Circulating tumour cells as biomarkers of prostate, bladder, and kidney cancer

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Abstract | Circulating tumour cells (CTCs) have been studied as biomarkers of a number of solid malignancies. Potential clinical applications for CTC analysis include early cancer detection, disease staging, monitoring for recurrence, prognostication, and to aid in the selection of therapy. In the field of urologic oncology, CTCs have been most widely studied as prognostic biomarkers of castration-resistant prostate cancer. Additionally, emerging data support a role for CTCs to help identify which patients are most likely to respond to novel androgen-pathway targeted therapies, such as abiraterone and enzalutamide. CTCs have also been studied as predictive biomarkers of bladder cancer, in particular as a means to identify patients whose disease has been clinically understaged. Less is known regarding CTCs in kidney cancer; this has been attributed to the fact that a minority of renal tumours express EpCAM, the epithelial cell surface protein commonly used by CTC assays for positive cell selection. However, alternative approaches using markers specific for kidney cancer are being explored.

Nearly 150 years ago, Thomas Ashworth reported the first observation of circulating tumour cells (CTCs) in the peripheral blood of a patient with metastatic cancer from an unknown primary site[1]. Since this early observation, tremendous strides have been made in our technical ability to isolate and analyse these rare cells. These advances have led to an improved understanding of basic cancer biology as well as a myriad of efforts aimed at exploring CTCs as cancer biomarkers.

CTCs enter the circulation by either passive shedding or through the dynamic processes of stromal invasion and subsequent intravasation into the blood stream[2,3]. Within the circulation, CTCs must survive sheer stress and evade the host immune system in order to extravasate at a distant site. Once at their new location, CTCs must adapt to the local microenvironment, where they can lay dormant in a quiescent state or undergo proliferation to develop into metastatic foci (FIG. 1).

CTCs can originate from either primary or metastatic sites of disease, providing a number of potential applications as cancer biomarkers, including early cancer detection, disease staging, monitoring for recurrence, prognostication, and therapy selection. Within the field of urologic oncology, CTCs have been explored as biomarkers of prostate, bladder, and kidney cancer. In this Review, we will explore the current state of CTC research in each of these diseases, summarizing the most clinically relevant publications as well as studies of emerging technologies that have achieved particular attention within the field.

Prostate cancer

CTCs have been extensively studied as biomarkers of prostate cancer. Publications on this topic range widely from small pilot studies describing initial feasibility with novel CTC technologies, to results of large phase III trials utilizing validated clinical-grade tests.

CellSearch

The CellSearch test (Janssen Diagnostics, USA) is currently the only CTC assay cleared by the FDA, having originally gained regulatory approval following the publication of several large diagnostic trials as an adjunctive method for monitoring patients with metastatic breast, colorectal, and prostate cancer[4–12]. The CellSearch test relies on the positive selection of cancer cells using antibodies against the epithelial cell adhesion molecule (EpCAM) antigen[4]. Blood samples are first incubated with ferroparticles coated in anti-EpCAM antibodies. Ferroparticle-bound cells are then captured in a magnetic field and stained with 4′,6-diamidino-2-phenylindole (DAPI) as well as fluorescently labelled antibodies against

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Key points

- Circulating tumour cells (CTCs) have been validated as prognostic biomarkers of prostate cancer. These data have been generated with a number of different methods for CTC detection including the FDA-approved CellSearch test
- Novel CTC assays have also been developed to help identify which prostate cancer patients are most likely to respond to androgen-pathway targeted therapies, such as abiraterone and enzalutamide
- CTCs have also been studied as prognostic biomarkers of bladder cancer. These data have mostly been generated using the CellSearch test
- One potential area of clinical application of CTCs in patients with urothelial carcinoma is the identification of patients with non-muscle-invasive bladder cancer who have been clinically understaged and are unlikely to benefit from intravesical therapy
- CTCs have been less well studied as biomarkers of kidney cancer. This is related to the fact that renal cell carcinoma expresses low levels of EpCAM, a cell surface maker that is used by many CTC isolation methods
- Alternative methods for CTC isolation that employ specific markers for kidney cancer have shown early promise

the leukocyte marker CD45, and cytokeratins 8, 18, and 19. After staining, CTCs are enumerated using the semi-automated Celltracks Analyzer II System. With this assay, a CTC is defined as any nucleated cell that is positive for cytokeratin expression but negative for CD45 (FIG. 2).

The CellSearch system has been evaluated in numerous studies of patients with metastatic prostate cancer[4,11–18]. In an early report of this test, Allard and co-workers[4] showed that 107 of 188 (57%) samples from 123 patients with metastatic prostate cancer contained ≥2 CTCs in a standard 7.5 ml blood draw. Notably, 14% of samples contained ≥50 CTCs. Based on these promising findings, two large prospective studies were performed to evaluate the prognostic utility of the CellSearch test in patients with metastatic castration-resistant prostate cancer (mCRPC)[11,12]. In the larger of these studies ($n = 231$), de Bono and colleagues[12] reported that 57% of patients had ≥5 CTCs per 7.5 ml of blood. Importantly, patients with ≥5 CTCs had a median overall survival nearly half that of patients with lower CTC counts (11.5 months versus 21.7 months; HR 3.3; $P < 0.0001$). These findings have since been validated in a number of other reports, including a recent phase III clinical trial evaluating the efficacy of atrasentan in combination with docetaxel chemotherapy[17]. Despite these seemingly favourable findings, this test has not translated into routine clinical use, an issue that has been attributed to the fact that the CellSearch test offers only prognostic information, and not truly actionable data that could influence the clinician to alter patient care. However, a phase III study to evaluate the CellSearch test in guiding the transition from first-line docetaxel to second-line cabazitaxel in men with mCRPC is planned[19], in which patients will be randomized to compare standard-of-care therapeutic decision making versus CTC-guided drug discontinuation using overall survival as the primary end point.

AdnaTest

Like CellSearch, the AdnaTest relies on the positive selection of CTCs from peripheral blood using anti-EpCAM antibodies. Cells are then lysed and RNA is extracted for downstream analysis with reverse-transcription (RT)-PCR. The standard AdnaTest includes primers against PSA, prostate-specific membrane antigen (PSMA) and the epidermal growth factor receptor (EGFR). A sample is defined as positive for the presence of CTCs if any of these genes are detected. In an early feasibility study of the AdnaTest, CTCs were detected in 11 of 16 patients (67%) with mCRPC before initiating docetaxel treatment[20].

