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CIRCULATING TUMOR CELLS IN GERM CELL TUMORS: ARE THOSE BIOMARKERS OF REAL PROGNOSTIC VALUE? A REVIEW

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Abstract

Analysis of circulating tumor cells from patients with different types of cancer is nowadays a fascinating new tool of research and their number is proven to be useful as a prognostic factor in metastatic breast, colon and prostate cancer patients. Studies are going beyond enumeration, exploring the circulating tumor cells to better understand the mechanisms of tumorigenesis, invasion and metastasis and their value for characterization, prognosis and tailoring of treatment. Few studies investigated the prognostic significance of circulating tumor cells in germ cell tumors. In this review, we examine the possible significance of the detection of circulating tumor cells in this setting.

Keywords: germ cell tumors, circulating tumor cells, biomarkers

Introduction

Germ cell tumors (GCTs) are frequent but highly curable cancers in young men. Long-term remissions are seen in about 90% of patients. As possible causes of the disease, genetic elements and the presence of isocromosome 12 have been described.

The vast majority of GTCs originate in the testicles with some extragonadal primary sites, such as retroperitoneal, mediastinal, pineal. GCTs are seminomas (40%), or non-seminomatous tumours (NSGCT, 40%), as well as "mixed" tumors (20%). The vast majority of tumors are mixed, while seminomas produce a uniform population of cells. NSGCT may contain structures as embryonal carcinoma, choriocarcinoma, yolk sac carcinoma or teratocarcinoma [1].

The tumor markers—a-fetoprotein (AFP), beta-human chorionic gonadotropin (β-HCG), and lactate dehydrogenase (LDH) - could be elevated in 80% of patients [2], but are within normal range in GCTs pure teratoma patients. Baseline evaluation comprises serum tumor markers, full histology report and computed tomography of the thorax, as well as the abdomen, and pelvis. There is a range of 10% to 20% and 30% of patients with stage I seminoma and nonseminoma testicular tumors respectively, with occult metastases, with a risk of 15-20% of relapse if surveillance method is used [3,4].

Elevated levels of AFP or HCG may be seen in about 80% of metastatic and 57% of stage I nonseminomatous tumors. Elevated AFP is usually due to the presence of yolk sac elements and may occur in all stages of disease [5].

Elevated HCG may be seen in both seminoma and NSGCT, with a sensitivity of 60% in metastatic NSGCT patients and of 20% in those with metastatic seminoma. It is found in patients with choriocarcinomatous components, and special round cells also found in pure seminomas [6].

Therefore, additional markers could improve staging methods and tailor the treatment.

Circulating tumor cells (CTCs) are described as malignant cells found in the peripheral blood, originating from primary or secondary sites of the tumor. Nowadays, there are several techniques developed to isolate and

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characterize the CTCs. CTC counts correlate with clinical outcome in several cancers: breast, prostate, colorectal, and lung cancer. The detection and characterization of tumor cells circulating in the peripheral blood have gained considerable attention over recent years. Research on the genotype and phenotype of disseminating cancer cells provides new insights into the biology of tumor cell dissemination in cancer patients and will open new ways for early detection of metastatic spread and its successful treatment. [7].

Efforts to improve the management of several cancers include finding better methods for the quantitative and qualitative analysis of CTCs. The detection and isolation of CTCs from the blood circulation can be difficult, due to the fact that they are few and due to the lack of reliable markers to identify these cells. Further biological and engineering-related research is required to improve the existing methods, i.e. finding more specific markers for CTCs as well as enhancing the sensitivity and functionality of current devices. [8].

Analysis of CTC subpopulationshighlights the importance of the epithelial to mesenchymal transition (EMT), a process that may be crucial for allowing tumors to invade into and grow at sites distant from the original tumor site. Similarly, the detection of CTCs expressing markers may also have important implications for treatment resistance. Genomic analysis of CTC might select novel therapeutic targets to combat treatment resistance. CTCs could become a valuable biomarker resource when tissue biopsies are unavailable. Cultures of patient-derived CTCs may allow for an evaluation of therapeutic strategies performed ex vivo and in real time [9]. This review article will focus on CTC detection and their use to date, then will explore the existing data concerning germ cell CTC subpopulations and their clinical relevance, genomic characterization, and avenues for future research.

