Fig. 27).

**Discussion**

Circulating tumor cells can be detected in the blood of many patients with different types of early or advanced cancer using antibody-based assays or molecular methods. In many studies the detection and quantification of CTC has been linked to unfavorable prognosis. CTC detection offers the opportunity for individualized risk assessment beyond that determined by TNM staging. CTCs are a heterogeneous population of cells with biological characteristics often different from those of their respective primary tumors cells. Pilot studies have shown that phenotyping of CTC could be used to predict response to targeted therapies. In the era of biological therapeutics, CTC characterization...
might become a valuable tool of refining prognosis and serve as a real-time tumor biopsy for individually tailored therapies (13).

Detection methods for CTC
Different markers have been used for the detection of CTC, based on their expression on epithelial cells (epithelial-specific markers). The cytokeratins (CKs) are intermediate filament keratins found in the cytoskeleton of epithelial cells and have been extensively used for this purpose (13).

We used an immunocytochemistry technique of staining with cytokeratin AE1/AE3.

Because CTCs are usually found at very low frequencies among the normal peripheral blood mononuclear cells (PBMCs), tumor cell enrichment techniques, including density gradient centrifugation (Ficoll-Hypaque separation), immunomagnetic or size filtration procedures are often used to enrich tumor cells before their detection (13).

Monoclonal antibodies directed against histogenic proteins and PCR-based molecular assays amplifying tissue-specific transcripts are two main approaches used for the detection of CTC. The molecular assays have generally been considered more sensitive, while immunocytochemistry has the advantage of allowing the morphological assessment of stained cells (13).

Recently, an automated immunomagnetic enrichment and staining system for CTCs (the CellSearch TM system) has been induced. This system performs automated immunomagnetic epithelial cell adhesion molecule (EpCAM)-based enrichment followed by CK staining of CTC in blood samples. Epithelial cells are distinguished from leukocytes using fluorescently labeled anti-leukocyte (CD45) and anti-epithelial (CK8, -18, -19)-specific monoclonal antibodies. This standardized method has been approved by the US Food and Drug Administration for in vitro enumeration of CTCs from blood samples of metastatic breast, colon and prostate cancer patients (14).

The AdnaTest Cancer Select/Detect is a new CTC detection system which uses a first step where cancer cells are enriched in vitro from cancer patients’ blood samples using magnetic bead-conjugated antibodies that are optimized for the specific cancer type. The isolated mRNA is transcribed into cDNA that can be amplified in a subsequent multiplex PCR, which analyses the tumor-associated gene expression of a variety of relevant tumor markers (15).

Another method for the detection of circulating epithelial tumor cells from whole unseparated blood uses laser scanning cytometry after staining with anti-EpCAM and anti-CD45 fluorescent antibodies (MAINTRAC TM) (16).

More recently, a microfluidic platform (‘CTC chip’) mediating the interaction of target CTCs with antibody EpCAM-coated microposts, under precisely controlled laminar flow conditions in whole blood, has been developed. Using this device, high numbers of CK-positive CTCs in nearly all tested patients with lung, prostate, pancreatic, breast and colon cancer, have been reported (17).

The EPISPOT assay is a completely different antibody-based approach, used to detect proteins released by CTCs. Only viable, protein-excreting cells are detected using this method (18).

The detection of CK19 mRNA-positive cells by real-time RT-PCR assay as surrogate for CTCs in women with early breast cancer has been developed and validated using the LightCycler TM system (19).

False-positive results
False positive results can be obtained using either nucleic acid-based or antibody-based assays. Contaminating genomic DNA during RNA extraction, illegitimate expression or stimulation of CTCs markers in normal leukocytes and the presence of CK19 pseudogenes, have been considered responsible for the false-positive results when using nucleic acid-based assays (20, 21). The specificity of the molecular methods may be increased by using quantitative RT-PCR, which can discriminate between the higher levels found in cancer patients and the low background expression of ‘normal’ cells by designing primers that do not amplify genomic DNA or pseudogenes (22). Similar limitations have been described using antibody-based techniques. Many of the antibodies designed for the epithelial cells, occasionally stain haematopoietic cells displaying illegitimate expression of CKs or mucin-1. Plasma cells can also be stained due to alkaline phosphatase reaction against the k and lambda light chains located onto the cell surface (23). Optimizing the antibodies and using the appropriate negative controls in staining experiments have been proposed to overcome these limitations. Very few studies have directly compared different methods of CTC detection and enumeration in the same blood samples and the lack of standardization technology hampers the implementation of CTC measurement in routine clinical practice.

