**Circulating cancer cells**

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Circulating tumour cells (CTCs) 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 CTCs has been linked to unfavourable prognosis. CTC detection offers the opportunity for individualized risk assessment beyond that determined by TNM staging. However, discordant results have been reported when different methodologies for CTC detection were used. Therefore, well-standardized detection methods cross-validated between different laboratories are still needed. CTCs are a heterogeneous population of cells with biological characteristics often different from those of their respective primary tumour cells. Pilot studies have shown that phenotyping of CTCs could be used to predict response to targeted therapies. In the era of biological therapeutics, CTC characterization at different time points during the course of disease may provide useful predictive information for the selection of the most appropriate treatment. Therefore, in the future, CTC detection and characterization might become a valuable tool to refine prognosis and serve as a real-time tumour biopsy for individually tailored cancer therapy. Prospective randomized studies are warranted to evaluate the utility of assessing and monitoring CTCs and modifying accordingly treatment strategies in order to improve the clinical outcome of cancer patients.

**Key words:** Circulating tumour cells, micrometastasis, prognosis

**introduction**

Circulating tumour cells (CTCs) are those cells present in the blood that possess antigenic and/or genetic characteristics of a specific tumour type [1]. CTCs are often detected in the blood of cancer patients. Many different methodologies have been used for the detection of CTCs with variable sensitivity and specificity. In many studies including patients with different types of cancer, the detection of CTCs in early and/or metastatic disease has been shown to correlate with unfavourable clinical outcome. When CTCs are present in patients with presumably localized disease, they are thought to contribute to disease relapse and therefore are obvious targets for adjuvant treatment strategies. In patients with metastatic disease, CTC enumeration and monitoring is thought to correlate with tumour load and may predict response to therapy. Moreover, the genetic and phenotypic profiling of CTCs often differs from that of the primary tumour and could be used to select the most effective targeted therapy. Consequently, the study of CTCs, apart from the impact on refining prognosis, has the exciting potential of individualizing treatment strategies for cancer patients.

**detection methods for CTCs**

Different markers have been used for the detection of CTCs, based on their expression on epithelial cells (epithelial-specific markers) or their specific expression on certain tissues (tissue-specific markers). The cytokeratins (CKs) are intermediate filament keratins found in the cytoskeleton of epithelial cells and have been extensively used among others for this purpose [2]. Especially for breast cancer, CK19 is among the most studied markers [3]. CK19 is stably and abundantly expressed on epithelial breast tumours but not on mesenchymal haemopoietic cells and has been successfully used for the detection of breast cancer cells in the bone marrow, lymph nodes and peripheral blood. CK18, mucin-1, carcinoembryonic antigen and mammaglobin have also been used separately or in combinations [4–6].

Because CTCs are usually found at very low frequencies among the normal peripheral blood mononuclear cells (PBMCs), tumour cell enrichment techniques, including density gradient centrifugation (Ficoll–Hypaque separation), immunomagnetic or size filtration procedures are often used to enrich tumour cells before their detection [7, 8].

Monoclonal antibodies directed against histogenic proteins and PCR-based molecular assays amplifying tissue-specific transcripts are the two main approaches used for the detection of CTCs [3–6]. The molecular assays have generally been considered more sensitive, while immunocytochemistry has the advantage of allowing the morphological assessment of stained cells [9–10]. One common problem with most of these methods is that they have not been adequately validated and standardized across different laboratories and therefore different studies have reported different sensitivities and specificities for CTC detection.

Recently, an automated immunomagnetic enrichment and staining system for CTCs (the CellSearch™ system) has been introduced. It performs automated immunomagnetic epithelial cell adhesion molecule (EpCAM)-based enrichment followed by CK staining of CTCs in blood samples. Epithelial cells are distinguished from leukocytes using fluorescently labelled anti-leukocyte (CD45) and anti-epithelial (CK8, -18, -19)–specific monoclonal antibodies. This standardized method has been associated with high intraobserver, interobserver and interinstrument accordance and has been approved by the US Food and Drug Administration for *in vitro* use.
enumeration of CTCs from blood samples of metastatic breast, colon and prostate cancer patients [11].

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. The multiplex PCR detection step analyses the tumour-associated gene expression of a variety of relevant tumour markers [12].

Another method for the detection of circulating epithelial tumour cells (CETCs) from whole unseparated blood uses laser scanning cytometry after staining with anti-EpCAM and anti-CD45 fluorescent antibodies (MAINTRAC™) [13].

More recently, a microfluidic platform (‘CTC chip’) mediated 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 [14].

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 [15].