Biology of CTCs

Tumor cell dissemination is an early event in tumorigenesis and is relevant for metastatic progression. These data have led to the introduction of disseminating tumor cells (DTCs) in international tumor classification systems. Significant technical advancements in immunological procedures and quantitative real-time PCR-based assays now allow DTCs to be identified and enumerated at frequencies of 1 per 106–107 nucleated blood or BM cells.

Sophisticated molecular techniques such as whole-genome analysis or gene expression profiling have been applied to obtain initial information on the molecular characteristics of DTCs. The current data indicate that most DTCs are dormant (non-proliferative) in situ. However, these cells are viable and can proliferate in cell culture in response to appropriate growth factors, such as the stem cell growth factors epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2). DTCs can express cancer stem cell profiles (such as CD44+/CD24– in breast cancer patients) and exhibit stem cell properties such as resistance to chemotherapy and long-term persistence in the bone marrow [10].

Their neoplastic origin was confirmed by demonstrating that almost all CTCs are aneuploid with multiple chromosomal aberrations [11]. CTCs are rare in healthy subjects as well as in patients with benign diseases, but have been isolated in metastatic carcinomas [12].

In an attempt to phenotype CTCs, investigators studied if activated signaling kinases might regulate cell migration. They used fluorescence in situ hybridization (FISH) to assess the status of HER2 gene and they found out those patients with HER2-negative breast cancer acquired HER2 gene amplification in CTCs if cancer progressed [13]. With immunofluorescent microscopy they demonstrated that individual CTCs showed hiperexpression of activated signaling kinases [phosphorylated phosphoinositide 3-kinase (PI3K)] and of HER2 [14].

With microarray technology investigators obtained CTCs global gene expression profiles from metastatic patients [15]. Using the Cell Point platform, mutations of the epidermal growth factor receptor (EGFR) were detected in CTCs isolated from lung cancer patients in treatment with gefitinib [16], showing that this method is feasible for using blood sample instead of tumor biopsy to monitor cell genotypes during treatment.

CTC profiling may be very important for identifying new targets and for eliminating minimal residual disease. This method is less invasive and feasible and this real-time monitoring system might give important prognostic and therapeutic solutions.

Biological characteristics of CTCs may offer new treatment options. In some studies investigators confirmed that there is a discordance in HER2 expression between primary breast tumors and CTCs: HER2-positive CTCs were isolated in HER2-negative primary tumors patients [17].

Regarding the role of CTCs in the course of cancer and their heterogeneous biological behavior, with serious prognostic and therapeutic implications, there are studies which confirmed the CTCs significant genetic and phenotypic heterogeneity [14,18]. It seems that not all patients who have detectable CTCs experience relapse, and that some patients relapse although they don`t have detectable CTCs [19,20,21,22,23]. Patients with breast cancer who had detectable CTCs following mastectomy had no relapse even after more than 20 years [24]. These observations raise challenging questions on the CTCs biology.

Methods of detection

There are several challenges associated with CTCs detection and characterization: these are rare cells and need enrichment before detection and capturing. CTCs heterogeneity may be often not feasible if the enrichment is

done on a preselected specific marker and, there might be a limitation in the blood volume to be analyzed. [25].

A review of the principles and techniques, as well as the trends seen in the development of these technologies can help researchers to recognize the potential improvements and alternative approaches.

The existing techniques for detection and isolation of CTCs have been classified as nucleic acid-based, physical properties-based and antibody-based methods. The review of the literature suggests that antibody-based methods, particularly in conjunction with a microfluidic lab-on-a-chip setting, offer the highest overall performance for detection and isolation of CTCs.

