

Circulating tumor cells in urological cancers

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Abstract

Circulating tumor cells (CTC) represent a very small subpopulation of the cancer cells found in the bloodstream of patients in the metastatic phase of neoplastic disease. Due to the timeline of the disease, they are regarded as a negative prognostic marker. This study focused on determining CTC percentages; these values vary between different types of cancer. In addition to their diagnostic use, CTCs may also be used to treat the disease. Calculating CTC population size and analyzing their biology in patients in advanced stages of cancer may prove valuable in creating a molecular profile for the disease. This would strongly encourage diagnostics and enable personalized treatment. We here present an analysis of recent data on CTCs in urological cancers and their potential uses. (*Folia Histochemica et Cytophiologica* 2017, Vol. 55, No. 3, 107–113)

Key words: CTCs; circulating tumor cells; prostate cancer; kidney cancer; bladder cancer; cultivation; *in vitro*; gene expression

Introduction

Disseminated cancer cells that are shed from the primary lesion or from metastatic foci into the peripheral blood are referred to as circulating tumor cells (CTCs). They are mostly found in patients in advanced metastatic stages of the disease, though very rarely they are also observed in premetastatic phases [1, 2]. Nevertheless, their number depends mostly on the phase of the disease and the type and localization of the malignancy. The analysis of their presence and characteristics provides a great amount

of data that cannot be obtained in any alternate way. First of all, CTCs may indicate the existence of micrometastases, despite negative results in diagnostic imaging. Moreover, the analysis of CTC biology may be used to verify chemoresistance and to evaluate the differences in primary and secondary tumor focus response. Additionally, the presence of CTCs following primary radical treatment suggests the risk of cancer reoccurrence [3]. In view of above outlined features, the application of various methods of CTC testing based on blood filtration may have great clinical impact. There are numerous studies being conducted at any given time to discover reproducible and reliable diagnostic techniques that would enable cells to be obtained for precise analysis and to determine their biological character. The most important goal is to create a reliable and reproducible tool for personalized medicine. The present manuscript presents the state of the art in the use of CTCs as individualized biomarkers in urological cancers.

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Biology of circulating tumor cells

On account of the many different processes they undergo, the morphology and biology of CTCs is heterogeneous and very complex. Generally, they enter the bloodstream and move to a location characteristic of the tumor type, where they create a metastatic focus. In a dynamically proliferating tumor, formation of blood vessels occurs rapidly. CTCs in these areas find their way into the bloodstream [4]. However, they are detected only very rarely among the billions of other blood cells, even in patients with advanced stages of disease [5]. The use of CTCs in diagnostics seems to be hindered because their presence and number may not correlate with the severity of disease. Besides their scarcity in circulation, CTCs are often highly heterogeneous due to metastatic subclone development in the expression of extraordinary mutational profiles during the progression of cancer [6]. In order to cross blood vessels walls, remain invisible to elements of the immune and coagulation systems, and disperse throughout whole body, CTCs must be capable of changing their phenotype [7]. The set of changes that needs to take place is called the epithelial–mesenchymal transition (EMT) [8]. When cells undergoing this transition gain the opportunity to metastasize, they lose the determinants typical of epithelial cells and begin to express antigens characteristic of cells of mesenchymal origin [9, 10]. First of all, cells undergoing EMT cease or lower the expression of epithelial cell adhesion molecule (EpCAM) and of cytokeratins (CKs). EpCAM is a transmembrane glycoprotein primarily responsible for cell-to-cell adhesion, which is also involved in cell signaling, migration, proliferation, and differentiation. CKs are intracytoplasmic proteins, elements of the cytoskeleton which help epithelial cells resist mechanical stress. Both particles are tissue-specific markers, observed only in cells originating from epithelial tissue [11, 12]. Simultaneously, the upregulation of mesenchymal markers such as vimentin (VIM), as well as markers of the EMT process, such as twist-related protein (TWIST), is observed. VIM is the major cytoskeletal component of mesenchymal cells, forming intermediate filaments. It plays a significant role in supporting and anchoring the position of organelles in the cytoplasm. TWIST works as a transcription factor, and is one of the hallmarks of EMT. Its activation upregulates N-cadherin and downregulates E-cadherin, as well as induces angiogenesis, extravasation, and chromosomal instability [11, 12].