The detection of CK19 mRNA-positive cells by real-time RT–PCR assay as a surrogate for CTCs in women with early breast cancer has been developed and validated using the LightCycler™ system [16].

False-positive results can be obtained using either nucleic acid-based or antibody-based assays. Contaminating genomic DNA or RNA extraction, illegitimate expression or stimulation of CTC markers in normal leukocytes and the presence of CK19 pseudogenes, have been responsible for the false-positive results when using nucleic acid-based assays [17, 18]. 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 [19]. Similar limitations have been described using antibody-based techniques. Many of the antibodies designed for epithelial cells, occasionally stain haematopoietic cells displaying illegitimate expression of CKs or mucin-1. Plasma cells can also be stained due to the alkaline phosphatase stain haematopoietic cells displaying illegitimate expression of CKs or mucin-1. Plasma cells can also be stained due to the alkaline phosphatase reaction against the \( k \) and \( \lambda \) light chains located on the cell surface [20].

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 from cancer patients. Reported results show substantial variability in the detection rates [21]. The lack of standardization of technology hampers the implementation of CTC measurement in routine clinical practice.

**Clinical relevance of CTCs**

Results from various studies demonstrating the clinical relevance of CTC detection in different tumour types are summarized in Table 1.

**Breast cancer**

Most studies reporting on the prognostic value of CTCs in patients with metastatic breast cancer (MBC) have used the CellSearch system. The presence of \( \geq 5 \) CTCs per 7.5 ml of whole blood in patients with MBC before a new treatment was started was an independent predictor of progression-free survival (PFS) and overall survival (OS) [22]. Moreover, CTC detection by the CellSearch system was a superior surrogate end point to the current radiology imaging studies for assessing response to treatment and predicting OS in MBC patients [36]. Based on these results and in order to evaluate whether CTC detection can improve clinical outcome in MBC, a phase III clinical trial has been initiated (ClinicalTrials.gov NCT00382018) to test the strategy of changing chemotherapy compared with continuing the same chemotherapy regimen for MBC patients who have elevated CTC levels at the first follow-up assessment.

In the early breast cancer setting, it was first reported that the detection by nested RT–PCR of CK19 mRNA-positive cells in the peripheral blood before the initiation of adjuvant chemotherapy was an independent prognostic factor for worse disease-free survival (DFS) and OS [23]. Later on, the same investigators developed a real-time RT–PCR for the quantification of CK19 mRNA transcripts, which was used to detect CK19 mRNA-positive cells in the peripheral blood of patients with axillary lymph node-negative breast cancer before the administration of adjuvant chemotherapy; their presence was an independent prognostic factor for worse DFS and OS [24]. Moreover, in an expanded cohort of 444 women with stage I–III breast cancer, CK19 mRNA-positive cells before adjuvant chemotherapy was an independent prognostic factor for short DFS and OS [25]. Interestingly, the presence of CK19 mRNA-positive CTCs predicted worse outcome in patients with oestrogen receptor (ER)-negative, triple-negative and HER2-positive subgroups, but not in the ER-positive/HER2-negative subgroup [25]. In the same patients, CK19 mRNA-positive cells detected after adjuvant chemotherapy were also independently associated with reduced DFS and OS [26]. To address the clinical utility of CTC detection during hormonal therapy, the same investigators monitored patients with hormone receptor-positive tumours treated with adjuvant tamoxifen, using the same real-time RT–PCR assay for CK19 mRNA. Failure of tamoxifen to eradicate CTCs was an independent prognostic factor for short DFS and OS [27].

In the SUCCESS trial, CTCs were detected by the CellSearch System in 1500 node-positive and high-risk node-negative early breast cancer patients before and after adjuvant chemotherapy. After a 12-month median follow-up, detection of >1 CTC per 23 ml of blood after but not before adjuvant chemotherapy was associated with shorter DFS and OS [28].

Using the MAINTRAC technology to identify CETCs in the blood of women with early breast cancer it was shown that CETCs could be detected in all patients and that a 10-fold increase in CETC numbers between blood samples drawn before and after adjuvant chemotherapy was an independent predictor of disease relapse [29].

Despite the obvious and significant differences in CTC detection rates between molecular methods, the CellSearch system and the MAINTRAC platform, CTC detection using any of these technologies has provided evidence of clinical relevance in refining prognosis of women with early breast cancer.

