Review Article on Circulating Tumor Cells

Circulating tumor cells isolation: the "post-EpCAM era"

Cristina Raimondi, Chiara Nicolazzo, Angela Gradilone

Dipartimento Medicina Molecolare, Sapienza Università di Roma, Roma, Italy

Correspondence to: Angela Gradilone. Dipartimento Medicina Molecolare, Sapienza Università di Roma, Viale Regina Elena 324, 00161, Roma, Italy. Email: angela.gradilone@uniroma1.it.

Abstract: Circulating tumor cells (CTCs) represent a submicroscopic fraction detached from a primary tumor and in transit to a secondary site. The prognostic significance of CTCs in metastatic cancer patients was demonstrated for the first time more than ten years ago. To date, it seems clear enough that CTCs are highly heterogeneous and dynamically change their shape. Thus, the inadequacy of epithelial cell adhesion molecule (EpCAM) as universal marker for CTCs detection seems unquestionable and alternative methods able to recognize a broader spectrum of phenotypes are definitely needed. In this review the pleiotropic functions of EpCAM are discussed in detail and the role of the molecule in the biology of CTCs is critically dissected.

Keywords: Epithelial cell adhesion molecule (EpCAM); circulating tumor cells (CTCs); epithelial-mesenchymal transition; EpICD

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Introduction

In the last decades, the interest of molecular oncologists has been focused on the evolving pathways of cancer metastasis, fuelled by the possibility to isolate circulating tumor cells (CTCs) in the blood of cancer patients (1-3).

Studies performed on cells forming a tumor have demonstrated that epithelial tumor cells exhibit epithelial properties and express on their surface molecules of epithelial origin (4). The ubiquitous expression of the epithelial cell adhesion molecule (EpCAM) in epithelial tumor cells allows to differentiate CTCs from blood cells and represents the rationale for the establishment of the so-called EpCAM based methods for the isolation of CTCs. To date, it seems clear enough that CTCs are highly heterogeneous and dynamically change their shape (5,6). Indeed, a large amount of date demonstrate that in cancer the expression of epithelial surface markers might be transiently lost during the epithelial to mesenchymal transition (EMT) process, to enable tumor cells to detach from primary tumor and to circulate into the bloodstream (7-9). Similarly, the same epithelial traits might be re-acquired during the reverse process of mesenchymal to epithelial transition (MET), to allow cell to cell interactions and cancer cell growth in distant organs (10). The inadequacy of EpCAM as universal marker for CTCs detection seems thus unquestionable and alternative methods able to recognize a broader spectrum of phenotypes are definitely needed. To date, it is possible to isolate cancer cells circulating in the blood basing on their biological and/or physical properties (11).

CTCs count and molecular evaluation may provide a source for molecular analysis over the time of tumors during the clinical management of patients, and this is supposed to facilitate both clinical investigations and cancer patient care. The prognostic significance of CTCs in metastatic cancer patients was demonstrated for the first time more than ten years ago, in the pivotal study by Cristofanilli *et al.* and paved the way for the Food and Drug Administration (FDA) clearance for the CellSearch system (12-14). This semi-automated platform relies on the expression of EpCAM on the surface of the CTCs, being thus limited in its performance by the intrinsic variability of these cells. In this review we illustrate the mechanisms by which EpCAM can be down-regulated in CTCs and dissect the functional consequences of EpCAM loss.

EpCAM: a mainstay in CTC detection

The EpCAM (CD326) is a cell surface glycoprotein, originally identified as tumor antigen, being highly expressed in epithelial cancers and at lower levels in normal epithelia (15). The contemporary discovery of this molecule by different research groups, led to the use of different names for the same molecule, based on monoclonal antibodies or cDNA clones (16). In 2007, in a two days meeting focused on EpCAMs, "EpCAM" has been unanimously suggested as exclusive name (17).