Biological characteristics of CTCs
Several investigators have tried to phenotype individual CTCs. By using fluorescence in situ hybridization for assessing the HER2 gene status it was
shown that patients with HER2-negative primary breast tumors had acquired HER2 gene amplification in their CTCs during cancer progression (24). Also, with immunofluorescent microscopy it was demonstrated that individual CTCs presented enhanced expression of activated signaling kinases (phosphorylated focal adhesion kinase, phosphorylated phosphoinositide 3-kinase (PI3K) as well as HER2 (25). These activated signaling kinases may regulate various cellular functions including cell migration, thus supporting the presumption of the malignant and metastatic nature of CTCs. The neoplastic origin of CTCs has been confirmed in studies showing that most CTCs are aneuploid cells and present multiple chromosome aberrations (26).

Using microarray technology it was feasible to obtain global gene expression profiles from CTCs of metastatic cancer patients and to create a list of CTC-specific genes (27). Furthermore, using the CellPoint platform, epidermal growth factor receptor (EGFR) mutations were detected in CTCs from patients with lung cancer treated with gefitinib (28). This study provided proof of the principle for using a blood sample instead of tumor biopsy for serial monitoring of tumor cell genotypes during treatment. It seems that CTC profiling may be crucial for identifying new targets that could be used to eliminate minimal residual disease or to a less invasive and therefore more feasible real-time monitoring system to assess the evolution of genetic and phenotypic changes on tumor cells with potential prognostic and therapeutic implications.

The evaluating of biological characteristics of CTCs may reveal new therapeutic options. A discordance between HER2 expression in primary tumor and corresponding CTCs has been confirmed since HER2-positive CTCs were observed in HER2-negative primary tumors (29). This was exploited in a pilot study where a short course of trastuzumab eliminated chemotherapy - and hormone therapy- resistant CK19 mRNA-positive CTCs in a majority of patients with early and MBC irrespective of the HER2 status of the primary tumor (30). Therefore, it would be interesting to test whether therapy directed against the biological characteristics of CTCs rather than those of the primary tumor cells would lead to a better clinical outcome.

A very important question regarding the role of CTCs in the course of cancer is their heterogeneous biological behavior, which has serious prognostic and therapeutic implications. Various studies have confirmed that CTCs present significant and phenotypic heterogeneity (13). Moreover, we know from reported clinical trials that not all patients have detectable CTCs or actually experience disease relapse, and that some patients do relapse although they do not present detectable CTCs (13). Immunophenotyping analysis of CTCs from patients with breast cancer revealed that a proportion of CTCs present phosphorylated receptors such as EGFR, HER2, PI3K, Akt, pFAK and vascular endothelial growth factor (VEGF), which may confer proliferative and survival advantage (31, 32).

**Clinical relevance of CTCs**

**Correlation to radiological response**

For our patients, the radiologic response after four and one cycle of chemotherapy, was partial response and stable disease, respectively, but with dramatic decrease in CTC number to zero. This finding suggests that imaging used to tailor future treatment may not be any more such a good predictor for PFS and OS.

Several studies in colorectal (33), breast (12, 34, 35), and prostate cancer (36) showed a correlation between radiologic response, CTC number and PFS and OS. This supports the clinical utility of serial CTC enumeration in conjunction with standard radiographic imaging to improve the ability to accurately assess treatment benefit and to identify effective treatment regimens for individual patients with measurable disease.

The data suggest that after the first cycle of therapy, the clinician can determine which patients are showing less than an optimal response and who should therefore receive an alternative therapy (4).

Measuring CTC after the first cycle of therapy has been shown to be an effective means of predicting very early on treatment efficacy. Effective therapies-ones that result in the elimination of CTC-can prolong survival as it has been shown by the improvement in overall survival in patients with MBC, MCRC and MPC that converted from unfavorable to favorable CTC count. The benefit was seen in patients independent of the line of therapy, indicating that treatment can still be effective in patients that have failed previous therapy. Using this information represents a major change in the current treatment paradigm used by most oncologists in the management of those malignancies (4).

The current standard of care calls for routine assessments of patient’s clinical status at or about one month interval depending on the type of therapy. Imaging assessments are usually performed at some intermediate time point and at the end of a line of therapy. Most clinicians do not change the treatment until several cycles of drug have been administered. Usually, two to three cycles of chemotherapy are often needed before clinical benefit may be evident. Thus the advan-
tages associated with changing treatment at a significant earlier point must be demonstrated. Clinical studies that explore whether or not an early change of therapy based on unfavorable CTC indeed can improve survival are underway. A prospective study using CTC counts at the end of the first cycle of chemotherapy to randomize patients with unfavorable CTC onto a change of treatment arm or continuation of the same arm, in order to demonstrate a survival benefit in patients changing to another treatment after a single cycle (4).

CTC can also be used as predictive biomarkers, further characterizing those entities at the gene and protein level to detect the presence of specific drug related targets. This information can be then used to tailor treatment to meet the individual patient needs at that particular point in time. Early phase drug development studies looking at specific gene and protein markers on CTC such as HER2, c-Myc, EGFR and IGF-1R, have been successful and have led to the inclusion of CTC analyses. But a patient must through necessity have CTC present for the assessment to be made. In cases where few CTC are detected, the technology challenge is to assure that the events detected are indeed tumor cells (4).

