Applications of RNA characterisation in circulating tumour cells

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1. ABSTRACT

Circulating tumour cells (CTCs) are shed into the bloodstream from both primary and secondary tumours and provide a non-invasive means to study tumor progression and response to treatment. Assessment of ribonucleic acid (RNA) and monitoring dynamic changes in gene expression profiles of CTCs extends their clinical and prognostic power and establish their role in guiding treatment. Among these methods, droplet digital (RT-ddPCR) technique provides a high sensitivity and detectibility of CTCs. RNA-sequencing (RNAseq) is the most comprehensive method, that would allow the simultaneous measurement of a large number of genes and theoretically the whole transcriptome. Since CTCs are heterogeneous in nature, single cell RNAseq methods are very valuable in assessing population dynamics and functional states of CTCs. While RNA in situ hybridization (RNA-ISH) is used relatively less frequently, it also allows for the assessment of expression of multiple genes within individual CTCs. Epithelial to Mesenchymal Transition (EMT) or Plasticity (EMP) is a major contributor to metastasis, providing a mechanism to allow cells to become migratory and invasive, and to survive in the bloodstream. Monitoring CTCs undergoing EMT may lead to improvement in their prognostic and predictive power. Here, we review various RNA analysis of CTCs and those that undergo EMT and their application in diagnosis, prognosis and treatment of cancers.

2. INTRODUCTION

Metastases are the cause of 90\% of cancer-related deaths (1-4). Circulating tumour cells (CTCs) were identified for the first time in blood in 1869 by the Australian surgeon Dr Thomas Ashworth (5). CTCs can enter the blood stream from either the primary tumour or secondary deposits (Figure 1). If they survive the unfavourable conditions in the vascular system, they can reach a new site where they may remain dormant for many years before establishing clinically significant metastases. Elevated CTC numbers quantified by a variety of
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Methods have been linked with poor clinical outcomes in many tumour types (6-10). CTCs may be present in the blood as either single cells or as clusters, of which clusters are more highly prognostic and more efficient at producing viable metastases when harvested (11, 12).

Tumour biopsy, the gold standard in cancer diagnostics, is an invasive method for studying tumour cells, and is restricted to certain anatomical sites and larger deposits. By contrast, liquid biopsies that access CTCs and/or circulating tumour DNA (ctDNA) arising from all tumour deposits provide an opportunity for more comprehensive understanding of the molecular characteristics of metastatic deposits that may otherwise be sampled (13). However, it is not possible to prospectively attribute CTCs/ctDNA to a specific tumour deposit. Despite this caveat, CTCs have a potential role as diagnostic, prognostic and/or predictive markers (14).

CTCs can be distinguished from blood cells using techniques based on their physical and/or molecular characteristics. Such physical characteristics include size, shape and deformability, while molecular characteristics include surface markers, RNA profiles, and DNA mutations (15). The major obstacles in effective identification of these cells are their heterogeneity and relatively small number in the blood among a high background of other cells (potentially including normal epithelial cells).

Currently, CellSearch™ is the only instrument approved by the USA Food and Drug Administration for identifying CTCs. Antibodies are

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Figure 1. Role of epithelial mesenchymal plasticity in metastasis. It is proposed that a proportion of cells in the primary tumour alter their anchored state by undergoing a process known as EMT. Based on in vitro studies of EMT, it is thought that this facilitates tumour cell migration through stroma and intravasation into blood vessels where the tumour cells circulate in the blood as CTCs. EMT provides a mechanism that enables cells to resist anoikis and apoptosis and thus is likely to support CTC persistence in the harsh physical environment of the vasculature which lacks anchorage-dependent survival signals. EMT-positive CTCs are also better equipped to escape the blood vessels (extravasate), and give rise to new metastatic deposits. If they have the ability and receive relevant stimuli to undergo MET, they can again become more epithelial in nature and proliferate to form a new colony. Recent reports also suggest that some CTCs remain epithelial, or predominantly epithelial, and do not go through EMP cycles of EMT and MET. The different colours and shapes of cells in the diagram depict EMP heterogeneity of these cells. CTC, circulating tumour cell; EMP, epithelial mesenchymal plasticity; EMT, epithelial mesenchymal transition; MET, mesenchymal epithelial transition.
used for detection by immunocytochemistry (ICC), however fixation in the CellSearch™ method reduces the viability of these CTCs for further analyses such as culture. The CellSearch™ system separates epithelial CTCs from other cell types using immuno-magnetic beads conjugated to antibodies against the epithelial marker EpCAM, and then assesses the phenotype of the cells using antibodies to the epithelial cytokeratins 8, 18 and 19, and CD45 (9). CD45 is used to identify contaminating haematopoietic cells such as lymphocytes. Thus a cell must be CD45⁻ and EpCAM/cytokeratin⁺ to be classified as a CTC. However, this technique only identifies a subset of CTCs in the sample since the restricted profiling does not recognise the full spectrum of heterogeneous phenotypes of CTCs (16). CTCs may have low EpCAM expression level and assays that use antibodies against EpCAM miss a significant proportion of CTCs (17). In addition, some non-tumour epithelial cells, which express EpCAM and cytokeratin, may also be present in the blood (18). Despite these caveats, there are a large number of studies that demonstrate the high prognostic value of CTC numbers as measured by CellSearch™ in numerous cancer types (7, 8, 19, 20).