**Colorectal cancer**

In metastatic colorectal cancer (mCRC) patients, CTC enumeration using the CellSearch system and a cut-off of \( \geq 23 \) CTCs per 7.5 ml of blood, before and during treatment, was an independent predictor of PFS and OS and could provide

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**Table 1.**

<table>
<thead>
<tr>
<th>Tumour Type</th>
<th>CTC Detection Method</th>
<th>Clinical Relevance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast Cancer</td>
<td>CellSearch system</td>
<td>Improved clinical outcomes, identified as an independent predictor of PFS and OS.</td>
</tr>
<tr>
<td>Colorectal Cancer</td>
<td>CellSearch system</td>
<td>Improved clinical outcomes, identified as an independent predictor of PFS and OS.</td>
</tr>
<tr>
<td>Metastatic Breast</td>
<td>CellSearch system</td>
<td>Improved clinical outcomes, identified as an independent predictor of PFS and OS.</td>
</tr>
</tbody>
</table>

**Note:** Table listing the clinical relevance of CTC detection in different tumour types with detailed references.
Table 1. Clinical relevance of CTC detection in different tumour types

<table>
<thead>
<tr>
<th>Ref</th>
<th>Tumor type/time of blood sampling</th>
<th>Number of patients</th>
<th>CTC detection method</th>
<th>Prognostic value</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>Metastatic breast cancer/ before and after chemo</td>
<td>177</td>
<td>CellSearch</td>
<td>PFS, OS</td>
</tr>
<tr>
<td>23</td>
<td>Early breast cancer/before chemo</td>
<td>148</td>
<td>Nested RT–PCR for CK19 mRNA</td>
<td>DFS, OS</td>
</tr>
<tr>
<td>24</td>
<td>Early breast cancer/node-negative, before chemo</td>
<td>167</td>
<td>Real-time RT–PCR for CK19 mRNA</td>
<td>DFS, OS</td>
</tr>
<tr>
<td>25</td>
<td>Early breast cancer/before chemo</td>
<td>444</td>
<td>Real-time RT–PCR for CK19 mRNA</td>
<td>DFS, OS</td>
</tr>
<tr>
<td>26</td>
<td>Early breast cancer/after chemo</td>
<td>437</td>
<td>Real-time RT–PCR for CK19 mRNA</td>
<td>DFS, OS</td>
</tr>
<tr>
<td>27</td>
<td>Early breast cancer/adjuvant tamoxifen</td>
<td>119</td>
<td>Real-time RT–PCR for CK19 mRNA</td>
<td>DFS, OS</td>
</tr>
<tr>
<td>28</td>
<td>Early breast cancer/before and after chemo</td>
<td>1500</td>
<td>CellSearch</td>
<td>DFS, OS (after chemo only)</td>
</tr>
<tr>
<td>29</td>
<td>Early breast cancer/before and after chemo</td>
<td>91</td>
<td>Maintrac</td>
<td>DFS</td>
</tr>
<tr>
<td>30</td>
<td>Metastatic colorectal cancer/ before and after chemo</td>
<td>430</td>
<td>CellSearch</td>
<td>DFS, OS</td>
</tr>
<tr>
<td>31</td>
<td>Colorectal cancer/ before surgery</td>
<td>132</td>
<td>Immunomagnetic enrichment and CK20 immunocytochemistry</td>
<td>Relapse, OS</td>
</tr>
<tr>
<td>32</td>
<td>Castration-resistant prostate cancer/before and after chemo</td>
<td>251</td>
<td>CellSearch</td>
<td>OS</td>
</tr>
<tr>
<td>33</td>
<td>Non-small-cell lung cancer/ before and after chemotherapy</td>
<td>67</td>
<td>Nested RT–PCR for CK19 mRNA</td>
<td>DFS, OS</td>
</tr>
<tr>
<td>34</td>
<td>Non-small-cell lung cancer/ before surgery, before chemo</td>
<td>143</td>
<td>RT–PCR ELISA for surviving mRNA</td>
<td>Relapse, survival</td>
</tr>
<tr>
<td>35</td>
<td>Esophageal squamous cell carcinoma/ before surgery, before chemo</td>
<td>108</td>
<td>RT–PCR ELISA for surviving mRNA</td>
<td>Relapse, survival</td>
</tr>
</tbody>
</table>

DFS, disease-free survival; OS, overall survival; PFS, progression-free survival.

prognostic information in addition to that of imaging studies [30]. Moreover, baseline CTC count was an important prognostic factor within specific subgroups defined by treatment or patient characteristics [37].

In another study the clinical significance of CK20-positive CTCs detected by a refined immunomagnetic enrichment assay in colorectal cancer patients was demonstrated for disease recurrence, metastasis and survival [31]. Furthermore, a recent meta-analysis of published studies on the prognostic value of CTCs in patients with colorectal cancer concluded that CTC detection is a significant prognostic factor for both recurrence-free survival and OS [38].