Enrichment techniques

CTCs are differentiated in the peripheral blood from normal cells due to their biologic properties. Challenges exist in identification of the CTCs and in differentiating them from hematologic and normal epithelial cells; that is why enrichment method can significantly improve the results. Enrichment methods are using specific characteristics of tumor cells: size, density, and protein expression. These methods are specific to patients with cancer, with none or rare cells in healthy subjects [12,26].

The most used methods for enrichment are: immunomagnetic techniques—MACS® Systems (Germany), RARE™ (Canada), AdnaTest (Germany), macroiron beads, magnetic beads, CellSearch® (Veridex) [27,28,29,30]. They separate tumor cells from other circulating cells using specific surface markers. Some use isolation of mononuclear cells with anti-CD45 antibody, and others use monoclonal antibody targeting epithelial markers of cancer cells [31]. These systems are more sensitive than those using density gradient [32]. Because there are not highly specific tumor antigens, some CTCs may be lost. False-negative results exist due to loss of tumor specific antigens by the CTCs.

CTC Identification

Techniques based on immunocytochemistry (ICC) in order to identify tumor-specific proteins or nucleic acid were used with the reverse-transcriptase polymerase chain reaction (RT-PCR) method [28,33]. The advantage of the first methods over the nucleic acid methods is the preservation of the cell, making possible molecular tests. ICC and RT-PCR differ significantly, and a comparison between results is difficult. Ring et al. [34] made a comparison between three techniques (two with ICC and one using RT-PCR) in metastatic breast cancer (MBC) patients and have reported significant different results in the prevalence of CTCs, and RT-PCR was the most sensitive. Same results were reported by Smith et al in MBC patients [35]. But the majority of clinical trials reported to date have used ICC methods because these techniques were more likely to identify intact cells.

In ICC techniques, monoclonal antibodies targeting epithelial antigens are used: cytokeratin (CK) and mammaglobin (breast cancer), carcinoembryonic antigen (CEA) and CK20 for colon cancer [33,36,37,38]. False-positive results were between 20%–60%, depending of what antibodies and staining techniques were used [38]. CTCs carrying epithelial markers can be identified easy enough, but there might be possible false-positive results due to nonmalignant cells expressing the same marker [27,39]. CTCs that have lost expression of epithelial surface markers during EMT are not captured [27].

Techniques using nucleic acids examine DNA/RNA changes which can be identified in tumor cells. RT-PCR is a very sensitive technique identifying minimal amounts of tumor-associated RNA, expressing an indirect evidence of CTCs in the blood. RT-PCR can give false-positive results recognizing some nonspecific gene expression from normal cells [40].

Automated methods which combine enumeration and identification techniques

The CellSearch® assay is a method approved by the U.S. FDA (Food and Drug Administration) for clinical use. For detection, 7.5 ml of blood is centrifuged, in order to separate the cells from the plasma and buffer layer. Then, CTCs are captured with ferrofluid covalently linked to an antibody against the surface epithelial cell adhesion molecule (EpCAM) and differentiated from leukocytes by labeling with a panel of monoclonal antibodies—pan-CK antibody (anti-CK8, anti-CK18, and anti-CK19 antibody), anti-CD45 antibody for WBCs, and nucleic acid dye (4′,6-diamidino-2-phenylindole) to detect intact cells. Then the sample is analyzed by an automated fluorescence detection system that identifies CTCs as nucleated cells expressing CK but not CD45 [41,42]. Allard et al. validated the system [12] analyzing more than 900 metastatic patients and it was demonstrated that the technique was accurate and highly reproducible, with a good correlation between laboratories [12,43], and with no difference in CTC number when samples were processed immediately after or after 72 hours of storage [43]. CTCs were found in 57% prostate cancer patients, 37% breast cancer patients, and 30% colorectal cancer (CRC) patients. CTCs in healthy volunteers and patients with benign diseases were rare [12]. The majority of clinical trials used a cutoff of more than three or five cells per 7.5 ml, but Goodman et al. [44] found a high predictive value for a cutoff of more than four cells per 7.5 ml in advanced prostate cancer. The optimal cutoff is still not known.