Moving beyond the simple readout of presence or absence of CTCs, Antonarakis *et al.*[21] modified the standard AdnaTest to include primers for the detection of the androgen-receptor splice variant 7 (AR-V7). This splice variant lacks a ligand-binding domain, resulting in constitutive activation of genes that are regulated by the androgen receptor (AR)[22,23]. Antonarakis and co-workers postulated that expression of *AR* splice variants, such as *AR-V7*, might enable resistance to drugs that target AR signalling. To test this hypothesis, the authors evaluated blood samples from 62 patients with the modified AdnaTest before initiating treatment with enzalutamide or abiraterone, enabling correlation of AR-V7 status with response to these drugs. Overall, 18 of 62 men (29%) had AR-V7-positive CTCs. More importantly, men with AR-V7-positive CTCs had markedly worse PSA responses and shorter periods of progression-free and overall survival as compared to men without this biomarker. Steinestel *et al.*[24] further modified the AdnaTest to include primers for AR-V7 status as well as point mutations in exon 3 and exon 5 of *AR*. With this modified assay, the authors identified *AR* variants in 19 of 37 patients (51%) and estimated a positive predictive value of nearly 94% for predicting response to AR-targeted therapy.

Extending this line of investigation further, Nakazawa *et al.*[25] used AdnaTest to explore the dynamics of CTC AR-V7 status in a cohort of 14 patients with metastatic prostate cancer as they transitioned between various therapies. Over a median follow-up period of 11 months, patients contributed a combined total of 70 blood samples. Conversions to AR-V7-positive status occurred while patients were receiving a host of therapies for prostate cancer including androgen deprivation, enzalutamide, abiraterone, or taxane chemotherapy. By contrast, reversions to AR-V7-negative status occurred only upon treatment with taxanes. Consistent with the earlier findings of Antonarakis *et al.*[21], patients with AR-V7-positive CTCs did not exhibit PSA responses while being treated with enzalutamide or abiraterone. These patients, however, did show responses to treatment with taxane chemotherapy. This finding has since been independently confirmed by another group who demonstrated that patients responded equally to treatment with cabazitaxel regardless of CTC AR-V7 status[26].

Taken together, the available data strongly suggest that AR-V7 status of CTCs can provide clinically actionable information to men with prostate cancer considering treatment with abiraterone or enzalutamide. We look forward to prospective trials that aim to determine if the selection of therapy based on AR-V7 status in CTCs leads to improved long-term patient outcomes.

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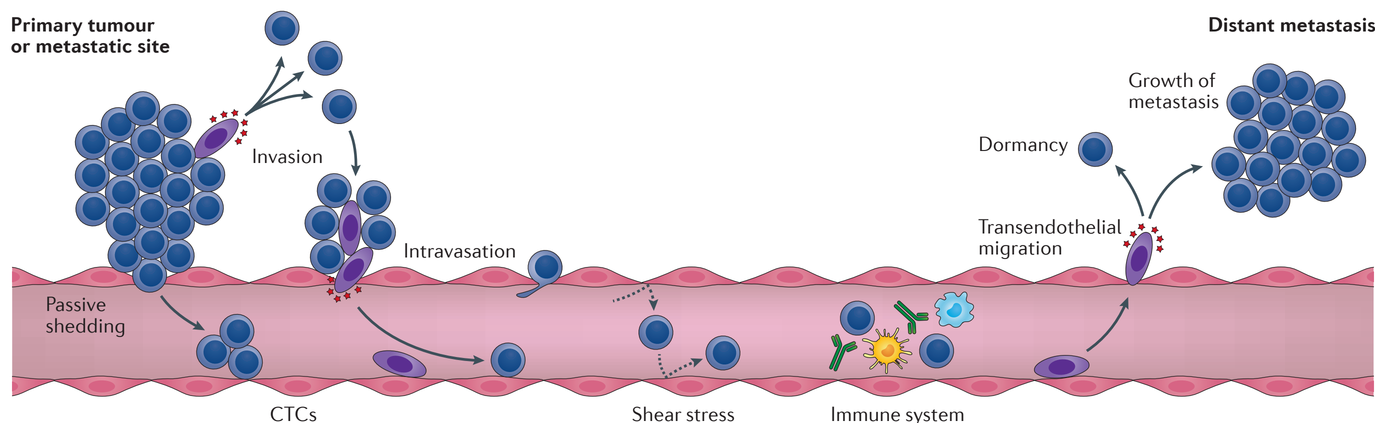

Figure 1 | **Schematic representation of CTCs entering the peripheral circulation and establishing a metastatic focus at a distant site.** Circulating tumour cells (CTCs) enter the circulation by either passive shedding or through the active process of invasion and intravasation. Within the bloodstream, CTCs must survive sheer stress and evade the host immune system. CTCs then undergo transendothelial migration to extravasate at a distant site. Once at their new location, CTCs can lay dormant or undergo proliferation.

Selection-free detection of CTCs

A common shortcoming of the CellSearch system and the AdnaTest is their reliance on positive selection of CTCs with antibodies against EpCAM. Thus, cells that lack or have low levels of this protein are excluded from capture and analysis[27,28] (FIG. 3). This group includes cells that predominantly express mesenchymal markers, a CTC population that is likely to be associated with an aggressive prostate cancer phenotype[29–31]. This limitation has lead several groups to develop selection-free methods for identifying and characterizing CTCs.

High-throughput imaging. A notable example of a selection-free approach for CTC detection relies on high-throughput imaging, as well as advanced segmentation and classification algorithms, to identify fluorescently labelled CTCs from a background of millions of white blood cells. This test, which was originally developed by investigators at The Scripps Research Institute[32], is now offered as a service of Epic Sciences in San Diego, CA[33,34]. In the first step of this assay, whole blood is incubated with an ammonium chloride solution to lyse and remove red blood cells. The remaining mononuclear cells are then pelleted and spread as a monolayer onto custom glass slides covered in a proprietary adhesive coating. At a plating density of 3 million cells per slide, a typical 7.5 ml blood sample is spread across 10–12 slides. Two of these slides are stained for DAPI, CD45 and cytokeratins, and the remainder are frozen for future biomarker analysis. Stained slides are scanned using fibreoptic array scanning technology (FAST) and acquired images are analysed by image-processing software that selects candidate CTCs. With this technology, data are reported on a number of CTC phenotypes including traditional CTCs, CTC clusters, small CTCs, cytokeratin-negative CTCs, and apoptotic CTCs (FIG. 4).