**other tumours**

The CellSearch system methodology for CTC detection using a cut-off of ≥5 CTCs per 7.5 ml of blood was applied in castration-resistant prostate cancer (CRPC) patients starting a new line of chemotherapy before treatment and monthly thereafter. CTC counts were the most accurate and independent predictors of OS (better than prostate-specific antigen decrement) [32].

CK19 mRNA was measured by nested RT–PCR in blood from 67 non-small-cell lung cancer (NSCLC) patients before and after chemoradiation given with curative intent [33]. CK19 mRNA detection after but not before treatment correlated with poor OS (P < 0.001) and PFS (P < 0.001). The worst survivals were seen in patients with persistently positive CK19 mRNA expression both before and after treatment. Multivariate analyses demonstrated that the positivity of CK19 mRNA after chemoradiation was an independent unfavourable prognostic factor for both OS and PFS [33].

The presence of survivin-expressing CTCs was detected using a RT–PCR ELISA assay in peripheral blood samples collected from 143 NSCLC patients [34]. CTC detection correlated with disease stage, relapse rate and survival. In multivariate analysis, the detection of survivin-expressing CTCs was found to be an independent predictor for cancer recurrence and survival [34]. Using the same methodology, surviving-expressing CTCs were detected in the blood of 108 patients with esophageal squamous
Indeed, various studies have already confirmed that CTCs present significant genetic and phenotypic heterogeneity [40, 47]. Moreover, we know from reported clinical studies that not all patients who have detectable CTCs actually experience disease relapse, and that some patients do relapse although they do not present detectable CTCs [23–27]. Patients with possible breast cancer dormancy have been described who had detectable CTCs on several occasions following mastectomy yet they had not relapsed even after a follow-up of >20 years [48]. These observations raise a challenging and critical question on the biology of CTCs. Recently, immunophenotypic 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 [49, 50]. Indeed, CTCs presenting HER2 were found to be prognostically more ominous [51]. Finally, given the recent discovery of tumour-initiating cells, it is important that the CD44high/CD24low–/CD24– phenotypes have recently been described in CTCs from patients with MBC [52]. All these intriguing data make the story of CTCs truly fascinating.

**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 tumours had acquired HER2 gene amplification in their CTCs during cancer progression [39]. Also, with immunofluorescent microscopy it was demonstrated that individual CTCs presented enhanced expression of activated signalling kinases [phosphorylated focal adhesion kinase, phosphorylated phosphoinositide 3-kinase (PI3K)] as well as HER2 [40]. These activated signalling 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 chromosomal aberrations [41]. CTCs are extremely rare in healthy subjects and patients with non-malignant diseases but present in various metastatic carcinomas with a wide range of frequencies [42].

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 [43]. Furthermore, using the CellPoint platform, epidermal growth factor receptor (EGFR) mutations were detected in CTCs from patients with lung cancer treated with gefitinib [44]. That study provided proof of principle for the feasibility of using blood sample instead of tumour biopsy for serial monitoring of tumour cell genotypes during treatment. Based on the above, it seems that CTC profiling may be crucial not only for identifying new targets that could be used to eliminate minimal residual disease but also as a less invasive and therefore more feasible real-time monitoring system to assess the evolution of genetic and phenotypic changes on tumour cells with potential prognostic and therapeutic implications.

The evaluation of biological characteristics of CTCs may reveal new therapeutic options for cancer patients. Several studies have confirmed discordance in HER2 expression between primary breast tumours and corresponding CTCs since HER2-positive CTCs have been observed in patients with HER2-negative primary tumours [45]. This was exploited in a pilot study where a short course of trastuzumab eliminated chemotherapy- and hormonotherapy-resistant CK19 mRNA-positive CTCs in a majority of patients with early and MBC irrespective of the HER2 status of the primary tumour [46]. Therefore, it would be interesting to test whether therapy directed against the biological characteristics of CTCs rather than those of the primary tumour 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 behaviour, which has serious prognostic and therapeutic implications. Indeed, various studies have already confirmed that CTCs

**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. Furthermore, CTC phenotyping and profiling may serve as a real-time tumour biopsy for individualized targeted therapies.

However, critical issues have to be addressed before CTCs could be used in the daily clinical practice. Detection of CTCs should be standardized and validated across different laboratories in a multicentre trial setting. Most importantly, future studies should demonstrate that using CTCs as a prognostic and/or predictive biomarker leads to improvement in clinical outcome of cancer patients.

**disclosure**

The author has declared no conflict of interest.

**references**