The microfluidic platform (CTC-Chip) isolate CTCs from the blood of common epithelial tumors patients [26,45]. This device transports peripheral blood through an array of microposts coated with anti-EpCAM, and Nagrath et al. [26] isolated CTCs in 99% of samples from metastatic lung, prostate, breast, pancreatic, or colorectal cancers patients, and in none in healthy volunteers. The method is able to sort cells directly from whole blood in a single step, with no need for other procedures (centrifugation, washing, incubation) and to perform additional molecular

and genetic tests.

A gold standard test is not yet available, many methods have not yet been validated, and data regarding their sensitivity and specificity are lacking.

Clinical applications in different neoplasias

We have analyzed results from several studies demonstrating the clinical relevance of CTCs detection in different tumor types.

The prognostic value of CTCs was shown in metastatic breast [46], prostate [47] and colon cancer [48]. An analysis on 2000 MBC patients, proved that the presence of CTCs detection was associated with worse outcome [49].

CTCs were evaluated as a surrogate of response to improve overall survival (OS) in a phase III trial in metastatic prostate cancer (MPC) and the authors demonstrated that CTCs and LDH, 12 weeks after the start of treatment was significantly associated with reduced OS. [50]

Metastatic breast cancer

The prevalence CTCs in MBC patients varies between 30%–50% depending on studies and detection methods [12,43,46,51,52,53]. Cristofanilli et al. [46] demonstrated that the number of CTCs before initiation of therapy was an independent predictor of progression-free survival (PFS) and overall survival (OS). The reduction of the number of CTCs 1 month after the start of the treatment was associated with longer PFS and OS [54]. Dawood et al. [51] confirmed the prognostic significance of CTCs in newly diagnosed MBC patients, and it was independent of the hormonal or human epidermal growth factor receptor (HER)-2/neu status and the location of metastases. Budd et al. [52] demonstrated that patients with evidence of progression on imaging scans and low CTC counts had a longer OS time than the others, suggesting that CTC adds prognostic information to imaging. CTCs might depend on the form of treatment: bevacizumab, combined with first-line chemotherapy modified the predictive value of CTCs, possibly due to impaired tumor-cell extravasation [55].

Early-Stage Breast Cancer

Ignatiadis et al. found CK19 mRNA positivity in the blood samples of 40% stage I–II breast cancer patients using RT-PCR [21]. It was associated with extensive nodal disease, higher rates of relapse and deaths due to cancer. DFS and OS were significantly shorter in the group with positive CK19 mRNA. They evaluated the expression of CK19, mammaglobin-A, and HER-2 mRNA in a similar group of patients [56] and found out that patients which expressed all three markers had a shorter DFS time than others. This suggests that RT-PCR method could be used for risk stratification early-stage breast cancer.

Evaluation of CTC in REMAGUS02 and in the 'GEPARQuattro' trial was an independent prognostic factor for shorter metastasis-free survival but did not correlate with pathologic complete response of the primary tumor [34,43,57].

Studies are ongoing in women with detectable CTCs to assess if vascular endothelial growth factor–directed therapy reduces relapses in women at high risk of recurrence [58].

Metastatic/Castration-Resistant Prostate Cancer

The CTCs in castration-resistant prostate cancer (CRPC) patients was found in 35%–65% of subjects [47,59,60,61,62,63]. Olmos et al. [62] evaluated CTCs before and after treatment and OS in CRPC patients: a high CTC level at baseline was associated with high-risk characteristics (elevated alkaline phosphatase, lower hemoglobin, elevated prostate-specific antigen (PSA), presence of bone involvement.

De Bono reported similar results: they prospectively studied more than 200 patients with CRPC and PSA ≥5 ng/ml at the start of a new therapy, assessing CTCs before treatment and monthly thereafter [47]. The stratification was in a favorable (CTCs <5/7.5 ml) or unfavorable (CTCs ≥5/7.5 ml) group. Patients in the unfavorable group CTC had a significantly shorter OS time than the others. Patients with an improvement in the CTC count after therapy had a longer OS time than the others. CTCs number during therapy was more predictive of clinical outcome than post-therapy changes in PSA at different time points.