EpCAM is a type I transmembrane glycoprotein of about 39-42 KDa, containing three N-glycosylation sites (6,10) all glycosylated as demonstrated in human and murine cell lines, although glycosylation of Asn198 seems to be of high importance for EpCAM's cell surface expression and protein stability (18).

EpCAM forms a complex called tetraspanin-enriched microdomains (TEMs) with tetraspanins CD9 and CO-029 (tetraspanin8), and with CD44v4-7variant isoform (19). This complex promotes EpCAM-specific functions as apoptosis resistance, cell proliferation and tumorigenicity (19,20). Moreover association of EpCAM, Claudin7 and TEMs, rather than the individual molecules promotes tumor progression and facilitates metastasis formation in colorectal cancer (21,22).

To date, our knowledge derived from more than 10 years of cancer research studies, indicates that EpCAM can act as either a tumor promoter or suppressor in human cancers depending on the type of cancer and the tumor microenvironment (23); however the presence of this molecule has been the main feature used to isolate CTC in the EpCAM era.

Although many EpCAM-based approaches have been developed and used in exploring CTCs, the detection rate of these rare cells seems critically dependent on the EpCAM clone used.

Antolovic *et al*. in 2010 demonstrated that the use of different anti EpCAM antibodies may lead to a heterogeneous detection of CTCs in patients with colorectal cancer employing immunomagnetic enrichment with mAb BerEP4 and mAb KS1/4 (24).

EpCAM era was characterized by studies performed by different assays all with the common aim to identify epithelial cells circulating within the blood through the expression of this surface EpCAM. Many assays have been widely developed for CTC enrichment and isolation each one with strength and weakness points.

CellSearch is the only FDA approved method used to obtain prognostic information through CTC count. CellSearch® assay was validated from Allard *et al*. for sensitivity, accuracy, and reproducibility (25). Using the CellSearch® assay, the prognostic value of CTC enumeration in metastatic breast (12,26-28), prostate (13,29) and colon cancer patients has been demonstrated (14,30). In patients with metastatic disease, superior survival was observed among patients with breast and colorectal cancer with a count of CTC fewer than five in a blood sample of 7.5 mL; for metastatic colorectal cancer patients the cut-off value was established <3 CTCs/7.5 mL. The clinical utility of this assay has also been demonstrated in metastatic small and non-small cell lung cancer (31,32), stomach cancer (33), pancreatic cancer (34), ovarian cancer (35), and in advanced (36) and non-muscle invasive bladder cancer (37,38).

According to the first CellSearch® training book, a CTC is characterized by positivity for EpCAM, cytokeratins (CKs), nuclear dye [DAPI (4',6-diamidino-2-phenylindole)] and negativity for CD45.

All images with delineated nuclear but speckled CK, or with cytoplasm area which does not surround the nucleus, are defined as "suspicious objects" and are not counted by the operator as CTCs. The predictive values of all types of suspicious objects were evaluated by Coumans *et al*. using the automated algorithm to identify and reanalyze all objects CD45−, EpCAM+, CK+, and/or DAPI+; all objects predicted OS in their cohort of 179 patients with castration-resistant prostate cancer (39).

Similar results were obtained in our laboratory using CellSearch analysis on renal cancer patients (40) and by other group in early and advanced NSCLC patients (41).

Another main strength of this assay is the possibility to perform an additional analysis with a monoclonal antibody of interest in the additional channel as first performed by Rossi *et al*. (42), using an anti M30 to recognize a neoepitope in CK-18 that becomes available at a caspase cleavage event during apoptosis (43,44). A device that collects the blood discarded after the EpCAM immunomagnetic detection by CellSearch system has been constructed to evaluate all EpCAM negative cells larger than 5 μm. These cells are filtered and immunostained to distinguish CTC from non-CTC. This innovative supplement offers the crucial advantage to recover all EpCAM-cells and to further perform immunostaining using different antibodies (45,46).