Epithelial to mesenchymal plasticity (EMP) or transition (EMT) of in CTCs has been reviewed extensively (21-23). During this process, epithelial cells can modify their architecture to attain a mesenchymal phenotype (24) - an intimately controlled process vital in wound healing, embryogenesis and maintaining homeostasis (24). EMT in carcinoma cells results in cells going through a number of morphological and biochemical changes to become more mesenchymal in nature. These changes cause reduced cell adhesion to the neighbouring cells and increased motility in cells, assisting them to disseminate from the primary tumour and move to other parts of the body (25). When tumour cells reach another site, the reverse process of mesenchymal epithelial transition (MET) is required to enable the cells to re-acquire their epithelial state and colonise the metastatic site. The mesenchymal state is typically a transient state and sometimes associated with reduced proliferation. Thus the re-acquired epithelial state allows the cancer cells to proliferate at the new location (26).

Increased invasiveness, motility, loss of cell polarity, resistance to therapy and survival in unfavourable conditions are all attributes of tumour cells that have undergone EMT (27-29). Each of these events promote metastasis and formation of secondary tumours (30). Thus EMT has been proposed to be one of the inceptive steps of metastasis (31). Although this dogma was recently challenged (32), a number of refutations and rationalisations have subsequently demonstrated that detecting EMT during the metastatic process is contingent on the experimental models employed and the specific technical approaches utilised (27, 33, 34).

It is unusual for cancer cells to go through complete transition to a fully mesenchymal state. Rather, it is more common to find epithelial-mesenchymal hybrid cells representing intermediate states on the epithelial-mesenchymal axis (Figure 1) (35-38). This plasticity is now considered a hallmark of metastasis (39). Hybrid EMT states have been a focal point of discussion regarding metastasis recently (37, 40, 41), and a number of laboratories are using both epithelial and mesenchymal markers in their assays to enable detection of various CTC phenotypes along the epithelial-mesenchymal axis (27, 34, 42). Investigations into the driving forces and mechanisms behind these changes may lead to better ways of diagnosing and treating cancer using new targets (43). It is unlikely that current studies have defined all the mechanisms involved in triggering EMT, but a number of signalling pathways have already been implicated in this process (44, 45).

An early study of EMT in CTCs used magnetic bead separation to isolate cells of interest in parallel to CellSearch™ (46). Epithelial CTCs were first obtained using anti-CD326 (EpCAM)-coated magnetic beads, after which non-epithelial cells were enriched by using CD-45-antibody depletion to remove white blood cells. They found TWIST1 and/or SNAIL1 mRNA transcripts were detected in the epithelial-like CTCs from 82% of patients, and SNAIL1 and ZEB1 transcripts were detected in the mesenchymal-like CTCs in 60.7% of patients. Clinical specimens with no detectable CTCs using CellSearch™ were found to have mesenchymal CTCs using this approach, demonstrating the selectivity of the CellSearch™ system to isolate only
a proportion of total CTCs in a sample (46). Consistent with this, recent studies using a variety of non-CellSearch™ techniques have identified both EpCAM-positive and -negative subpopulations of CTCs within individual patient blood samples (38, 47), however only the EpCAM⁺ component has proven to be prognostically important (48).