In an early report using this novel selection-free approach, Marrinucci and colleagues[35] found >5 CTCs per 1 ml of blood in 80% of tested patients with metastatic prostate cancer. When compared to the CellSearch test, their assay detected ≥1 CTC in over twice as many patients (40% versus 93%)[35]. This test has since gone through rigorous technical and analytical validation[33,34] and is now currently offered as a Clinical Laboratory Improvement Amendments (CLIA) certified assay[36].

The strength of the above test does not lie solely in its ability to detect more CTCs than CellSearch. Additional notable features include the ability for the user to introduce disease-specific and pharmacodynamic markers as well as to perform downstream molecular assays on identified cells. An example of these aspects of the test were prominently featured in a study by Dago *et al.*[37] in which the authors explored AR protein levels and genomic alterations present in CTCs of a single patient undergoing systemic therapy for mCRPC. Using this technique in combination with measurement of genomic copy number variation profiles, the authors were able to identify three distinct CTC lineages arising in response to systemic therapy. Punnoose and colleagues[38] also demonstrated the potential of the Epic Sciences test with the development of an assay that includes fluorescence *in situ* hybridization (FISH) for the *PTEN* tumour suppressor. These authors found that men with *PTEN* loss detected in CTCs had worse outcomes compared with patients with preservation of this gene. Finally, this assay has also been applied to patients with neuroendocrine prostate cancer in order to determine unique staining and morphological criteria for detecting CTCs in this patient population[39]. Notably, compared to CTCs from patients with mCRPC, CTCs from patients with neuroendocrine prostate cancer were found to be of smaller size and contain abnormal nuclear and cytoplasmic features. In addition, neuroendocrine patients had a higher prevalence of low-cytokeratin-expressing CTCs.

Epic Sciences is not the only company to use high-throughput imaging for CTC detection. The AccuCyte CyteFinder system is a similar imaging-based platform that has been commercialized by RareCyte, Seattle, WA[40]. With this system, density-based separation is first used to isolate nucleated blood cells from

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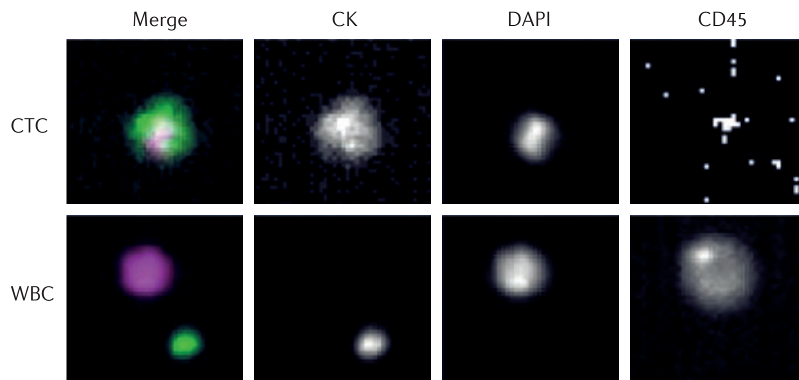

Figure 2 | **Representative images of a CTC and WBC detected using the CellSearch test.** Circulating tumour cells (CTCs) are nucleated cells that are positive for cytokeratins (CK) but negative for CD45. White blood cells (WBCs) are nucleated and positive for CD45. Figure provided by Dr Daren Davis, *et al.* (ApoCell Inc., USA).

red blood cells using the AccuCyte kit. The isolated cell fraction is then smeared onto glass slides and stained with fluorescently labelled antibodies of the investigator's choosing. High-throughput imaging is employed to identify candidate CTCs with the CyteFinder scanning microscope and image analysis tools. A unique feature of the system is the integration of a single-cell micromanipulator termed the CytePicker. Using this retrieval device, single cells can be retrieved from stained glass slides for downstream molecular analysis, including DNA sequencing and array comparative genomic hybridization. In contrast to the Epic Sciences test, the RareCyte platform is a distributed technology for local laboratory investigation of CTCs rather than a service provided by a commercial company.

In an initial report describing the RareCyte system, Campton *et al.*[40] showed >90% recovery efficiency across tested cell lines, including the PC3 and LNCaP prostate cell lines. In a head-to-head comparison with the CellSearch system, RareCyte identified on average 34% more CTCs in 17 patients with metastatic prostate cancer[41]. Similar results were also observed in samples from patients with lung and breast cancer. The authors attribute these differences in CTC counts to the ability of the RareCyte system to detect CTCs with low EpCAM levels (FIG. 3). At the 2015 Prostate Cancer Foundation 22nd Scientific Retreat, Kaldjian *et al.*[42] presented an updated version of the RareCyte platform that offers simultaneous six-colour staining. These additional channels greatly expand the phenotyping potential of this assay.

Microfluidic devices

Microfluidic devices offer yet another approach for CTC isolation[43–52]. The herringbone-chip (HB-chip) is a notable example of a microfluidic device that has been used for the analysis of CTCs in patients with prostate cancer[44,45]. This device uses microgrooves that generate microvortices to direct CTCs towards anti-EpCAM antibodies coating the chip's surface. CTCs are then stained, imaged, and analysed directly on the device. Using this technology, Stott *et al.*[44] identified CTCs in 14 of 15 patients (93%) with metastatic prostate cancer and demonstrated the feasibility of downstream molecular characterization using RT-PCR analysis for the *TMPRSS2–ERG* translocation.

In a second study using the HB-chip, Miyamoto *et al.*[45] developed an assay for detecting AR signalling status of prostate cancer CTCs. Taking advantage of the fact that PSA and PSMA are inversely regulated in response to AR signalling, the authors were able to classify CTCs as AR-on (PSA$^+$ and PSMA$^-$), AR-mixed (PSA$^+$ and PSMA$^+$), or AR-off (PSA$^-$ and PSMA$^+$). Using this designation of AR-signalling status, the authors showed that CTCs in patients with untreated metastatic prostate cancer were consistently of the AR-on phenotype. Additionally, they observed that CTCs consistently switched to an AR-off status upon initiating androgen-deprivation therapy. In applying this same assay to patients with mCRPC, the authors found marked interpatient and intrapatient heterogeneity in AR status, both at baseline and in response to second-line hormonal therapy. Interestingly, patients with a baseline level of >10% AR-mixed CTCs experienced worse overall survival than patients with lower numbers of these cells, suggesting a possible role for this test in guiding therapy in men with mCRPC.