The clinical utility of CellSearch analysis as a prognostic test was definitively confirmed by Bidard *et al*., who recently

published the first European pooled analysis on clinical validity of CTC in 1,944 metastatic breast cancer patients treated between 2003 and mid-2012 in 17 centers. This is the largest pooled analysis, aimed to assess the clinical validity of CTC count by the standardized CellSearch® technique. The obtained results led to the conclusion that CTC count is an independent prognostic marker of PFS and OS while CEA (carcinoembryonic antigen) and CA15-3 (cancer antigen 15-3) levels at baseline and during therapy did not add further significant information (47). Despite the prognostic value of CTC enumeration test, the lack of predictive ability to guide decision-making still represents the major obstacle for its validation in clinical practice. The randomized Phase III SWOG S0500 trial was designed to test whether persistently high CTC levels after the first cycle of therapy could be used as an early indicator of disease progression and to determine whether switching at that early point to an alternate chemotherapy regimen would result in improved survival and time to progression among patients with metastatic breast cancer. Although the study confirmed the prognostic value of CTCs, it failed to demonstrate the clinical utility of counting CTCs to evaluate the effectiveness of frontline chemotherapy in metastatic breast cancer patients (48).

Among the molecular-based techniques developed, AdnaTest is a series of commercially available assays that combines the immunomagnetic enrichment of tumor cells and a subsequent multiplex RT-PCR. The potential advantage of this approach is the possibility to simultaneously characterize cells for several additional markers.

In the first step, magnetic bead conjugated with antibody cocktail optimized for breast, colon, ovarian, prostate or EMT/stem cell are used. In the second, the expression of a set of molecular tumor markers is analyzed at RNA level.

Several studies have been performed with this technique, although the results appear discordant (7,49,50).

Although AdnaTest offers an enrichment step based to an additional marker, which is specific for tumor type and the advantage to deepen the molecular pattern of the enriched cells, the real value of this test, seems currently quite limited.

CTCs are isolated by immunomagnetic beads labelled with antibodies against MUC1 and EpCAM. However, MUC1 and EpCAM are also expressed by activated leukocytes, and the mRNA expression of these markers is, therefore, not restricted to CTCs (51) leading to false-positive findings. This test, as others commercially available for CTC detection, has not the potential to evolve as real fluid biopsy, due to its inability to offer live cells for morphological analysis.

For a long time, immunomagnetic separation (IMS) was performed using Dynabeads (52,53). These are magnetic beads coated with antibodies against specific cell surface antigens. Hardingham *et al*. used for the first time Dynabeads as EpCAM based enrichment followed by reverse transcription-polymerase chain reaction (RT-PCR) for detecting CTCs in cancer patients (54).

Furthermore, enrichment of CTCs can be achieved by immunomagnetic depletion of leukocytes with magnetic beads coated anti-CD45 antibody (55,56).

The first generation of microfluidic devices for CTCs capture was called CTC-Chip (57); it is a silicon chamber which holds 78,000 anti EpCAM-functionalized microposts to enhance cell-surface interactions. After this first Chip developed in the Massachusetts General Hospital by a trend group on CTC capture by this devices, others platform were generated: in 2011, one year later, the Herringbone chip has been developed in order to enhance the CTC recovery (58).

Other alternative devices as IsoFlux (59), MagSweeper (60) and GILUPI (61), all EpCAM based and with similar limitations to those of CellSearch, have been used, although some of them provide live CTCs, suitable for molecular analysis and *in vitro* expansion.