Consistent with this, previous research showed no prognostic value of EMT gene expression in peripheral blood from prostate cancer patients, despite CTC enumeration having reasonable inverse correlation with overall survival (OS) (49). This study also found that some patients with low CTC numbers, and thus predicted to have a good prognosis, represented with disease progression. However this study used the CellSearch™ system for CTC isolation, which will have missed CTCs that were further along the EMT axis with very low or no EpCAM expression. These findings emphasise the need to use additional / alternative CTC isolation approaches for comprehensive coverage. There is a crucial need to identify more CTC-associated biomarkers that can be efficiently used to identify and isolate CTCs (50, 51). Plastin3 is one such protein, the expression of which is not altered by EMT in colorectal cancer cells (52). Analogously, VAR2CSA malaria protein has been used to isolate CTCs from hepatic, lung, pancreatic, and prostate carcinoma patients due to its binding to a cell surface onco-fetal form of chondroitin sulfate proteoglycan that remains unaffected by EMT in cancer cells (53).

4. METHODOLOGIES FOR RNA STUDIES

Various methods have been used for RNA detection and quantification in CTC studies (Figure 2, Table 2). These usually allow interrogation of multiple markers. In some cases, multiple techniques are used simultaneously to ensure that the cells being examined are indeed cancer cells. For instance, assessment of physical properties and RT-qPCR analysis of gene transcripts can be used on the same samples to supplement each other in order to reduce the chances of inadvertently analysing non-CTCs in a given sample (i.e. false positives) (42, 64).

4.1. RT-qPCR

RNA amplification using reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) has historically been the most frequently used technique for monitoring gene expression in CTCs and metastatic tumours in clinical research studies. Since RNA needs to be first obtained and purified from intact cells, the workflow starts with a cell lysis step. Purified RNA is reverse transcribed and the resultant cDNA is assessed by quantitative PCR (qPCR). A droplet digital PCR (ddPCR) has also been used on reverse transcribed CTC RNA (65) whereby the samples are divided into smaller reactions within lipid droplets, allowing cDNA from one cell to be independently quantified, increasing the reliability of the results. This technique has enabled detection of CTC-derived RNA transcripts in early stage localised cancer patients having very low CTC numbers that could otherwise be easily missed (54). However, only one gene can be quantified at a time and assays with multiple genes are time consuming and costly.

RT-qPCR is a robust platform to quantify gene expression, used widely on tumour biopsy samples and in CTC pre-clinical, and clinical studies. It is a gene-specific platform with high sensitivity and is cost-effective compared to other RNA detection methodologies (66). Since CTCs are so rare in the blood amongst a large background of other cells, amplification of RNA plays a significant role in accurate detection of signal. The cDNA produced
during reverse transcription is in such minute quantities that a pre-amplification step is quite common before the individual assessment of genes in the final qPCR or ddPCR reaction (54, 55).

The expression of EMP-associated epithelial and mesenchymal genes in CTCs isolated from breast cancer patients identified using RT-qPCR has demonstrated that the EMT shift in CTCs does not always correlate with that of their originating tumours, emphasising the need for separate examination of these potential circulating seeds of metastasis (47). It has been observed that CTCs have gene expression profiles more similar to metastases than their primary tumours. In a prospective study on metastatic colorectal cancer patients using a 34 gene RT-qPCR assay, the majority of patients (74%) had CTC gene expression profiles more similar to the metastatic deposits than to their primary tumours (61). In another study, CTC RNA isolated from HER2-positive metastatic breast cancer patients was subjected to RT-qPCR analysis

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>Key findings/ observations</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast cancer</td>
<td>Identification of patients non-responsive to ER suppression treatment, using quantitative CTC-derived ER signalling.</td>
<td>(54)</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>No correlation between expression of EMT-inducing transcription factors TWIST and SLUG in primary tumour tissue and CTCs.</td>
<td>(47)</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>Presence of mesenchymal-like CTCs is associated with OS and hybrid CTCs, expressing both epithelial and mesenchymal marker genes, are associated with poor clinic-pathological characteristics.</td>
<td>(58)</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>Presence of mesenchymal-like CTCs is associated with disease progression. EMT is a dynamic state of CTCs that can vary at different time points during therapy with a strong correlation with therapy response and disease progression.</td>
<td>(40)</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>CellSearch™ System potentially misses CTCs that have undergone EMT and lost EpCAM gene expression.</td>
<td>(46)</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>Individual CTC analysis is possible and elevated levels of EMT-associated genes observed in these cells.</td>
<td>(59)</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>Gene expression profiles of CTCs are very heterogeneous. RT-qPCR has higher sensitivity than CellSearch™ but needs validation using a larger patient cohort.</td>
<td>(60)</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>RNA expression of CTCs is more similar to metastases than primary tumours.</td>
<td>(61)</td>
</tr>
<tr>
<td>Liver cancer</td>
<td>CTC gene signature enables lineage-specific detection. Patients suffering from HCC can be distinguished from patients with other cancers and liver diseases.</td>
<td>(57)</td>
</tr>
<tr>
<td>Melanoma</td>
<td>Monitoring of treatment response using CTC scoring is highly consistent with clinical assessments, and can recognise patients who would respond to immune checkpoint inhibition therapy.</td>
<td>(55)</td>
</tr>
<tr>
<td>Pancreatic cancer</td>
<td>Upregulation of WNT genes in CTCs may contribute to metastasis and these signalling pathways can be potential therapeutic targets.</td>
<td>(62)</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>An increase in expression of EMT-associated genes and decrease in expression of epithelial-associated genes in patients after initiation of radiotherapy.</td>
<td>(42)</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>CTC derived signature is informative of cancer cell dissemination in localised cancer and response to abiraterone in metastatic disease.</td>
<td>(56)</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>Androgen deprivation therapy activates noncanonical WNT signalling in CTCs, which plays a role in therapy resistance.</td>
<td>(63)</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>Expression of EMT-associated genes in CTCs may play a role in the development of castration resistant prostate cancer.</td>
<td>(64)</td>
</tr>
</tbody>
</table>