Although the HB-Chip has proven to be highly amenable to the study of prostate cancer CTCs, its reliance on EpCAM-based cell capture prompted the development of the CTC-iChip[49,50]. This novel microfluidic technology enables either positive or negative selection of CTCs following three sequential processing steps: first, separation of nucleated cells from red blood cells and platelets using deterministic lateral displacement; second, alignment of nucleated cells within a microfluidic channel using inertial focusing; and third, deflection of magnetically labelled cells into a collection channel. In initial testing, this device enabled an average 3.8-log depletion of white blood cells and a 97% recovery rate of rare cells[50]. Miyamoto and co-workers[51] used the CTC-iChip to perform RNA-sequencing of 77 intact CTCs from 13 men with prostate cancer and showed that AR-targeted drug resistance was associated with activation of noncanonical Wnt signalling.

Bladder cancer

Although not as vast as the body of work that has been generated in prostate cancer, several studies have explored CTCs as biomarkers in patients with bladder cancer. With the exception of several early reports using RT-PCR-based approaches[53–57], nearly all studies to date have employed the CellSearch test[58–65].

To our knowledge, the first study to evaluate CellSearch in patients with bladder cancer was performed by Naoe and co-workers in 2007 (REF. 58). Before assaying patient samples, the authors performed recovery experiments by spiking HT1197 bladder cancer cells into blood samples of healthy donor controls. Consistent with the performance characteristics of CellSearch in other malignancies, this test demonstrated a recovery rate of approximately 80%. The feasibility of using CellSearch was then tested in patients with localized ($n = 12$) and metastatic ($n = 14$) urothelial carcinoma. In total, 10 (71.4%) patients with metastatic disease had ≥1 detectable CTC

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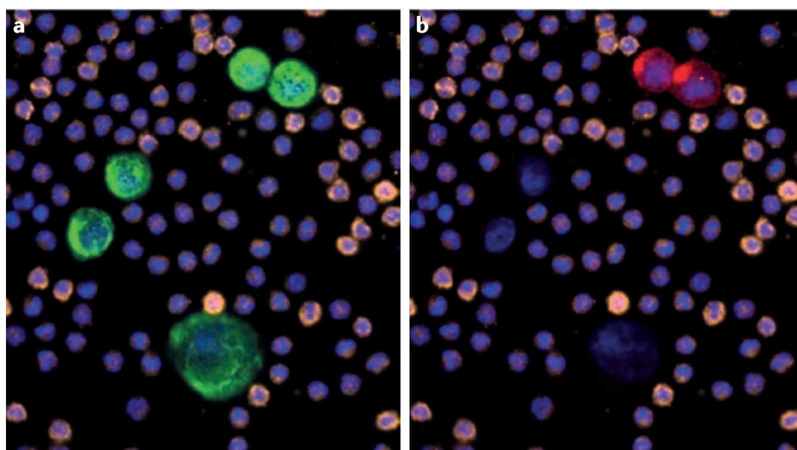

Figure 3 | **PC3 prostate cancer cells spiked into white blood cells. a** | The PC3 cells stain positively for cytokeratin (green), whereas the white blood cells stain positively for CD45 (orange). **b** | The cells were additionally stained using a fluorescently labelled antibody for epithelial cell adhesion molecule (EpCAM; red). Note, the marked heterogeneity in the EpCAM staining pattern of the PC3 cells despite the universally strong cytokeratin staining. Circulating tumour cells (CTCs) with low EpCAM expression would likely not be detected with methods that positively select cells using this cell-surface protein. Thus, many in the field of CTC research now favour selection-free approaches for CTC detection. Figure provided by Dr Eric Kaldjian, *et al.* (RareCyte Inc., Seattle, WA).

per 7.5 ml of blood, whereas no patient with localized bladder cancer had any detectable. Notably, the majority of the cohort with nonmetastatic disease (66.7%) had non-muscle-invasive bladder cancer (NMIBC), limiting the likelihood of occult micrometastatic disease in this small patient cohort.

In another early study, Gallagher and co-workers[59] evaluated the performance of the CellSearch test in 33 patients with untreated or progressive metastatic urothelial carcinoma. This cohort included 20 (61%) patients with a primary tumour of the bladder and 12 (36%) with a primary tumour of the upper urinary tract (in one patient the primary was unknown). In total, 14 (42%) patients had ≥1 detectable CTC (range 0–87) per 7.5 ml of blood. A significantly higher number of CTCs were observed in patients with ≥2 metastatic sites (3.5 versus 0 CTCs, $P = 0.04$), a finding that has since been replicated by other researchers[60].

Rink *et al.*[61] next evaluated the prognostic utility of the CellSearch test in 55 patients undergoing radical cystectomy for urothelial carcinoma of the bladder. This cohort included 50 patients with clinically localized bladder cancer and five patients with distant metastatic disease. In total, 30% of patients with localized bladder cancer had ≥1 CTC per 7.5 ml of blood (median 1, range 1–11). By contrast, all five patients with metastatic disease had detectable CTCs (median 2, range 1–372). Notably, the authors observed that patients with ≥1 CTCs had worse oncological outcomes than those with <1 CTC, including shorter times to disease recurrence and cancer-specific death. These findings were later validated in a larger cohort of 100 patients with nonmetastatic bladder cancer undergoing radical cystectomy[62]. After controlling for standard clinicopathological features, the authors observed adjusted hazard ratios of 4.60 (95% CI 1.95–10.8), 5.52 (95% CI 1.85–14.73), and 3.50 (95% CI 1.52–8.02) for disease recurrence, cancer-specific death, and overall mortality, respectively. Although the presence of CTCs provides prognostic information in patients undergoing cystectomy, the presence of these rare cells does not seem to correlate with adverse pathological features, such as extent of local tumour invasion or lymph node involvement[61–63].

The CellSearch test has also been evaluated in patients with NMIBC[64,65]; Gazzaniga *et al.*[64] showed that CTCs were detectable in 18% of such patients. Notably, the subset of patients with ≥1 CTC were at a higher risk than patients with no detectable CTCs in terms of tumour recurrence (87.5% versus 36%) and progression to muscle-invasive disease (87.5% versus 0%). However, the study included a heterogeneous population of patients with varying tumour stages and grades. In a second study that included a more homogeneous cohort of patients with high-grade T1 bladder cancer, Gazzaniga *et al.*[65] observed that patients with ≥1 detectable CTC were at a markedly higher risk of disease recurrence (HR 2.92, 95% CI 1.38–6.18) and progression (HR 7.17, 95% CI 1.89–27.21) than those without detectable CTCs. Combined, the two studies demonstrate the potential of the CellSearch test to identify patients with clinically understaged bladder cancer who might benefit from radical cystectomy and/or systemic treatment instead of intravesical therapy.