The exceptional heterogeneity of CTCs is clearly visible in advanced breast cancer [13]. Due to their individual cancer-associated marker expression and tumor-seeding potential originating from their phe-

notypic characteristics, primary and secondary foci may require different handling. Nowadays, the golden standard in breast cancer diagnostics involves only sampling the primary tumor. In view of the above, CTCs may survive endocrine treatment in patients diagnosed as estrogen receptor (ER)-positive, because CTCs often lack ER expression [13, 14]. In male tumors, decreased therapy effectiveness as a result of CTC presence has also been shown. In prostate cancer, CTCs expressing mutated androgen receptor (AR) and linking variants, such as AR-V7, present antiandrogenic therapy resistance [15, 16].

Detection of circulating tumor cells

There are two main challenges to the detection of CTCs: their rare occurrence in the blood and difficulties in identifying the appropriate cells for further isolation [15, 17]. To comprehend this extremely small quantity of cells, we need to realize that a 7.5 mL blood sample obtained from a patient with a solid malignancy contains approximately 1 CTC alongside 10 million white blood cells. Direct identification is based on a combination of negative and positive immunological selection of nonepithelial and epithelial cells, respectively. On the other hand, indirect detection mainly involves the analysis of epithelial-specific mRNA transcripts by RT-PCR. Most existing methods of CTC capture are based on immunoaffinity to EpCAM, which is generally overexpressed in various cancers; it is present in 97% cases of colon cancers, though only 41.7% of patients with primary malignant breast cancer show increased EpCAM expression [18, 19]. On the other hand, EpCAM expression appears to be downregulated in CTCs, and the relationship between EpCAM expression at the primary breast cancer focus and in CTCs is unclear [20]. The occurrence of the EMT process with all its features may be a reason for the high rate of false positives in CTC detection based on EpCAM enrichment techniques. The ideal method of CTC detection would be isolation based on cell size, meaning that tumor cells would be captured in accordance with their size from different segments of the peripheral blood. An additional benefit would be the possibility of *in vitro* cultivation, which would enable differentiation between malignant and benign epithelial cells [21–25].

Use of circulating tumor cells

The term *biomarker* encompasses a broad group of determinants, such as clinical, laboratory, genetic, molecular and imaging-based approaches [26]. They are primarily employed in diagnostics to differentiate healthy specimens from pathological specimens, mostly in doubtful cases. They give additional data about

the advancement of the disease and about survival rates. Moreover, treatment efficacy and recurrence risk may be monitored through marker analysis. The specificity of CTCs as biomarkers is that they represent the individual tumor cells of individual patients and reflect the heterogeneity of possible metastatic sites.

Prostate cancer

The selection of the most suitable therapy for prostate cancer is currently based on the Gleason score (histopathological analysis) and the serum level of prostate-specific antigen (PSA). Unfortunately, the low specificity of PSA is well known. It can be similarly increased in benign hyperplasia, indolent lesions of epithelial origin (IDLE), and even aggressive lesions [27]. In addition, it is inappropriate for therapy monitoring, due to its low accuracy in treatment response verification.

Nowadays, CTCs are generally accepted as prognostic biomarkers in prostate cancer. The prognostic value of the baseline CTC level has been evaluated in numerous studies in which patients were treated with chemotherapy and androgen receptor (AR) signaling inhibitors [28–33]. Various studies have shown an association between baseline CTCs levels and clinical outcomes in metastatic patients [28, 29, 32, 34]. Decreased levels of CTCs in the bloodstream of patients after therapy correlated with longer overall survival (OS), which was subsequently observed as decreased PSA level and radiographic response [28, 35]. Most importantly, alterations in CTCs levels predate PSA changes, suggesting the usefulness and high accuracy of method for monitoring cancer treatment [29].

Numerous prospective clinical trials have assessed presence of CTCs as an intermediate end point. SWOG S0421 was a phase III double-blind, randomized placebo-controlled trial evaluating patients with metastatic castration-resistant prostate cancer (mCRPC) starting first-line docetaxel chemotherapy with or without atrasentan [30]. Atrasentan is an endothelin receptor antagonist that blocks endothelin-induced cell proliferation. CTCs were found to provide additional discriminatory value (independent prognostic marker) over the PSA level and other factors. Atrasentan had no influence on OS [30].