Correlation to serum markers/chemotherapy response

The enumeration of CTCs was done before chemotherapy, at the end of four cycles of BEP for the first patient, with a dramatically decrease from 175,000/7.5 mL to zero, and, for the second patient, after just one cycle, a decrease from 16,500/7.5 mL to zero.

This decrease in CTC numbers correlated with the serum markers decrease, better than with radiologic response, and may be a future viable predictor factor of PFS and OS, as shown in several studies. In prospective studies assessing CTCs as a predictor and prognostic factor in breast (12, 34, 37), colorectal (33), and prostate cancer (36, 38), a prediction of PFS and OS before initiation of therapy, after the first cycle of therapy and at the first reassessment of disease status, a very significant correlation was evidenced between the number of CTCs (five or more in 7.5 mL of blood) and the patients’ outcome.

Data suggest that CTCs are a significant surrogate marker for treatment efficacy and prognosis, particularly in candidates for chemotherapy. Again, this technique might have a value in the identification of chemotherapy-resistant patients who could benefit from early treatment change and/or more investigational approaches.

To sum up, these data suggest that detection of CTCs in germ cell tumors may correlate with the prognosis at baseline and early in the treatment and may be more predictive than traditional measures of treatment efficacy (e.g. partial remission or stable disease by Response Evaluation Criteria in Solid Tumors Group criteria).

Future perspectives

The study of CTCs may provide new insight into the biology of cancer and the process of metastasis. In the future, CTC detection may become a valuable tool to refine prognosis in cancer patients. Circulating tumor cells phenotyping and profiling may serve as a real-time tumor biopsy for individualized targeted therapies. However, critical issues have to be addressed before CTCs could be used in daily clinical practice. Detection of CTCs should be standardized and validated across different laboratories in a multicenter trial setting. Future studies must demonstrate that using CTCs as a prognostic and/or predictive biomarker leads to improvement in clinical outcome of cancer patients.

There are several scenarios for which CTCs could have utility in germ cell tumors. The data presented suggest that CTCs may be used as a stratification factor in future advanced and localized disease treatment studies. The current list of prognostic factors is short and the prognostic system made in 1997, with further (not yet) validated attempts to create new prognostic models. Further studies should prospectively address whether modification of treatment based on unfavorable CTCs early in the course of treatment will result in improvement of PFS or OS, or, for the localized disease and favorable CTCs setting, fewer cycles of chemotherapy or just no chemotherapy to avoid unnecessary toxicity.

However, all those intriguing data make the story of circulating tumor cells truly fascinating.

Conclusion

Although this pilot study has demonstrated the ability to detect the presence of circulating tumor cells in patients with high-risk germ cell patients, and a correlation between the number of CTC and the serum markers levels all together with the radiologic response before and after chemotherapy, further studies are required to fully investigate this line of inquiry for possible prognostic values. We believe that this is a tentative, but important beginning, because patients who are newly diagnosed and about to start treatment will derive the most benefit from appropriate risk stratification.
and treatment planning. Furthermore, an early prediction of treatment efficacy could have an impact in their quality of life.

The study of CTC may provide new insight into the biology of cancer and the process of metastasis, and in the future, this may become a valuable tool to refine prognosis. However, detection of CTC should be standardized and validated in a multicenter trial, and future studies should demonstrate the hypothesis that improvement in clinical outcome of cancer patients is due to prognostic and predictive biomarker role of CTC.

In many studies including patients with different types of cancer, the detection of CTC in early or metastatic disease has been shown to correlate with unfavorable outcome. When CTC are present in patients with presumably localized tumors, they are thought to contribute to disease relapse, and therefore an adjuvant treatment might be required. In extended disease, CTC enumerating and monitoring is thought to correlate with tumor load and might predict response to therapy. The genetic and phenotypic profiling of CTC could be used to select the most effective targeted therapy. Finally, the study of CTC has the exciting potential of refining prognosis and individualizing treatment strategies.

Prospective randomized studies are required to evaluate the utility of assessing and monitoring circulating tumor cells and modify accordingly treatment strategies in order to improve the clinical outcome of cancer patients.

References:


Disclosure: Disclosure and Potential Conlicts of Interest.

Author contributions:

Conception and design: Cristina Ligia Cebotaru
Provision of study materials or patients: Cristina Ligia Cebotaru, Rares Buiga, Alexandra Alina Lazar, Adriana Placintar
Collection and assembly of data: Cristina Ligia Cebotaru, Rares Buiga
Data analysis and interpretation: Cristina Ligia Cebotaru, Rares Buiga
Manuscript writing: Cristina Ligia Cebotaru, Rares Buiga
Final approval of manuscript: Cristina Ligia Cebotaru, Rares Buiga, Nicolae Ghilezan