Kidney cancer

Compared with prostate and bladder cancer, CTCs have been much less well studied in kidney cancer. As with bladder cancer, early attempts at detecting CTCs in patients with renal cell carcinoma (RCC) focused on RT-PCR-based approaches. For example, in 1999 McKiernan *et al.*[66] described a method for detecting CTCs on the basis of carbonic anhydrase IX (*CAIX*) expression, a gene that is ubiquitously expressed by the clear cell subtype of RCC (ccRCC). In their report, the authors tested blood from 36 patients with ccRCC (27 with localized RCC and nine with metastatic disease) and found a detectable signal in 18 (50%) of patients. Demonstrating the specificity of their assay, signal was detected in zero of five patients with a benign renal tumour and in only one of 55 (1.8%) healthy donor controls. In a follow-up study, Gilbert *et al.*[67] went on to report that the detection of *CAIX* by RT-PCR in the blood of patients with localized renal tumours was associated with a higher rate of disease recurrence (5-year disease-free survival 39.5% versus 88.1%, $P = 0.048$). Similarly, work from other groups has investigated using RT-PCR for detecting cadherin 6 and showed comparable rates of CTC detection in both the localized and metastatic setting[68–70]. Although promising, these techniques have failed to be clinically validated beyond these early reports and concerns remain regarding the amplification of contaminating nonspecific transcripts.

Cellular-based assays have also been employed for the detection of CTCs in patients with kidney cancer. For example, in the initial report validating the CellSearch system, blood was tested from 11 patients with RCC[4].

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Unlike the experience with prostate and bladder cancer, a mean of only one CTC was detected in patients with metastatic kidney cancer, and only 25% of patients had ≥2 CTCs. Consistent with this observation, Gradiloine *et al.*[71] reported detecting ≥1 CTC with the CellSearch test in only 16% of patients with metastatic RCC. This low frequency of CTCs detected by CellSearch is likely explained by the observation that only 20–40% of ccRCC tissue specimens express the EpCAM surface protein, despite the epithelial origin of this malignancy[72,73].

The lack of EpCAM expression on ccRCC has prompted the development of alternative approaches for CTC detection in this malignancy, including methods of negative selection[74,75], size exclusion[76], and positive selection using RCC-specific markers[77]. Perhaps most promising is the method described by Liu and co-workers[77] using a microfluidic device known as the NanoVelco chip, which is composed of chaotic mixer and a nanowire substrate coated with antibodies against *CAIX* and *CD147*. Before implementing this positive-selection-based method, the investigators validated the expression of these two markers in ccRCC tissue specimens, demonstrating that when used in combination 97.1% of tumours were positive for these markers, in sharp contrast to their observation that only 18.6% of tumours expressed EpCAM. Using these markers for positive selection, Liu *et al.*[77] were able to detect cytokeratin-positive CTCs in 72 of 76 (94.7%) of patients with ccRCC. In comparison, no cells were detected in 13 of 15 (86.7%) healthy donors. Furthermore, they observed that the number of detected CTCs was associated with clinical stage, with 1.2-fold more CTCs present in patients with stage III and IV disease, compared with those with stage I and II cancer. Additionally, the team found that concomitant expression of vimentin (a mesenchymal marker) was associated with higher stage disease, suggesting the potential of this marker to aid in disease prognostication.