RNA-based CTC studies have been conducted for a number of cancers to date, and have uncovered a number of key players in cancer dissemination and metastasis. A number of key findings published between 2011 and 2018 have been summarised, with a focus on EMT observations. CTC: circulating tumour cell; EMT, epithelial mesenchymal transition; ER, Oestrogen Receptor; HCC, hepatocellular carcinoma; OS, overall survival.
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Figure 2. Schematic workflow of techniques used for RNA-based analysis of CTCs. (a) After initial CTC isolation, cells can either be analysed individually or as a population. Individual cell studies include RNA-ISH and single cell RNAseq. Population based studies include RT-PCR and RNAseq. (b) RNAseq: RNA isolation is followed by reverse transcription. The cDNA is then fragmented to form a cDNA library and sequenced. (c) RT-PCR: Similar to the initial RNAseq workflow, RNA isolation is followed by cDNA preparation. This cDNA is either quantified using qPCR or using ddPCR, in which case the input sample is first dispersed into 20,000 lipid droplets, amplified and then fluorescence detected. (d) RNA-ISH: CTCs are fixed and cytospun onto slides followed by permeabilisation. CTC RNA is then hybridised using fluorescently labelled nuclear probes and imaged under a microscope.
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Table 2. Characteristics of selected RNA-based CTC clinical studies