In a Southwest Oncology Group (SWOG) trial, CTCs were enumerated at baseline and three weeks after the first dose of the treatment and a correlation with prognostic markers (PSA, alkaline phosphatase, bone pain, liver disease, hemoglobin) was done. Baseline CTC count was associated with subsequent PSA declines and with objective responses. The median survival for patients with less than 5 CTCs/7.5 mL of blood at baseline was better than for the others (26 v 13 months), and the baseline CTC count was strongly associated with survival (hazard ratio, 2.74; P< .001). A rising CTC count after a first dose of treatment was independently associated with worse outcomes, reflecting chemotherapy resistance [64].

Early Prostate Cancer

In a study conducted by Davis, [65] investigators studied if there is a correlation between CTCs and tumor volume, pathological stage, and Gleason score in localized prostate cancer, but they found no correlation. The same negative results was obtained using RT-PCR with primers specific to the PSA gene with respect to clinical stage, PSA, or Gleason score [66].

Metastatic Colorectal Cancer

Cohen et al. [67] isolated CTCs from the peripheral blood of metastatic CRC (mCRC) patients using an immunomagnetic technique. Another prospective study of more than 400 patients with a new line of therapy tested the hypothesis that the CTC count at baseline and on treatment is of prognostic significance [48,68]. There was a group of unfavorable (CTCs ≥3/7.5 ml blood) and one of favorable (CTCs< 3/7.5 ml blood) prognosis and it was demonstrated that it was a shorter PFS and OS in the unfavorable group

(9.4 versus 18.5 months; p< .0001). CTCs at baseline and during treatment were independent predictors of PFS and OS in mCRC patients.

In the CAIRO 2 trial, CTCs were prospectively collected from almost 500 patients, comparing patients with mCRC treated with capecitabine, oxaliplatin, and bevacizumab with or without cetuximab [69], and the results were very similar to those found by Cohen et al. [48]. There were significant differences in PFS and OS between patients with three or more CTCs versus those with less than three CTCs. Furthermore, the CTC value at any point during therapy was a better predictor of PFS and OS than site of disease, lactate dehydrogenase or treatment regimen.

In a meta-analysis the prognostic relevance of CRC-derived CTC: CTC detection is associated with poor recurrence free [HR = 3.24 (95%CI: 2.06-5.1)] and overall survival [HR = 2.28 (95%CI: 1.55-3.38)] [43]. In a prospective study including 200 patients it was demonstrated significantly higher CTC counts in the mesenteric venous blood compartment as compared to the central venous blood compartment. This finding strongly supports the theory of continuous CTC shedding from the primary tumor into the bloodstream as well as the theory of the liver acting as a filter for CTC, a putative reason for the liver as the most common site for CRC metastases [70].

Early-Stage Colorectal Cancer

Investigators used quantitative PCR for detection, CEA and CK20 transcripts in blood and peritoneal lavage for almost 40 patients referred to curative resection of CRC [71] and found out that patients with positive quantitative PCR had shorter DFS and OS times than the others. Bessa et al. [72] found no prognostic value of CTCs 24 hours after surgery detected by RT-PCR for CEA mRNA in 66 CRC patients.

In a study conducted by Bork a total of 287 patients with potentially curable CRC were enrolled, including 239 patients with UICC stage I–III. CTC were measured with the CellSearch system preoperatively and on postoperative days 3 and 7. CTCs were detected more frequently in patients with metastatic disease and it was significantly associated with worse overall survival in the non-metastatic group (UICC I–III), as well as in the complete cohort. On multivariate analysis CTCs were the strongest prognostic factor in non-metastatic patients. [73].

Evaluation of CTCs was investigated in many other cancers, including lung cancer, ovarian cancer, pancreatic cancer, melanoma, bladder cancer and others [74,75,76,77,78,79], but there are few studies in germ cell tumors (GCTs).