The possibility to capture, from large volumes of whole blood rare CTCs with both epithelial and non-epithelial characteristics has been object of study by Ozkumur *et al*. in 2013; in this work an inertial focusing-enhanced microfluidic CTC capture platform, termed "CTC-iChip" was described (62). The innovative characteristic of this chip is the ability of isolating CTCs using strategies that are either antigen dependent or independent and thus virtually applicable to all cancers. The authors demonstrated in patients with prostate, breast, colon, pancreatic, and lung cancer a very high sensitivity for the posCTC-iChip, particularly critical in patients with a lower CTC burden. Furthermore, the iChip is able to isolate cells in suspension, and their immobilization on a standard glass slide for clinical cytopathological examination and high-resolution imaging. The negative depletion of aberrant cells normally present in blood, which can be performed by iChip, allows the employ of this device, virtually to all cancers. Results obtained by the comparison between the posCTC-iChip and the CellSearch® system, demonstrated for the microfluidic device a higher sensitivity in capturing low

CTC numbers suggesting that EpCAM-low CTCs were missed by the CellSearch® bulk-processing approach. An alternative approach for CTC capture which eliminates the issue of phenotypic surface marker heterogeneity is the Size based isolation (63,64). The advantage of this method is the possibility to use isolated CTC for genomic or proteomic analysis; for this purpose ScreenCell®, ISET filters and more recently lithographic microfilters have been employed (65-68). Isolation size based tests have the advantage to isolate live cells, which can be expanded *in vitro* or isolated to be further analyzed at molecular level. Mach *et al*. investigated on CTC trapping sensitivity and efficiency on cancer cells (69). In 2011 an inertial microfluidic size and deformability-based cell device for CTC enrichment was developed (70) and later, a Vortex Chip specific for high-purity extraction of cancer cells from blood sample was used (71). A novel three dimensional microfilter device that can enrich viable CTCs from blood from cancer patients was reported by Zheng *et al*. The device efficiency was investigated by cultured tumor cells spiked in blood with immunofluorescent staining, confocal and scanning electron microscopy (72).

EpCAM: ianus bifrons in the biology of circulating tumor cells (CTCs)

EpCAM and EMT: a dynamic phenotype switching

Since 2004, it has become evident that CTCs deserve attention as a biomarker for cancer disease and progression (12). From a biological perspective, CTCs represent a submicroscopic fraction detached from a primary tumor and in transit to a secondary site.

Similarly to intratumor heterogeneity, a certain degree of intercellular heterogeneity can be also envisaged within CTCs (5,6). One could imagine that CTCs recapitulate the Darwinian evolution of cancer, through the stepwise acquisition of genetic and epigenetic variations, followed by selective outgrowth of the fittest clones. Several lines of evidence have recently demonstrated that CTCs may adopt different strategies to protect themselves from the cell death fate, changing their phenotype from epithelial to mesenchymal, grouping into cell clusters or switching between the cancer stem cell state and the differentiated state of cancer cells (73). The dynamic evolution of CTCs phenotype might impair their detection, when antigen-dependent methods are used. Indeed, the EpCAM-based approach for the isolation of CTCs might underestimate the real CTCs burden, being unable to catch cells with a down-regulated expression of epithelial markers, as it happens when the EMT program is activated (74). A recent study by Yu *et al*. provided evidence of EMT in human breast cancer specimens, both in rare cells within primary tumors and in a significant number of CTCs, supporting a close link between EMT, CTCs and metastasis as components of a continuous multistep process (75). The aberrant activation of the EMT program leads to the down-regulation of proteins that support the epithelial architecture. As such, epithelial cancer cells loose cell–cell adhesion and polarity to become invasive and motile mesenchymal cells. Once detached from primary tumors, CTCs migrate as single cells or as part of cell clusters and then stay as dormant tumor cells or grow as a distant metastasis by the reverse process of EMT, the MET. The transient nature of the molecular changes that CTCs display during their lifetime leads to the hypothesis that EMT is sustained by reversible epigenetic regulatory mechanisms rather than permanent genetic alterations (76). The epithelial mesenchymal transition in CTCs might thus be conceived as a global reprogramming process, through which cells not only acquire invasion and migration competence, but also resistance to programmed cell death and stem cell-like functions (77).