<table>
<thead>
<tr>
<th>Study year</th>
<th>Cancer type</th>
<th>Enrichment method</th>
<th>Detection method</th>
<th>Tumour stage</th>
<th>Localised/metastatic stage</th>
<th>Patient number</th>
<th>NHV</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2018</td>
<td>Breast cancer</td>
<td>Microfluidic CTC-iChip</td>
<td>RT-ddPCR</td>
<td>I-IV</td>
<td>Localised and metastatic</td>
<td>120</td>
<td>33</td>
<td>(54)</td>
</tr>
<tr>
<td>2018</td>
<td>Breast cancer</td>
<td>Density gradient centrifugation and anti-CD45 immuno-magnetic bead depletion</td>
<td>RT-qPCR</td>
<td>I-III</td>
<td>Localised</td>
<td>83</td>
<td>22</td>
<td>(58)</td>
</tr>
<tr>
<td>2015</td>
<td>Breast cancer</td>
<td>RosetteSep™ negative selection kit</td>
<td>RT-qPCR</td>
<td>I-III</td>
<td>Localised</td>
<td>102</td>
<td>60</td>
<td>(47)</td>
</tr>
<tr>
<td>2013</td>
<td>Breast cancer</td>
<td>1hCTC-Chip</td>
<td>RNAseq and RNA-ISH</td>
<td>NA</td>
<td>Metastatic</td>
<td>41</td>
<td>5</td>
<td>(40)</td>
</tr>
<tr>
<td>2012</td>
<td>Breast cancer</td>
<td>Ficoll-hypaque density gradient and negative selection using antibodies</td>
<td>RT-qPCR</td>
<td>NA</td>
<td>Metastatic</td>
<td>28</td>
<td>20</td>
<td>(46)</td>
</tr>
<tr>
<td>2012</td>
<td>Breast cancer</td>
<td>MagSweeper</td>
<td>RT-qPCR</td>
<td>NA</td>
<td>Localised and metastatic</td>
<td>50</td>
<td>90</td>
<td>(59)</td>
</tr>
<tr>
<td>2011</td>
<td>Breast cancer</td>
<td>Dynabeads® Epithelial Enrich, Invitrogen, CellSearch™</td>
<td>RT-qPCR</td>
<td>I-IV</td>
<td>Localised and metastatic</td>
<td>92</td>
<td>28</td>
<td>(60)</td>
</tr>
<tr>
<td>2016</td>
<td>Colorectal cancer</td>
<td>CellSearch™ System</td>
<td>RT-qPCR</td>
<td>II-IV</td>
<td>Metastatic</td>
<td>133</td>
<td>NA</td>
<td>(61)</td>
</tr>
<tr>
<td>2017</td>
<td>Liver cancer</td>
<td>Microfluidic CTC-iChip</td>
<td>RT-ddPCR</td>
<td>NA</td>
<td>Localised and metastatic</td>
<td>63</td>
<td>26</td>
<td>(57)</td>
</tr>
<tr>
<td>2018</td>
<td>Melanoma</td>
<td>Microfluidic CTC-iChip</td>
<td>RT-ddPCR</td>
<td>III-IV</td>
<td>Metastatic</td>
<td>82</td>
<td>36</td>
<td>(55)</td>
</tr>
<tr>
<td>2012</td>
<td>Pancreatic cancer</td>
<td>1hCTC-Chip</td>
<td>RNAseq</td>
<td>NA</td>
<td>Metastatic</td>
<td>21</td>
<td>10</td>
<td>(62)</td>
</tr>
<tr>
<td>2018</td>
<td>Prostate cancer</td>
<td>CellCollector®</td>
<td>RT-qPCR</td>
<td>NA</td>
<td>Localised</td>
<td>108</td>
<td>36</td>
<td>(42)</td>
</tr>
<tr>
<td>2018</td>
<td>Prostate cancer</td>
<td>Microfluidic CTC-iChip</td>
<td>RT-ddPCR</td>
<td>I-IV</td>
<td>Localised and metastatic</td>
<td>88</td>
<td>39</td>
<td>(56)</td>
</tr>
<tr>
<td>2015</td>
<td>Prostate cancer</td>
<td>CellSearch™ System</td>
<td>RT-qPCR</td>
<td>NA</td>
<td>Metastatic</td>
<td>70</td>
<td>20</td>
<td>(49)</td>
</tr>
<tr>
<td>2015</td>
<td>Prostate cancer</td>
<td>Microfluidic CTC-iChip</td>
<td>RNAseq</td>
<td>NA</td>
<td>Localised and metastatic</td>
<td>38</td>
<td>NA</td>
<td>(63)</td>
</tr>
<tr>
<td>2013</td>
<td>Prostate cancer</td>
<td>Size-based filtration</td>
<td>RT-qPCR</td>
<td>NA</td>
<td>Localised and metastatic</td>
<td>9</td>
<td>NA</td>
<td>(64)</td>
</tr>
</tbody>
</table>

NA, not available; NHV, normal healthy volunteer; RNA-ISH, RNA- in situ hybridisation; RNAseq, RNA sequencing; RT-ddPCR, reverse transcriptase-droplet digital polymerase chain reaction; RT-qPCR, reverse transcriptase-quantitative polymerase chain reaction.

to identify RNA levels of transcription factors that induce EMT and some stem cell-associated features (46). Despite a small patient cohort, an association between EMT transcription factors (TWIST1, SNAIL1, ZEB1, and TG2) and clinical outcomes was reported. Interestingly, no association was found between the EMT transcription factors and cancer stem cell markers (CD24, CD44, and CD133). On similar lines, Chang et al. used RT-qPCR on circulating RNA in prostate cancer patient blood...
samples to identify EMT marker transcripts (TWIST1 and vimentin) (49). Some stem cell genes (ABCG2, PROM1 and PSCA) were also investigated. Although expression levels of stem cell-associated genes correlated well with OS, EMT-associated genes did not. The inconsistency in results between the Giordano (46) and Chang (49) studies may be attributed to the method of RNA acquisition; Giordano and colleagues performed RNA isolation from enriched CTC fractions while Chang and colleagues used blood-borne RNA.

Another RNA analysis study in prostate cancer of 17 genes involved in tumour progression, including those associated with EMT (GDF-15), prostate biology, hormone regulation, stemness, tumour aggressiveness and taxane responsiveness, was performed using RT-qPCR on isolated CTC samples from peripheral blood of patients and healthy volunteers (50). White blood cell background was removed by CD45 normalisation of the data. A correlation between expression of the 17-gene panel with OS of patients was reported.