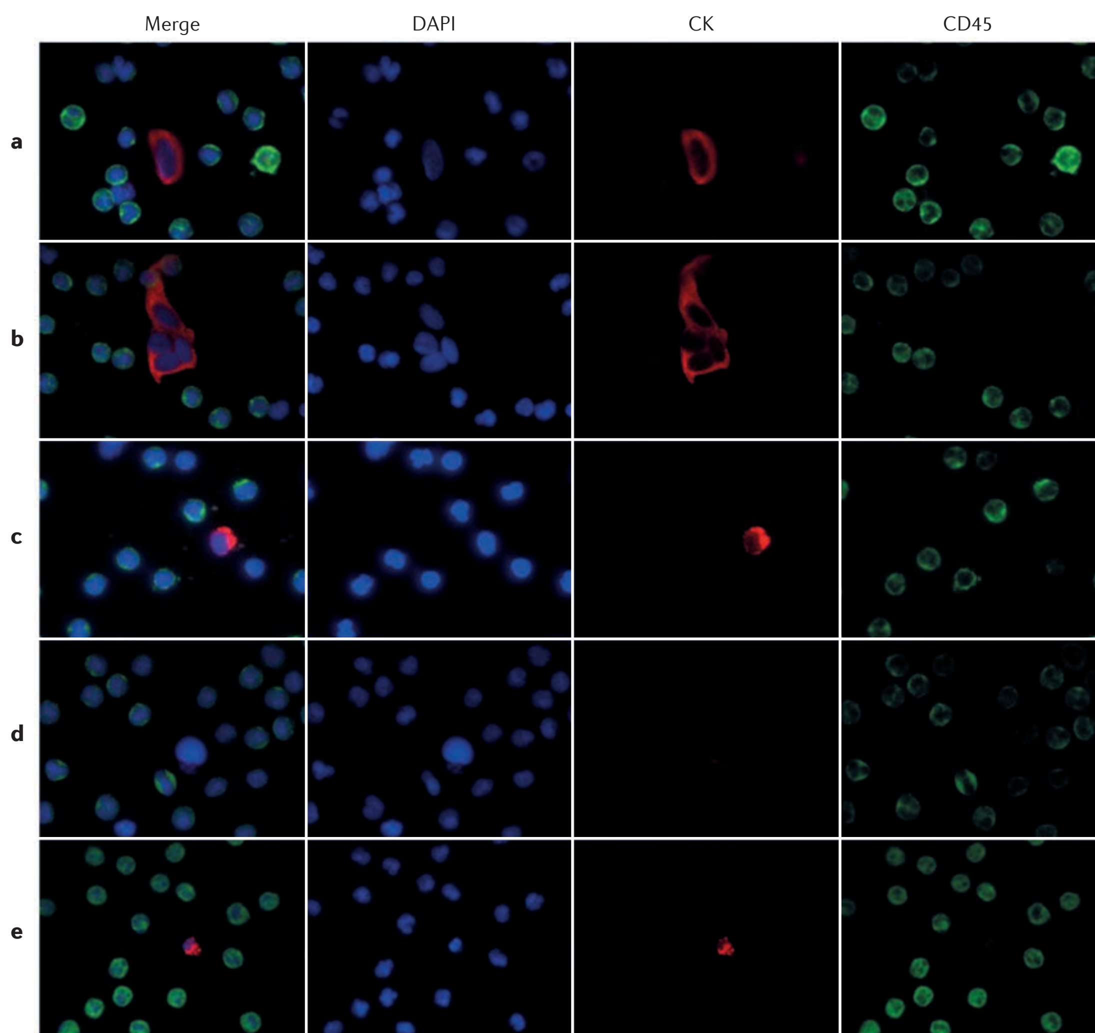

Figure 4 | **Representative CTC subtypes detected by the Epic platform. a** | Traditional circulating tumour cells (CTCs) (4',6-diamidino-2-phenylindole (DAPI)+, cytokeratin (CK)+, CD45–). **b** | CTC clusters (≥2 adjacent traditional DAPI+, CK+, CD45– CTCs that share cytoplasmic boundaries). **c** | Small CTCs (DAPI+, CK+, CD45– with similar nuclear size to surrounding white blood cells). **d** | CK-negative CTCs (DAPI+, CK–, CD45–). **e** | Apoptotic CTCs (CK+, CD45–, with DAPI staining pattern consistent with chromosomal condensation and/or nuclear fragmentation). Figure provided by Dr Peter Kuhn and co-workers (University of Southern California, USA).

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Conclusions

CTCs have been explored as biomarkers of prostate, bladder and kidney cancer. To date, the majority of work has been performed using the CellSearch test. However, other novel CTC technologies have had a sizeable effect on the field, particularly in terms of informing our understanding of basic cancer biology. For prostate cancer, the CellSearch test has been shown to provide prognostic information in several large clinical trials. Studies aimed at using CTCs to identify individuals with mCRPC who harbour AR variants, such as AR-V7 and are unlikely to respond to second-line androgen-pathway-targeted therapies, such as abiraterone and enzalutamide, might, perhaps, have a greater effect on patient outcomes. CTCs have also shown promise as prognostic biomarkers in patients undergoing radical cystectomy for bladder cancer. Additionally, emerging data support a potential role for CTCs to help identify patients with NMIBC who could harbour unrecognized advanced disease. Finally, CTCs have also been studied as biomarkers of kidney cancer, but the data for this disease are the least mature of the three malignancies covered in this Review. Based on available data, non-EpCAM-based approaches seem necessary in this disease owing to low levels of EpCAM expression by RCC.

