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Typical and Atypical Circulating Tumour Cells in Bladder Cancer. Why Improve our Knowledge?

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Abstract

Liquid biopsy is the new frontier in cancer biology: easy to perform at every stage of disease course, low invasive, and not painful, it looks amazing. In this field can recognize two main characters: circulating tumour cells (CTCs) and circulating tumor DNA (ctDNA). They can be considered as a patient's individual tumour fingerprint, promising an easiest way to follow cancer progression. Taken together they all seem bright and sparkle, in reality they find low application in clinics, especially in bladder cancer where only the CTC count has been correlated to clinical outcomes. Since stage bladder cancer is difficult to manage due to chemotherapy resistance, recurrences and few therapeutics protocols, we need to go over simply count of CTCs, and use them to improve the patient's management therapy and find potential new biomarkers for innovative pharmacological treatments. Here we provide an overview about the potential employment of CTCs in clinics highlighting the relevance of non-clinically detectable CTCs, named "atypical CTCs", which may be responsible for recurrences and chemotherapy resistance.

Liquid biopsies in bladder cancer, why so important?

The first time the presence of CTCs has been reported was in 1869, 150 years ago, and now they are considered the newest frontier in cancer biology. CTCs are cells originated from either a primary or a metastatic site, which could develop metastatic foci in a distant area or district. To achieve this, they must both survive to sheer stresses and escape to the host immune system, and then they have to extravasate and adapt themselves to a new local microenvironment. Liquid biopsy relying on analysis of such cells, together with ctDNA, has increased hopes to finally decrypt the metastatic process. So both CTCs and ctDNA could play a pivotal role in precision and personalized medicine, providing important information as cancer biomarkers, prognostic data and for therapy design [1,2].

Table 1: Tests approved by FDA for detection of bladder cancer [6,7].

Test	Protein	Assay type	Sensitivity	Specificity
BTA stat	Complement factor H – related protein and complement factor H	Immunoassay or point-of-care	57 – 83%	60-92%
BTA TRAK	Complement factor H – related protein and complement factor H	Sandwich ELISA	66%	65%
NMP22 Bladder Chek	Nuclear mitotic apparatus protein	Sandwich ELISA or point-of-care	47-100%	69-79%
uCyt+	Carcinoembryonic antigen, two bladder tumorcell-associated mucins	Immunocytochemistry	50-100%	69-79%
UroVision	Alterations in chromosomes 3, 7, 17, and 9p21	FISH	41-70%	80%

Bladder cancer is the most common malignancy of the urinary tract and the ninth most common cancer worldwide, and in advance stages is difficult to treat and manage because of its high recurrence rates, rapid progression, poor response to chemotherapy, and lack of novel targeted therapeutics [3-5].

Diagnostic protocol for bladder cancer begins with cystoscopy and computed tomography (CT) images. The first is considered the golden standard radical treatment, whereas the latter one is recommended as surveillance method. Indeed, 50% of recurrence cases will occur within 5 years, possible answers can be found in undetected micro metastatic diseases after cystectomy. Although unsettling, it is tempting to hypothesize that micrometastatic disease is responsible for recurrences, so their implementation in surveillance can be more effective than CT imaging alone, optimizing patient's management. Scientists are now involved in urinary biomarkers research in order to strengthen standard bladder cancer detection protocols, however, few of them are used in clinics and have received Food and Drug Administration (FDA) approval (Table 1) [6-8,10]. The same for blood-based tests, or "liquid biopsies", for micrometastasis or metastasis detection in post-cystectomy bladder cancer, where they could provide important evidences and information about possible residual disease, before conventional image detection, and even more sensitive and precise than them.

Even if there are still no valid demonstrations in clinical practices, implementing bladder cancer with novel liquid biopsies analysis can improve patient's life condition and expectancy. Unfortunately, CTCs applied to bladder cancer reported conflicting results [3,11-16]. CTCs have been detected

in patients with localized muscle invasive bladder cancer, but also in patients with non-muscle invasive bladder cancer [12-14]. No association between CTC count and extravesical disease has been demonstrated [12]. However, there are findings regarding CTCs and bladder cancers demonstrating that the presence of CTCs is correlated with advance tumour stage, histological grade, metastasis and regional lymph node metastasis [3,11-14,17-23]. The presence of even only one CTC in cancer patient's blood sample is correlated with low survival and worse prognosis [13-15].

Most of these data are provided by using the CellSearch[®] technology (Janssen Diagnostics). CellSearch[®] is, until now, the only CTC assay approved by the FDA for breast [24], prostate [25] and colorectal cancers [26]. Mainly, CellSearch[®] relies on immune-magnetic positive selection of cancer cells using antibodies against EpCAM antigen. As CellSearch[®] there are other tests as AdnaTest (AdnaGen AG) [27], which works in the same way as CellSearch[®] with further RNA evaluation. These assays allow detection and the analysis of only EpCAM positive cells. EpCAM protein is strongly expressed in epithelia and most carcinomas, thus it is involved in several cellular processes such as proliferation, migration and differentiation [27-30]. In addition to EpCAM expression CTCs are also cytokeratin (CK) positive and CD45 negative responding to "CTC-criteria" [31]. Nevertheless, there are several evidences coming not only from urogenital cancers field, but also from breast, lung, esophageal and colorectal cancers, which have demonstrated that there are other "atypical" CTCs which express heterogenous markers panel on their surface and could be correlated with worse outcomes (Table 2) [16,32-42]. Not all metastatic cells express EpCAM resulting in potential false negative, decreasing CellSearch[®] predictive value. Chalfin

Table 2: Atypical CTC markers.