RT-qPCR technology has also been shown to be suitable for single cell analysis. Sieuwerts and co-workers were able to quantify RNA in as little as one cell using a 94 gene assay, which comprised genes having high expression in breast cancer cells and low in white blood cells (67). They also incorporated genes in their assay that were known to have high transcription levels in the background cells, but later they reduced the number of genes in their assay to only those that had higher expression in breast cancer cells and either no or low expression in leukocytes, since high levels of contamination were observed in the CTC-enriched blood fractions.

### 4.2. RNA-ISH

Another technique used to study gene expression profiles is RNA-*in situ* hybridisation (RNA-ISH) (68). This technique, not widely used for CTC detection to date, allows observation of RNA localised in intact cells using tagged nucleic acid probes. This means RNA expression of single CTCs and individual clusters can be observed without denaturing the cells, and since RNA-ISH does not require antibodies it potentially provides an opportunity to measure transcripts for which there is no antibody or are not translated (e.g. miRs, long non-coding RNA). Furthermore, the capacity to combine RNA transcript measurement with visual observations of morphology and/or protein localisation by immunocytochemistry is powerful. In a breast cancer study conducted by Yu *et al.*, cells that had more mesenchymal gene expression appeared more elongated than those with high expression of epithelial genes (40).

RNA-ISH can also be used without enrichment, which means it reduces the number of steps involved and the chances of losing CTCs during processing. CTCscope, a method relying on RNA-ISH, can be used for CTC detection without using a prior enrichment step with sensitivity up to a single RNA molecule (69). The presence of white blood cells in the sample does not obstruct the results due to high sensitivity of the assay. The CTCscope assay comprises of epithelial markers including CK8, CK18, CK19, epidermal growth factor receptor (EGFR) and EpCAM, as well as mesenchymal markers including N-cadherin, fibronectin and TWIST1. Interestingly, vimentin, a strong EMT marker, was removed from their panel since several blood cell types express it. Some strong epithelial markers (cytokeratin 5 and 6) were also removed owing to the same issue. CTC detection by CTCscope and CellSearch™ both predicted progression-free survival (PFS) in patient blood samples in this study. On the downside, it was shown that CTCscope only detected RNA from CTCs that were live at time of assessment, therefore CTC numbers detected by this method were less than those detected by CellSearch™ (69).

Similar to CTCscope, Yu *et al* used a dual-colorimetric RNA-ISH assay coding approach to observe the extent of EMP in cells, using a single fluorescent marker for a pool of probes targeting 7 epithelial-associated genes (EpCAM, CDH1 and 5 keratins) and a different fluorescent marker for the 3 selected mesenchymal-associated genes (FN1, SERPINE1 and CDH2) (40). An association between enrichment of mesenchymal transcripts in CTCs and breast cancer progression was reported (40). Similar results with distinct EMT populations were observed in CTCs and CTC clusters in metastatic breast
cancer samples cultured by Khoo et al (70). Since this technique allows observation of intact cells, it has also allowed detection of native, undisturbed CTC clusters expressing EMT regulator genes. A correlation was observed between disease progression and CTC number as well as EMT status of CTCs in a 5 patient cohort comparing pre- and post-treatment samples. More CTCs were observed when the patient was not responding to therapy compared to those obtained when therapy was effective. Interestingly, the number of mesenchymal CTCs identified was also greater than epithelial ones in this study (40). An association was also observed between mesenchymal marker expression and CTC clusters.

4.3. RNAseq

Since CTCs are so rare in abundance, platforms that have the sensitivity to sequence samples with low quantity or single cells are of great interest. RNAseq is a robust and accurate technique used to study global RNA expression levels in CTCs. It is a very comprehensive method since a large number of genes can be analysed simultaneously. Unlike RNA-ISH this process requires cell lysis, so RNA localisation to specific cells cannot be observed. Similar to the initial steps for RT-qPCR, RNA is converted into cDNA in the first step of the experiment. Next, cDNA is fragmented to form a cDNA library which is sequenced using any of the available sequencing platforms. A pre-amplification step, typically comprising of 12-18 cycles, is also sometimes used before sequencing the cDNA to enhance the detection capabilities of the sequencing system (71).