Circulating tumor cells in germ cell tumors

There are few data about the presence of CTCs in GCTs patients. Some studies assessed the presence of tumor-specific mRNA or whole cells in apheresis products of patients undergoing peripheral stem cell transplantation [80,81,82]. Investigators detected CTCs in the peripheral blood of GCTs by reverse transcriptase PCR (RT-PCR) using as tumor markers AFP and human chorionic gonadotropin–specific mRNA [83,84].

In the study conducted by Nastaly et al, the authors analyzed one hundred and forty-one patients with testicular GCTs and 2 with primary mediastinal GCTs, treated between 2011-2013. Investigators used a new assay with a label-free enrichment technique based on physical properties of tumor cells and including epithelial cell markers (keratins 8, 18, 19 and EpCAM) and germ cell markers (SALL4 andOCT3/4). For comparison, authors used the CellSearch system, this technique being the only assay approved by the U.S. Food and Drug Administration (FDA) for CTC detection, following the clinical studies in metastatic breast, prostate, and colon cancer patients [46,47,48].

Authors found out that almost 10% of 143 patients were positive for CTCs. The method of enrichement was by Ficoll density gradient centrifugation and the technique of detection was by staining with SALL4/keratins and/or OCT3/4/EpCAM. With the CellSearch system, fourteen (11.5%) of 122 patients were found positive. Irrespective of the method, CTCs were detected in 25 (17.5%) /143 patients.

CTCs were detected more frequently in patients with nonseminomatous tumors than in pure seminomas. Patients with higher percentages of yolk sac tumor and teratoma components within the primary tumor were more frequently positive for CTCs.

This study shows that CTCs were found in the peripheral blood in 18% germ cell tumors patients, using both systems: CellSearch and an assay using an enrichment method and a combination of immuno-cytochemical markers. The presence of CTCs is associated with a more aggressive histology, disseminated tumors, increased serum markers, and chemotherapy refractory relapsed disease. [85]

In another study, by establishing sensitive nested reverse transcription-PCRs for the detection of mRNA of a-fetoprotein (AFP) and b human chorionic gonadotropin (bhCG), authors investigated the presence of CTCs in the peripheral blood of 119 patients with germ-cell tumor. A total of 336 blood samples obtained before and during therapy were examined with regard to clinical applicability. The overall ratio of positive PCR results was 26.5% and was independent of the serum concentration of AFP and hCG/bhCG. No correlation of the positivity for AFP-mRNA to serumAFP level was found. In contrast, positive results in bhCG-PCR were twice as frequent in patients with elevated serum hCG/bhCG levels as in those with normal serum hCG/bhCG levels (P=0.012). To develop a valid correlation to tumor stage, tumor histology, and serum level of tumor markers, a subgroup of 36 patients was evaluated before definite therapy. The subgroup

revealed an overall ratio of 33.3% positive PCR results. The serum level of both of the markers did not correlate with the detection of corresponding mRNA in peripheral blood samples. However, positive bhCG-PCR results were found exclusively in patients with elevated serum hCG/bhCG (6 of 18 versus 0 of 18; P 0.019). Patients with stage IIC/III germ-cell tumor demonstrated nearly twice the frequency of positive PCR results as patients with stage I tumor [7 (41.2%) of 17 versus4 (23.5%) of 17] in this subgroup. With regard to histology, positive PCR results were found mostly in embryonal carcinoma. Based on the experiences in other tumor entities, the value of circulating tumor cells as a prognostic factor is worth being discussed [86]. Further clinical follow-up will be mandatory. In patients with clinical stage I, our data (2 of 7 NSGTs) may correspond to the known tumor recurrence rate in patients who undergo a watch-and-wait-strategy in this tumor stage. The authors findings of AFP- and/or bhCG-mRNA detection in the peripheral blood of germ-cell tumor patients strongly suggest the presence of circulating tumor cells. These findings are dependent on tumor stage and seem to be associated with tumor histology and serological data for hCG/bhCG. The detection of circulating germ-CTCs may have other implications in tumor management. Whether these tumor cells have the capacity to contribute to recurrence and metastasis remains to be determined. The significance of PCR for identifying patients with risk of recurrence needs further study, especially concerning the follow-up during chemotherapy, the watch-and-wait strategy in patients with stage I tumor, and the correlation with histological cell type [84].