Marker	Cancer type	Clinical impact	References
CK+, CD45+, EpCAM+/-	Breast cancer	Prognostic and predictive value for OS	[32,33]
	Pancreatic cancer	Correlated with advance stages of the disease	
CK+, EpCAM+, CD14+, CD11c+, CD45+/-	Breast	Increased risk of disease progression, number affected by treatment	[34–36]
	Pancreatic		
	Prostate		
CD14+, CD68+, CK+, EpCAM+, CD163+, CD204/6+	Melanoma	Not determined	[37]
CSV+, CD14+, CD68+, CD45-	Sarcoma (including gastrointestinal stromal tumors)	Predict metastasis	[38]
CD14+, CD68+, CD163+	Colon	Not determined	[39]
	Breast		
	Ovarian		
	Colorectal cancer		
CD133+, N-cadherin+, CK-	Renal	Shortened progression free survival	[40]
CK +/-, PD-L 1	Bladder	Decrease OS	[16]

and co-workers demonstrated the presence of EpCAM negative CTCs in patients with muscle invasive, non-muscle invasive and metastatic bladder cancer [43]. Also, CTCs negative for CK have been detected in patients with muscle-invasive and metastatic bladder cancer [44].

So, what about the negative or “atypical” circulating cell population?

In breast cancer, evidences show that EpCAM and CD45 negative cells are associated with significantly decreased overall survival (OS) [32], EpCAM negative and CK/CD45 positive cells are suggested to be correlated to tumour-associated macrophages with worse outcome [32]. Taken together, many experimental sets and clinical investigations have described the presence of EpCAM-negative and undetectable CTCs, but few were able to address their presence with clinical relevance. Moreover, several findings have demonstrated that CTC clusters have increased metastatic potential compared to a single cell, with shortened survival [41,45,46]. This may be correlated with epithelial-to-mesenchymal transition (EMT), in which the cell loses epithelial characteristics gaining mesenchymal and invasive features. EMT is assumed not only to increase tumour invasion, but also it contributes directly to therapy resistance, enable cell to escape from death acquiring stem cell capabilities and increase the aggressiveness of the tumour [41]. In addition to that, it could be useful in some cases the molecular marker expression analysis using digital droplet PCR (ddPCR) and next generation sequencing (NGS) in order to better classify CTCs and distinguish them from tumour-associated hematopoietic cells, as cancer associated macrophages-like cells or tumour-associated neutrophils [34,47,48].

In order to detect these “atypical” CTCs, it is required an improvement in clinical and laboratory techniques aimed to improve the sensitivity of the method, enriching CTC pools allowing the detection of possible useful new biomarkers through molecular biology assays by genetic, epigenetic and transcriptomic approaches.

There are many available techniques, which provide the isolation of all CTCs in patient’s blood, independently on their marker expression. Today the scene is currently dominated by the density-based gradient tools as the FicollParque[®] and the novel OncoQuick[®]. This could overcome heterogenous marker expression problems, but findings demonstrated that there is effectively cell loss in the blood sample, maybe because of differential cell loss in CTCs densities. Other label-free separation technologies are based on the biophysical properties of CTCs such as deformability and electrical properties (Parsortix system, ANGLE). Therefore, there are several CTCs assays, which allow studying from a different point of view these cells making possible future CTC applications in clinical medicine. Improvement in CTCs approaches could be useful to better classify and stratify patients and to divide them for pharmacological treat which may have more neoadjuvant chemotherapy benefit respect systemic chemotherapy treatment guiding for individual targeted therapies [3,17,49,50].

Conclusion

Since CTCs are detected in 50% of metastatic urothelial cancer, CTCs approaches has to be implemented with possible further prognostic role in recurrences detection, promising also preclinical, clinical, pharmaceutical and real-time surveillance data [12,13,19,41,51,52]. There is the need to determine an appropriate study designs within clinical evaluations of CTCs. However, an interventional study regarding CTCs, the “gold standard” for evaluating a new discovery and its causal impact on an outcome, as the randomized controlled trials, has some disadvantages. Among them, the applicability of the study results to real-world situations may be limited by the study population characteristics, procedures implemented, outcomes measured and above all the time needed to arrive at an answer [53]. Undoubtedly it can be useful to include liquid biopsies in bladder cancer patients’ management alongside standard diagnostic protocols. There is the necessity to improve the research and detection of typical CTCs, mesenchymal-like CTCs and in general atypical CTCs, which could have lost some epithelial cell features. Addition of extra markers specific for CTCs can aid to discriminate tumor cells from other events and creates a narrower definition, which will decrease the intra-reader variation and improve the identification of true CTCs. Indeed, as highlighted by Tibbe et al., when researchers exploit the number of CTCs analyzing the standard volume of liquid biopsy (7.5 ml) to stratify patients, the Poisson distribution has to be applied and the probability of over/underestimation is very high [54]. For all these reasons CTCs’ enumeration itself should be avoided giving way to a new perspective. After detection CTCs can be further analyzed at the DNA, RNA and protein level to obtain global information on tumor biology and targets relevant to cancer therapy [54].

In conclusion, the analysis of CTCs complementary to other liquid biopsy biomarkers such as ctDNA or exosomes has the potential to improve the management of individual cancer patients and contribute to the vision of personalized medicine decreasing healthcare cost, ameliorating patient management and therapeutic design care.

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