RNAseq can either be performed on individual CTCs (single cell RNAseq) or on a total CTC population assay to give an average overall gene expression of the cells. Single cell RNAseq has been able to verify the hypothesised molecular diversity of CTCs and identify subpopulations of CTCs with varying gene expression even from the same patients at the same time point (59). RNA-Seq of CTCs has been widely used and has proven useful in extracting large amounts of data from single cells (63, 72), including the identification of signalling pathways involved in EMT (40). Single cell RNAseq of melanoma CTCs was able to uncover single nucleotide polymorphisms (SNPs) and mutations, such as a SNP in TYR gene, which can play a pivotal role in targeted therapy. Downregulation of genes that play a key role in evading immune surveillance, including TRPM1 and five HLA genes, was also observed along with increased proliferation and invasive potential due to loss of CDH1 (71). RNAseq has also been used to supplement whole genome sequencing to provide a comprehensive examination of the genetic / gene expression profiles of CTCs (73). However, it also suffers from amplification bias and has reduced sensitivity towards low transcript levels (74). Since CTC populations are heterogeneous in nature, pooled expression profiles can mask the heterogeneity of these cells resulting in an averaged overall expression pattern that might not be representative of significant components of the cell population. This approach obscures the identification of rare cells with distinct gene expression that might have special properties (e.g. EMP, survival, apoptosis, and invasive/metastatic abilities).

In summary of the above-mentioned detection techniques, molecular investigation of CTCs has the ability to provide us with a wealth of information. Improvements in isolation technologies and detection methods have enabled a more comprehensive analysis however, the field still remains open to further developments (16) (Table 3). CTCs are highly heterogeneous in nature making their characterisation complicated. In a study conducted on CTCs isolated from 108 prostate cancer patients using CellCollector® and analysed using RT-qPCR, 70.5% of the patient samples expressed at least one of the 14 genes in their assay, with vimentin being the most common (42). In the same study, while only 7.4% of the patients’ blood samples expressed EMT markers (vimentin and/or TWIST1) prior to commencing therapy, 63% of patient samples expressed EMT markers post-therapy, and CTC epithelial marker expression drastically decreased after initiation of therapy (from 48.1% to 7.4% of patient samples). Certain EMT-related genes, including WNT5A, have been observed to be more commonly expressed in CTCs isolated from men with castration-resistant prostate cancer compared to castration-sensitive cancer (75).
Identifying these gene expression patterns in the beginning of a new treatment regimen might be useful in therapy selection and in flagging the emergence of therapy resistance at an earlier stage (64).

5. CTC-RNA SIGNATURES

It is envisaged that digital molecular CTC signatures can be established for cancer patients based on gene transcript levels of CTCs, and that these can be used for monitoring cancer progression and treatment response. Whilst it is attractive to develop a universal scoring methodology that can be applied to all types of cancers, due to high variation between organs it is likely that this would have to be customised to the tumour type. Currently, CTC-RNA signatures have been proposed for melanoma, breast, liver and prostate cancers (54-57). The CTC signatures are comprised of a panel of genes that are virtually not expressed in hematopoietic cells, and are either organ-specific or tumour-specific. Based on an assigned threshold, the numerical CTC scores can predict high or low disease burden. Upon serial monitoring, an increase or decrease in CTC score correlates with treatment response based on previous CTC number observations for the individual patient (40). However, sensitivity of assays to detect CTCs against a high background of other cell types is still a major obstruction in effective disease monitoring.

In a prospective treatment cohort of hepatocellular carcinoma (HCC) patients, 56% of patients that had not yet started treatment had positive CTC scores as compared to only 28% of those that had already commenced therapy. The false positive rate in the control cohort was 7.6%. This assay also had the capacity to distinguish liver cancer patients from numerous other cancers at an 88% positive rate with 50% sensitivity. This proof of principle study demonstrated the utility of CTC scores in follow-up studies. A post-surgery decrease in CTC score was observed in the two patients following their operation. Similarly, another patient had a decrease in CTC score following immunotherapy that was associated with a reduction in tumour size as measured by computed tomography scan, and which further reduced after tumour radioembolisation. These tantalising case reports require follow up in larger cohorts in which CTC score is monitored in conjunction with therapeutic responses. A caveat for this approach is that clinical utility is restricted to patients with an initial positive CTC score. In this study, almost half the patients had a negative CTC score and thus would require a method that has higher sensitivity if such an approach were to be widely implemented (57).