The dynamic regulation of EpCAM expression

Human EpCAM is a transmembrane glycoprotein of 314 amino acids (aa), that functions as a homophilic, epithelial-specific intercellular cell-adhesion molecule, involved in the regulation of cell proliferation and differentiation. It consists of a large extracellular domain (N-terminal) of 242 aa, a single-spanning transmembrane domain of 23 aa and a short cytoplasmic domain of 26 aa (C-terminal) (78). Discovered as a dominant antigen on epithelia and invasive carcinomas, EpCAM was initially considered a mere cell adhesion molecule and became one of the most commonly used membrane-associated proteins for the isolation of CTCs from peripheral blood. Nevertheless, the observation that EpCAM can be downregulated during the dissemination of cancer cells from primary tumor through the bloodstream and that many epithelial tumors may lack EpCAM expression, rapidly suggested that EpCAM-based methods could be inadequate in the enrichment step of CTCs capture (79). That EpCAM expression is highly dynamic into the bloodstream was originally demonstrated in xenograft mouse models with EpCAM-expressing breast cancer cell lines, where

the intravenous injection of EpCAM expressing cells caused EpCAM downregulation 4 hours after the injection (7). Further studies widely demonstrated that EpCAM negative CTCs with mesenchymal cell like phenotype and downregulation of epithelial markers are frequently derived from EpCAM-positive primary tumors (80). Data are accumulating on the biology of EpCAM and increasing evidence indicates that the expression of EpCAM and its functional consequences are controlled by fine-tuned regulatory mechanisms. EpCAM can be transcriptionally downregulated by methylation of DNA at cytosine residues within CpG islands or can be mutated, although less frequently. Post-translational changes, such as glycosylation, have also been reported to influence the stability of EpCAM expression on epithelial cells membrane. The proteolytic cleavage of EpCAM is a further mechanism to fine-tune the dynamics of EpCAM expression. The membrane full length EpCAM protein, indeed, is subject to γ-secretase-dependent regulated intramembrane proteolysis (RIP), and proteasome-mediated degradation. Finally, endocytosis might be an additional means by which the EpCAM expression can be regulated (79,81).

Regulated intramembrane proteolysis (RIP) and EpCAM cleavage

The finding of a proteolytic cleavage of the EpCAM molecule in cancer cells led to the demonstration that a mechanism of RIP is able to activate EpCAM as a mitogenic signal transducer (82). Thus, a new role for EpCAM as mediator of proliferative signalling has been proposed. The cleavage of EpCAM, which is sequentially catalysed by TACE and presenilin-2, leads to the cleavage of the extracellular domain (EpEX) of the EpCAM molecule and to the consequent release of the short-lived intracellular domain (EpICD). After RIP, the intracellular EpICD is released in the cytoplasm and shuttles into the cell nucleus in a complex with the scaffold protein FHL2 and β-catenin, inducing transcription of target genes, including c-myc, cyclins, stemness-inducing genes and genes related to cell proliferation (83). Shedding of EpEX during activation of EpCAM signalling produces a soluble ligand that can promote generation of EpICD in an autocrine or paracrine fashion. EpCAM expression and cleavage are both tightly regulated and only occur in case of a temporary need for cell proliferation. The nuclear localization of EpICD was first reported in human colon cancer and in different subtypes of thyroid cancers (84), where its presence was associated with some tumor aggressiveness and poor prognosis of patients. Hence, there is now growing evidence that subcellular compartmental accumulation of EpICD may be involved in development of epithelial carcinomas (85). Using anti-EpEX and anti-EpICD antibodies staining on tumour specimen, Fong *et al*. identified two EpCAM variants: the membrane-bound full-length protein (EpCAM Membranous full-length; EpCAMMF) and its truncated variant (EpCAM Membranous truncated; EpCAMMT) which lacks the intracellular domain but still has a remnant transmembranous and integral extracellular domain (86). Authors found that the ratio between EpCAMMF and EpCAMMT changed significantly depending on the tumor type. Proteolysis of EpCAM analyzed in different cancer types revealed strongest cleavage in cancers of the endometrium and the bladder, intermediate cleavage in gastrointestinal cancers, and low cleavage in lung, ovarian, breast and prostate tumors. Increased release of EpEX enhances EpICD cleavage resulting also in activation of epithelial-mesenchymal transition genes suggesting that EpICD might contribute to tumor initiation and progression. One of the consequences of EpCAM proteolysis is the withdrawal of the extracellular domain from the plasma membrane, which might account for the lack of EpCAM expression in CTCs from patients with aggressive tumor types. Being that the antibodies used for CTCs capture usually target the ectodomain of the antigen, they cannot discriminate between membrane-bound full-length EpCAM and cleaved variants. Indeed, the status of nuclear EpICD in CTCs lacking EpCAM ectodomain, thus being missed using EpCAM-based methods, has never been investigated and deserves further attention.