1. Ashworth, T. R. A case of cancer in which cells similar to those in the tumors were seen in the blood after death. *Aust. Med. J.* **14**, 146–149 (1869).
2. Loberg, R. D. *et al.* Detection and isolation of circulating tumor cells in urologic cancers: a review. *Neoplasia* **6**, 302–309 (2004).
3. Joosse, S. A., Gorges, T. M. & Pantel, K. Biology, detection, and clinical implications of circulating tumor cells. *EMBO Mol. Med.* **7**, 1–11 (2015).
4. Allard, W. J. *et al.* Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. *Clin. Cancer Res.* **10**, 6897–6904 (2004).
5. Cristofanilli, M. *et al.* Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *N. Engl. J. Med.* **351**, 781–791 (2004).
6. Cristofanilli, M. *et al.* Circulating tumor cells: a novel prognostic factor for newly diagnosed metastatic breast cancer. *J. Clin. Oncol.* **23**, 1420–1430 (2005).
7. Hayes, D. F. *et al.* Circulating tumor cells at each follow-up time point during therapy of metastatic breast cancer patients predict progression-free and overall survival. *Clin. Cancer Res.* **12**, 4218–4224 (2006).
8. Budd, G. T. *et al.* Circulating tumor cells versus imaging — predicting overall survival in metastatic breast cancer. *Clin. Cancer Res.* **12**, 6403–6409 (2006).
9. Cohen, S. J. *et al.* Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. *J. Clin. Oncol.* **26**, 3213–3221 (2008).
10. Shaffer, D. R. *et al.* Circulating tumor cell analysis in patients with progressive castration-resistant prostate cancer. *Clin. Cancer Res.* **13**, 2023–2029 (2007).
11. Danila, D. C. *et al.* Circulating tumor cell number and prognosis in progressive castration-resistant prostate cancer. *Clin. Cancer Res.* **13**, 7053–7058 (2007).
12. de Bono, J. S. *et al.* Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. *Clin. Cancer Res.* **14**, 6302–6309 (2008).
13. Okegawa, T., Nutahara, K. & Higashihara, E. Prognostic significance of circulating tumor cells in patients with hormone refractory prostate cancer. *J. Urol.* **181**, 1091–1097 (2009).
14. Leversha, M. A. *et al.* Fluorescence *in situ* hybridization analysis of circulating tumor cells in metastatic prostate cancer. *Clin. Cancer Res.* **15**, 2091–2097 (2009).
15. Helo, P. *et al.* Circulating prostate tumor cells detected by reverse transcription-PCR in men with localized or castration-refractory prostate cancer: concordance with CellSearch assay and association with bone metastases and with survival. *Clin. Chem.* **55**, 765–773 (2009).
16. Goodman, O. B. *et al.* Circulating tumor cells in patients with castration-resistant prostate cancer baseline values and correlation with prognostic factors. *Cancer Epidemiol. Biomarkers Prev.* **18**, 1904–1913 (2009).
17. Goldkorn, A. *et al.* Circulating tumor cell counts are prognostic of overall survival in SWOG S0421: a phase III trial of docetaxel with or without atrasentan for metastatic castration-resistant prostate cancer. *J. Clin. Oncol.* **32**, 1136–1142 (2014).
18. Crespo, M. *et al.* Androgen receptor expression in circulating tumour cells from castration-resistant prostate cancer patients treated with novel endocrine agents. *Br. J. Cancer* **112**, 1166–1174 (2015).
19. Mehra, N., Zafeiriou, Z., Lorente, D., Terstappen, L. W. & de Bono, J. S. CCR 20th anniversary commentary: circulating tumor cells in prostate cancer. *Clin. Cancer Res.* **21**, 4992–4995 (2015).
20. Todenhöfer, T. *et al.* Preliminary experience on the use of the Adnatest® system for detection of circulating tumor cells in prostate cancer patients. *Anticancer Res.* **32**, 3507–3513 (2012).
21. Antonarakis, E. S. *et al.* AR-V7 and resistance to enzalutamide and abiraterone in prostate cancer. *N. Engl. J. Med.* **371**, 1028–1038 (2014).
22. Dehm, S. M., Schmidt, L. J., Heemers, H. V., Vessella, R. L. & Tindall, D. J. Splicing of a novel androgen receptor exon generates a constitutively active androgen receptor that mediates prostate cancer therapy resistance. *Cancer Res.* **68**, 5469–5477 (2008).
23. Hu, R. *et al.* Ligand-independent androgen receptor variants derived from splicing of cryptic exons signify hormone-refractory prostate cancer. *Cancer Res.* **69**, 16–22 (2009).
24. Steinestel, J. *et al.* Detecting predictive androgen receptor modifications in circulating prostate cancer cells. *Oncotarget* http://dx.doi.org/ 10.18632/oncotarget.3925 (2015).
25. Nakazawa, M. *et al.* Serial blood-based analysis of AR-V7 in men with advanced prostate cancer. *Ann. Oncol.* **26**, 1859–1865 (2015).
26. Onstenk, W. *et al.* Efficacy of cabazitaxel in castration-resistant prostate cancer is independent of the presence of AR-V7 in circulating tumor cells. *Eur. Urol.* **68**, 939–945 (2015).
27. Gorges, T. M. *et al.* Circulating tumour cells escape from EpCAM-based detection due to epithelial-to-mesenchymal transition. *BMC Cancer* **12**, 178 (2012).
28. de Wit, S. *et al.* The detection of EpCAM$^+$ and EpCAM$^-$ circulating tumor cells. *Sci. Rep.* **17**, 12270 (2015).
29. Nauseef, J. T. & Henry, M. D. Epithelial-to-mesenchymal transition in prostate cancer: paradigm or puzzle? *Nat. Rev. Urol.* **8**, 428–439 (2011).
30. Li, P., Yang, R. & Gao, W.-Q. Contributions of epithelial-mesenchymal transition and cancer stem cells to the development of castration resistance of prostate cancer. *Mol. Cancer* **13**, 55 (2014).
31. Jadaan, D. Y., Jadaan, M. M. & McCabe, J. P. Cellular plasticity in prostate cancer bone metastasis. *Prostate Cancer* **2015**, 651580 (2015).
32. Krivacic, R. T. *et al.* A rare-cell detector for cancer. *Proc. Natl Acad. Sci. USA* **101**, 10501–10504 (2004).
33. Werner, S. L. *et al.* Analytical validation and capabilities of the epic CTC platform: enrichment-free circulating tumour cell detection and characterization. *J. Circ. Biomarkers* http://dx.doi.org/10.5772/60725 (2015).
34. Lu, D. *et al.* Detection and characterization of circulating tumour cells from frozen peripheral blood mononuclear cells. *J. Circ. Biomarkers* http://dx.doi.org/10.5772/60745 (2015).
35. Marrinucci, D. *et al.* Fluid biopsy in patients with metastatic prostate, pancreatic and breast cancers. *Phys. Biol.* **9**, 016003 (2012).
36. Epic Sciences receives CLIA certification for its cancer diagnostics laboratory. *EpicSciences* [online]. http://www.epicsciences.com/news-events/press-releases/epic-sciences-receives-clia-certification-its-cancer-diagnostics-laboratory/ (2016).
37. Dago, A. E. *et al.* Rapid phenotypic and genomic change in response to therapeutic pressure in prostate cancer inferred by high content analysis of single circulating tumor cells. *PLoS ONE* **9**, e101777 (2014).
38. Punnoose, E. A. *et al.* PTEN loss in circulating tumour cells correlates with PTEN loss in fresh tumour tissue from castration-resistant prostate cancer patients. *Br. J. Cancer* **113**, 1225–1233 (2015).
39. Beltran, H. *et al.* The initial detection and partial characterization of circulating tumor cells in neuroendocrine prostate cancer. *Clin. Cancer Res.* http://dx.doi.org/10.1158/1078-0432.CCR-15-0137 (2016).
40. Campton, D. E. *et al.* High-recovery visual identification and single-cell retrieval of circulating tumor cells for genomic analysis using a dual-technology platform integrated with automated immunofluorescence staining. *BMC Cancer* **15**, 360 (2015).
41. Stilwell, J. L. *et al.* Clinical performance of the AccuCyte®–CyteFinder® System, a dual-technology platform for comprehensive collection and high resolution imaging of circulating tumor cells [abstract]. *Cancer Res.* **75**, 1601 (2015).
42. Kaldjian, E. *et al.* Multi-level analysis of circulating tumor cells in advanced prostate cancer using AccuCyte®–CyteFinder®. *Proc. 22nd Annu. Prostate Cancer Found. Scientif. Retreat* [online] http://www.pcf.org/prostate-cancer-research/2015-scientific-retreat/abstracts (2015).
43. Nagrath, S. *et al.* Isolation of rare circulating tumour cells in cancer patients by microchip technology. *Nature* **450**, 1235–1239 (2007).
44. Stott, S. L. *et al.* Isolation of circulating tumor cells using a microvortex-generating herringbone-chip. *Proc. Natl Acad. Sci. USA* **107**, 18392–18397 (2010).
45. Miyamoto, D. T. *et al.* Androgen receptor signaling in circulating tumor cells as a marker of hormonally responsive prostate cancer. *Cancer Discov.* **2**, 995–1003 (2012).
46. Gupta, V. *et al.* ApoStream™, a new dielectrophoretic device for antibody independent isolation and recovery of viable cancer cells from blood. *Biomicrofluidics* **6**, 24133 (2012).
47. Casavant, B. P. *et al.* A negative selection methodology using a microfluidic platform for the isolation and enumeration of circulating tumor cells. *Methods* **64**, 137–143 (2013).
48. Casavant, B. P. *et al.* The VerIFAST: an integrated method for cell isolation and extracellular/intracellular staining. *Lab. Chip* **13**, 391–396 (2013).
49. Ozkumur, E. *et al.* Inertial focusing for tumor antigen-dependent and -independent sorting of rare circulating tumor cells. *Sci. Transl Med.* **5**, 179ra47 (2013).
50. Karabacak, N. M. *et al.* Microfluidic, marker-free isolation of circulating tumor cells from blood samples. *Nat. Protoc.* **9**, 694–710 (2014).
51. Miyamoto, D. T. *et al.* RNA-Seq of single prostate CTCs implicates noncanonical Wnt signaling in antiandrogen resistance. *Science* **349**, 1351–1356 (2015).
52. Gogoi, P. *et al.* Development of an automated and sensitive microfluidic device for capturing and characterizing circulating tumor cells (CTCs) from clinical blood samples. *PLoS ONE* **11**, e0147400 (2016).
53. Lu, J. J. *et al.* Detection of circulating cancer cells by reverse transcription-polymerase chain reaction for uroplakin II in peripheral blood of patients with urothelial cancer. *Clin. Cancer Res.* **6**, 3166–3171 (2000).