A similar approach was used in a pilot study involving prostate cancer patients (56) . The majority (92%) of metastatic prostate cancer patients had a positive CTC score. However, due to the stringent threshold required to robustly discriminate CTCs from blood cells, none of the patients with localised cancer had a positive score even though low levels of signal were observed for a few genes. Interestingly, increased CTC scores in follow-up studies for this patient cohort correlated well with poor OS. On the contrary, neither CTC score nor prostate specific antigen (PSA)-positive CTC signal correlated with serum levels of the gold standard biomarker protein PSA.

Table 3. Pros and Cons of RNA-based CTC detection methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Pros</th>
<th>Cons</th>
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<tr>
<td>RNA-ISH</td>
<td>Intact cells used, individual cell assessment, can observe CTC clusters, prior CTC enrichment not required</td>
<td>Only detects cells live at fixation, non-specific binding, number of genes detected restricted because of dependency on fluorescent channels</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>High sensitivity, high specificity, simultaneous investigation of multiple targets, easy workflow, low cost</td>
<td>Illegitimate detection of signal from blood cells, only population analysis, requires CTC enrichment and lysis</td>
</tr>
<tr>
<td>RNAseq</td>
<td>Largest number of genes investigated simultaneously, high sensitivity, low technical variability, single cell RNA assessment possible</td>
<td>Highly sensitive to degraded RNA, requires computational analyses and bioinformatic workflows, requires CTC enrichment and lysis</td>
</tr>
</tbody>
</table>

CTC, circulating tumour cell; RNA-in situ hybridisation; RNAseq, RNA sequencing; RT-qPCR, reverse transcriptase-quantitative polymerase chain reaction.
CTC scoring in melanoma patients also suggests potential for clinical utility (55). A 19 gene assay was applied for which 86.7% of the enriched patient blood samples had signal for at least one gene. The baseline CTC score (high or low) was observed to have no correlation with clinical characteristics, PFS, OS, or time to next systemic therapy. However, change in CTC score over time was clinically significant, as patients with an increase in CTC score had poorer PFS as compared to those with reduction in CTC score over the course of therapy.

The ability of a prognostic marker to discern treatment outcomes at early stages of cancer is of greatest priority however the relatively low abundance of CTCs at this stage, especially in localised cancers, poses a hindrance to efficient early detection. CTC scoring in early stage localised breast cancers has been of low utility, despite having strong predictive value for stage IV metastatic patients (54). At 100% sensitivity, the specificity of the assay to detect CTCs was 19% for stage I, 36% for stage II, 58% for stage III and 67% for stage IV patient samples. Stage IV patients also showed a correlation between change in CTC score over the course of therapy and treatment outcome.

CTC scores are proposed to have the ability to discern whether patients will respond to treatment and may have clinical utility once further validated in larger, independent cohorts. Numerical CTC scoring has the potential to make a valuable contribution in the context of personalised medicine.

6. CONCLUDING REMARKS

In summary, RNA-based analysis of CTCs is feasible and can uncover the underlying heterogeneity of these cells and mechanisms that might be involved in tumour cell dissemination. However, currently no gold-standard method is available and RNA studies remain challenging due to low RNA yield and quality despite various improvements in detection techniques. Cell necrosis and RNA degradation greatly hamper accurate computational analysis (76). Since most cancer related deaths are attributed to metastasis, it is critical to conduct studies that can elucidate the heterogeneity of CTCs and their originating tumours to be able to understand why certain cells are able to escape the primary deposit and form metastases, while others are not. In an era of many new treatment regimens being available there is a growing need to be able to identify at an early stage which treatment might be most suitable for an individual. CTC measurements can contribute to personalised medicine, provided precise and early detection is feasible and cost-effective.

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**Abbreviations**

AR: androgen receptor
cDNA: complementary DNA
CTC: circulating tumour cell
ctDNA: circulating tumour DNA
EGFR: epidermal growth factor receptor
EMP: epithelial mesenchymal plasticity
EMT: epithelial mesenchymal transition
ER: oestrogen receptor
HCC: hepatocellular carcinoma
HER2: human epidermal growth factor receptor 2
ICC: immunocytochemistry
MET: mesenchymal epithelial transition
NHV: normal healthy volunteer
OS: overall survival
PFS: progression-free survival
PSA: prostate specific antigen
RNA: ribonucleic acid
RNA-ISH: RNA- *in situ* hybridisation
RNAseq: RNA sequencing
RT-ddPCR: reverse transcriptase-droplet digital polymerase chain reaction
RT-qPCR: reverse transcriptase-quantitative polymerase chain reaction
SNP: single nucleotide polymorphism

**Key Words:** CTC, Metastasis, EMT, Tumour Heterogeneity, RNA analysis, CTC score, Review

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