In another study by Ruf, in order to select the most appropriate markers for CTCs detection, the expression of epithelial and germ cell markers was studied in 4 different TGCT cell lines (TCam-2/2102Ep/NCCIT/NT2) as well as in 12 histologically different testicular cancers. Peripheral and testicular vein blood samples from 73 and 12 patients, respectively, were collected and examined for CTCs. All samples were enriched for mononuclear cells using Ficoll density gradient centrifugation and CTCs were detected by alkaline phosphatase (AP) enzymatic activity and immunocytochemistry using anti-keratin, anti-EpCAM and anti-SALL4-antibodies. Additionally, peripheral blood samples were tested for the presence of CTCs by the automated CellSearch® system. Patients with >1 CTC/sample were classified as CTC-positive to compare results for testicular and peripheral blood, CTC yields were calculated as number of CTC per 1 ml of blood. Based on the previous analyses, double immunofluorescent staining for SALL4 and keratin, as well as analysis of AP activity were performed to detect CTCs. According to both detection systems the one based on selected markers and automated CellSearch®, 7 (58.3%) testicular vein and 13 (17.8%) peripheral blood samples showed presence of at least one CTC. The CTC number ranged from 5 to 10^8/ml and 0.13 to 2.18/ml of testicular vein and peripheral blood, respectively. CTC were detected in seminoma and non-seminoma, in clinically metastasized and non-metastasized stages.

This study is the first to demonstrate CTC detection in TGCT patients. The proposed detection systems seem to be either specific and also with high sensitivity for the identification of CTC [87].

Only 10% to 60% in nonseminomas, 10% to 40%, and 40% to 60% of patients, respectively, have elevated concentrations of tumor serum markers (AFP, hCG and LDH, respectively) at primary diagnosis. In this study, CTCs were significantly associated with elevated serum markers. High levels of tumor markers after orchiectomy are associated with worse outcome in metastasized nonseminomatous tumors. The association between elevated serum markers and the presence of CTCs is an indicator for the prognostic significance of CTCs, but CTCs were found also in 4 marker-negative patients. It suggests that the detection of CTCs could help minimize the diagnostic gap of conventional tumor markers. This is the first study investigating intra-operatively collected blood from the testicular vein of GCTs patients. In the testicular vein the deoxygenated blood from the testis is carried to the inferior vena cava and it might be the first path of hematogenous spread in GCTs and these results seem to support this hypothesis [85].

In a small pilot study, we prospectively enumerated CTCs at baseline, during treatment and at the end of chemotherapy in the peripheral blood of two high-risk GCT patients,. A correlation with serum markers and the radiological response was made. We used a density gradient centrifugation separation method and immunocytochemistry technique of staining with cytokeratin AE1/AE3. The CTCs enumeration correlated with serum marker decrease and radiologic response. CTCs might provide additional information to prognostic scores. [88].

Future perspectives

The study of CTCs is a new exciting tool of research for the biology of cancer cells and the metastatic process. CTC detection might be a tool for establishing prognosis in cancer patients. CTCs profiling may serve as a real-time tumor biopsy for individualized targeted therapies.

Further studies with a higher number of patients and longer follow-up periods are needed to evaluate the association of CTCs with outcome and in particular survival of patients with germ cell tumors. Molecular characterization of CTCs might help improve our knowledge about metastasis formation in germ cell tumors and may serve as a "liquid biopsy" assessing potential targets for therapy.

Critical issues have to be raised before CTCs could be used in the daily practice. Detection of CTCs should be standardized and validated across different laboratories. Future studies should demonstrate that using

CTCs as a prognostic and/or predictive biomarker leads to improvement in the outcome of cancer patients.

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