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54. Retz, M. *et al.* Cytokeratin-20 reverse-transcriptase polymerase chain reaction as a new tool for the detection of circulating tumor cells in peripheral blood and bone marrow of bladder cancer patients. *Eur. Urol.* **39**, 507–515 (2001).
55. Osman, I. *et al.* Detection of circulating cancer cells expressing uroplakins and epidermal growth factor receptor in bladder cancer patients. *Int. J. Cancer* **111**, 934–939 (2004).
56. Ribal, M. J. *et al.* Molecular staging of bladder cancer with RT-PCR assay for CK20 in peripheral blood, bone marrow and lymph nodes: comparison with standard histological staging. *Anticancer Res.* **26**, 411–419 (2006).
57. Gradilone, A. *et al.* Prognostic significance of survivin-expressing circulating tumour cells in T1G3 bladder cancer. *BJU Int.* **106**, 710–715 (2010).
58. Naoe, M. *et al.* Detection of circulating urothelial cancer cells in the blood using the CellSearch System. *Cancer* **109**, 1439–1445 (2007).
59. Gallagher, D. J. *et al.* Detection of circulating tumor cells in patients with urothelial cancer. *Ann. Oncol.* **20**, 305–308 (2009).
60. Okegawa, T., Hayashi, K., Hara, H., Nutahara, K. & Higashihara, E. Immunomagnetic quantification of circulating tumor cells in patients with urothelial cancer. *Int. J. Urol.* **17**, 254–258 (2010).
61. Rink, M. *et al.* Detection of circulating tumour cells in peripheral blood of patients with advanced non-metastatic bladder cancer. *BJU Int.* **107**, 1668–1675 (2011).
62. Rink, M. *et al.* Prognostic role and HER2 expression of circulating tumor cells in peripheral blood of patients prior to radical cystectomy: a prospective study. *Eur. Urol.* **61**, 810–817 (2012).
63. Guzzo, T. J. *et al.* The presence of circulating tumor cells does not predict extravesical disease in bladder cancer patients prior to radical cystectomy. *Urol. Oncol.* **30**, 44–48 (2012).
64. Gazzaniga, P. *et al.* Prognostic value of circulating tumor cells in nonmuscle invasive bladder cancer: a CellSearch analysis. *Ann. Oncol.* **23**, 2352–2356 (2012).
65. Gazzaniga, P. *et al.* Circulating tumor cells detection has independent prognostic impact in high-risk non-muscle invasive bladder cancer. *Int. J. Cancer* **135**, 1978–1982 (2014).
66. McKiernan, J. M. *et al.* The detection of renal carcinoma cells in the peripheral blood with an enhanced reverse transcriptase-polymerase chain reaction assay for MN/CA9. *Cancer* **86**, 492–497 (1999).
67. Gilbert, S. M. *et al.* Detection of carbonic anhydrase-9 gene expression in peripheral blood cells predicts risk of disease recurrence in patients with renal cortical tumors. *Urology* **67**, 942–945 (2006).
68. Shimazui, T. *et al.* Detection of cadherin-6 mRNA by nested RT-PCR as a potential marker for circulating cancer cells in renal cell carcinoma. *Int. J. Oncol.* **23**, 1049–1054 (2003).
69. Shimazui, T. *et al.* The level of cadherin-6 mRNA in peripheral blood is associated with the site of metastasis and with the subsequent occurrence of metastases in renal cell carcinoma. *Cancer* **101**, 963–968 (2004).
70. Li, G. *et al.* Cadherin-6 gene expression in conventional renal cell carcinoma: a useful marker to detect circulating tumor cells. *Anticancer Res.* **25**, 377–381 (2005).
71. Gradilone, A. *et al.* Circulating tumor cells and 'suspicious objects' evaluated through CellSearch® in metastatic renal cell carcinoma. *Anticancer Res.* **31**, 4219–4221 (2011).
72. Went, P. *et al.* Expression of epithelial cell adhesion molecule (EpCam) in renal epithelial tumors. *Am. J. Surg. Pathol.* **29**, 83–88 (2005).
73. Zimpfer, A. *et al.* Prognostic and diagnostic implications of epithelial cell adhesion/activating molecule (EpCAM) expression in renal tumours: a retrospective clinicopathological study of 948 cases using tissue microarrays. *BJU Int.* **114**, 296–302 (2014).
74. Blümke, K. *et al.* Detection of circulating tumor cells from renal carcinoma patients: experiences of a two-center study. *Oncol. Rep.* **14**, 895–899 (2005).
75. Bluemke, K. *et al.* Detection of circulating tumor cells in peripheral blood of patients with renal cell carcinoma correlates with prognosis. *Cancer Epidemiol. Biomarkers Prev.* **18**, 2190–2194 (2009).
76. El-Heliebi, A. *et al.* Are morphological criteria sufficient for the identification of circulating tumor cells in renal cancer? *J. Transl Med.* **11**, 214 (2013).
77. Liu, S. *et al.* Combined cell surface carbonic anhydrase 9 and CD147 antigens enable high-efficiency capture of circulating tumor cells in clear cell renal cell carcinoma patients. *Oncotarget* http://dx.doi.org/10.18632/oncotarget.10979 (2016).

Author contributions

M.A.G. and J.V. researched data for the article and wrote the manuscript. All authors made a substantial contribution to discussion of content and reviewed and edited the manuscript before submission.

Competing interests statement

K.J.P. is a member of the advisory board to Celsee Diagnostics. The other authors declare no competing interests.

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