COU-AA-301 was a phase III double-blind randomized placebo-controlled trial evaluating whether CellSearch-based CTCs enumeration could be used as a surrogate efficacy-response biomarker of OS [32]. Patients with mCRPC who had previously been treated with docetaxel received abiraterone with prednisone versus prednisone alone. Abiraterone is a steroidal CYP17A1 inhibitor and, by extension,

an androgen synthesis inhibitor. Baseline favorable versus unfavorable CTCs counts measured on the CellSearch platform were associated with better OS (26 vs. 13 months, HR = 2.74, $p = 0.001$). Any increase in CTCs count after one cycle of docetaxel treatment was significantly associated with worse OS, whereas a decrease in CTCs counts pointed to an insignificant trend towards improved OS [36]. These data suggest that the detection of rising CTCs counts during docetaxel chemotherapy may be used for clinical verification of the therapy. Moreover, 1195 men with mCRPC who had previously received docetaxel benefited from abiraterone acetate treatment [37]. Thus, CTCs enumeration could be used as a surrogate efficacy-response biomarker of OS [32].

A study analyzing levels of PSA, LDH (lactate dehydrogenase), and CTCs in patients treated with abiraterone showed interesting relationships. Abiraterone treatment (HR = 0.70, $p < 0.0001$), baseline LDH concentration (HR = 2.98, $p < 0.0001$), and CTC count (HR = 1.19, $p < 0.0001$) were prognostic for survival, while PSA level was not (HR = 1.04, $p = 0.1797$) [38]. A “CTCs biomarker panel”, composed of CTCs count and LDH serum activity, categorized subjects as low risk (CTCs count ≤ 4 cells per 7.5 mL of blood, any LDH), intermediate risk (CTCs count ≥ 5 , LDH ≤ 250 U/L), and high risk (CTCs count ≥ 5 , LDH > 250 U/L). The CTCs biomarker panel discriminated survival time and satisfied the four Prentice criteria for surrogacy [38], unlike the CTCs count or LDH as individual variables [32]. These prospective phase III data from SWOG S0421 and COU-AA-301 trials are encouraging and require validation by ongoing, independent phase III clinical trials.

The majority of mCRPC patients are diagnosed in the bone involvement phase. In this case, the Response Evaluation Criteria in Solid Tumors cannot be applied for interpretation. New treatment response biomarkers are thus acutely needed for CRPC patients. The Prostate Cancer Working Group 2 criteria rely on bone scintigraphy and changes in the PSA levels to evaluate response to treatment in these patients [39]. However, progression according to bone scintigraphy is not evaluable before 16 weeks of treatment, and most studies evaluating decrease in PSA levels as a surrogate of survival have yielded negative results; treatment based solely according to PSA values is not recommended [39–42].

Verification of radiological progression free survival (PFS) cannot currently be acquired before at least 12–16 weeks of treatment, and is difficult in the evaluation of widespread bone involvement [43]. An additional *post hoc* analysis of data for patients

in prospective IMMC-38 (chemotherapy) and COU-AA-301 (abiraterone) trials with baseline CTCs ≥ 5 cells per 7.5 mL was performed in 2016, and evaluated the value of a 30% CTCs decline from baseline at 4, 8, and 12 weeks of treatment [33]. The OS in patients with mCRCP after abiraterone and chemotherapy is associated with a 30% decrease in CTC level from the original number of ≥ 5 cells per 7.5 mL. Further prospective studies are needed to evaluate this promising surrogate.

Urothelial cancer

There is still lack of diagnostic biomarkers with satisfactory prognostic and predictive value in urothelial cancers. Besides, all the tests analyze urine samples, and so cannot be used in extravesical tumor diagnostics. The greatest advantages of serum markers are their minimal invasiveness and their possible use in the monitoring of treatment effectiveness, mostly in comparison to surgical biopsies of metastatic sites. There are studies outlining the use of CTCs in superficial and invasive urothelial cancers [44, 45].