The functional consequences of EpCAM loss

From a theoretical point of view, the dynamic expression of EpCAM in cancer cells might be both the result of an aberrant activation of the EMT program, which in turn leads to an overall downregulation of epithelial markers, or can represent itself the initial trigger for the phenotype switching. The properties of EpCAM as a mitogenic signaling might favor this latter hypothesis (87). Indeed, among its plethora of functions, at least two might be advocated to support the hypothesis of EpCAM as a driving force for the phenotypic changes in the EMT/MET switch. EpCAM has been initially proposed as a homotypic cell adhesion molecule, contributing to the integrity of epithelial tissues. However, it has been demonstrated that

EpCAM can weaken E-cadherin-mediated intercellular adhesion, decreasing overall the strength of intercellular adhesive junctions (16). It has been also demonstrated that EpCAM directly regulates the induction of EMT, through the expression of Snail, Slug and vimentin (88). The induction of EMT has been shown to generate stem-like cells (89), which is somehow consistent with the observation that nuclear translocation of EpICD participates in stemness genes modulation, to maintain cell renewal and cell survival. The positive autoregulation of the EpCAM loop, maintained through the soluble fraction EpEX, which enhances the EpCAM cleavage and triggers the EpICD signaling, may ensure that EpCAM provides a sustained signal for proliferation, self-renewal, anchorage-independent growth and invasiveness (23). Overall, these observations lead to the hypothesis that the pleiotropic and apparently contradictory functions of EpCAM, might instead be tightly controlled to allow cancer cells to acquire phenotypic beneficial changes, which favor alternatively cell-cell adhesion and active proliferation or motility and stemness properties along the metastatic cascade.

Take home messages

CTCs are rare and multifaceted cells travelling from primary tumors to secondary sites. The dynamic changes in CTCs phenotype are increasingly being recognized and it is definitely apparent that CTCs represent a heterogeneous entity that lies beyond a univocal definition. Originally identified as a dominant antigen in epithelial cancers, EpCAM has been considered since the beginning the ideal marker for the detection of CTCs in the peripheral blood of cancer patients. However, over the last few years, a growing body of evidence has arisen supporting the plasticity of CTCs phenotype. As a consequence, a kaleidoscopic definition of CTCs is required and the establishment of a universal marker for their detection seems merely a vision. Conversely, the pleiotropic functions of EpCAM have been recently clarified and the definition of the biological role of this molecule in CTCs became complicated. So far, the experimental evidence of the dynamic expression of EpCAM in CTCs is limited to studies performed in order to demonstrate the limited sensitivity of EpCAM-based methods in the detection of CTCs in pre-defined subtypes or settings of disease. It is rather conceivable that the functional consequences of EpCAM loss need to be investigated to understand what happens to CTCs when they undergo to the global reprogramming process that includes the epithelial to mesenchymal switch and the transient acquisition of stemness properties. Some new technologies, mainly CTC-chip and high-definition (HD)-CTC assays, relying upon EpCAM independent enrichment of the entire CTC population, seem the optimal candidates for future fluid biopsies, being able to measure gene expression, DNA mutations and to capture live cells for conventional histological analysis.