It has been shown that CTC-positive patients who have undergone radical cystectomy had a higher risk of recurrence and reduced OS [46, 47]. Moreover, CTC positivity was found to be an isolated risk factor of reduced OS [47, 48]. Alarmingly, the presence of a single CTC in a patient's bloodstream was correlated with reduced survival in patients with urothelial carcinoma of the bladder [46]. Apart from research that examined the quantity of CTCs, there are also studies on their quality. Osman *et al.* compared CTCs with and without *uroplakin/EGFR* mRNAs in patients who had undergone radical cystectomy [49]. The first group (*uroplakin/EGFR+*) had a higher risk of recurrence. Gudemann *et al.* analyzed the presence of CKs in CTCs, revealing cancer development potential in CTCs that expressed CK20, which was also associated with clinical stage/disease burden [50].

Renal cancer

A small number of studies have been published on the detection of CTCs in metastatic renal cell carcinoma (mRCC). An association was found between the presence of CTCs, lymph node involvement, and the presence of metastases at the time of diagnosis in mRCC [51]. Studies have also shown that the presence and quantity of mesenchymal and stem-cell-like CTCs is associated with poor treatment response. The presence of stem-cell-like CD133+ cells or mesenchymal N-cadherin+/CK- cells correlates with shortened PFS.

Moreover, in cases with detectable CD133+ cells, N-cadherin+/CK- cells were often found [52].

Molecular characterization of circulating tumor cells

The ability to detect CTCs in patients with various diseases has given clinicians the opportunity to improve the diagnostic screening and monitoring of the therapy employed. Moreover, to determine the full informative potential of CTCs, the laboratory tests include DNA, RNA, and protein analysis. Pooled CTCs analyses offer the potential to assess the dominant circulatory clone at a given point in time in a specific patient, and allow the tracking of clonal selections during systemic therapies. The evolution of the molecular development of circulating cells from the primary or metastatic sites could be reproduced by personalized CTCs profiling, which could lead to the ability to detect uncommon resistant clones. For example, AR has recently been a deeply studied area in prostate cancer, also in view of CTCs. The level of expression of complete AR or its splice variants correlates directly with clinical outcome and response [16, 53]. It has been shown that patients with the expression of AR-V7 (the most frequent variant) present abiraterone and enzalutamide resistance [16]. The clinical importance of circulating AR-V7 as a marker of resistance to inhibition of the androgen/androgen receptor axis has been supported by whole-blood PCR assays detecting *AR-V7* mRNA without prior CTC enrichment [54]. Similarly, Anonarakis *et al.* studied patients with overexpressed AR-V7 who were resistant to abiraterone treatment, showing no influence on PSA level [16]. The detection of AR-V7 in mCRPC patients may lead to a reduction in ineffective abiraterone and enzalutamide treatment, which would be healthful for patients and cost-beneficial to healthcare providers. AR-V7 testing in clinical phase is presently ongoing [54]. However, Bernemann *et al.* have indicated that patients with mCRPC and positive AR-V7 CTCs should not be excluded from either next generation androgen deprivation therapy or abiraterone or enzalutamide treatment as long as no appropriate alternative therapy has been introduced [55].

In prostate cancer patients, examination of CTCs revealed mutations in the erythroblastosis virus 26 oncogene homolog gene (*ERG*). This leads to the synthesis of an *ERG* oncogene with the AR-driven *TMPRSS2* promoter and has been identified in more than 50% of hormone-sensitive prostate cancers with preserved further tumor progression [56, 57]. Recognizing this as a potential predictive factor, patients

with specific ERG mutations can be expected to be more sensitive to abiraterone therapy [58].

The routinely preformed analysis of proliferation marker Ki67 seems to also have diagnostic potential in prostate cancer. Its expression in CTCs positively correlates with expression levels and nuclear localization of ARs, and likewise with the stage of advancement of prostate cancer [59]. Patients with positive Ki-67 CTCs during therapy are theoretically resistant to treatment, and therefore Ki-67 has the potential to be used as marker of therapy effectiveness.

Conclusion

In summary, it is essential to undertake larger and more detailed analyses of the molecular characteristics of CTCs in urological tumors. Understanding the biology of CTCs will provide a new quality of diagnostic and therapeutic strategies, which will enable effective personalized treatment and prevention of metastatic process.

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