Even in the current post EpCAM era the crucial role of EpCAM molecule in CTC detection cannot be neglected. The prognostic significance of EpCAM positive CTCs, which was recently confirmed by the first European pooled analysis in metastatic breast cancer, is a undisputable evidence. Nevertheless, the increasing evidence of CTCs heterogeneity, which, similarly to what described in primary tumors, likely evolves during the disease course, needs to be further investigated.

In addition to difficulties to pinpoint an antibody combination to cover the complex heterogeneity of CTCs, avoiding false negative results, there are at least three more questions to be addressed: (I) some systems are very expensive; this bias automatically exclude some research groups; (II) each system used for the enrichment step has different sensitivity and (III) there is necessity to standardize each method. In the post-EpCAM era we have the feeling that no single marker will be sufficient to isolate the entire pool of CTC, as well as no marker combination will be sufficient to cover the extreme heterogeneity of these cells in different tumor types.

Whether EpCAM cleavage might represent a beneficial change for CTCs to survive, proliferate and acquire stem-like features deserves to be deepened.

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Footnote

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References

1. Ashworth TR. A case of cancer in which cells similar to those in the tumours were seen in the blood after death. Aus Med J 1869;14:146-9.
2. Engell HC. Cancer cells in the circulating blood; a clinical

study on the occurrence of cancer cells in the peripheral blood and in venous blood draining the tumour area at operation. Acta Chir Scand Suppl 1955;201:1-70.
3. Zhe X, Cher ML, Bonfil RD. Circulating tumor cells: finding the needle in the haystack. Am J Cancer Res 2011;1:740-51.
4. Trzpis M, McLaughlin PM, de Leij LM, et al. Epithelial cell adhesion molecule: more than a carcinoma marker and adhesion molecule. Am J Pathol 2007;171:386-95.
5. Martelotto LG, Ng CK, Piscuoglio S, et al. Breast cancer intra-tumor heterogeneity. Breast Cancer Res 2014;16:210.
6. Hiley C, de Bruin EC, McGranahan N, et al. Deciphering intratumor heterogeneity and temporal acquisition of driver events to refine precision medicine. Genome Biol 2014;15:453.
7. Gorges TM, Tinhofer I, Drosch M, et al. Circulating tumour cells escape from EpCAM-based detection due to epithelial-to-mesenchymal transition. BMC Cancer 2012;12:178.
8. Punnoose EA, Atwal SK, Spoerke JM, et al. Molecular biomarker analyses using circulating tumor cells. PLoS One 2010;5:e12517.
9. Königsberg R, Obermayr E, Bises G, et al. Detection of EpCAM positive and negative circulating tumor cells in metastatic breast cancer patients. Acta Oncol 2011;50:700-10.
10. Takai Y, Miyoshi J, Ikeda W, et al. Nectins and nectin-like molecules: roles in contact inhibition of cell movement and proliferation. Nat Rev Mol Cell Biol 2008;9:603-15.
11. Joosse SA, Gorges TM, Pantel K. Biology, detection, and clinical implications of circulating tumor cells. EMBO Mol Med 2014;7:1-11.
12. Cristofanilli M, Budd GT, Ellis MJ, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med 2004;351:781-91.
13. de Bono JS, Scher HI, Montgomery RB, et al. Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. Clin Cancer Res 2008;14:6302-9.
14. Cohen SJ, Punt CJ, Iannotti N, 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 2008;26:3213-21.
15. Herlyn M, Steplewski Z, Herlyn D, et al. Colorectal carcinoma-specific antigen: detection by means of monoclonal antibodies. Proc Natl Acad Sci U S A 1979;76:1438-42.
16. Schnell U, Cirulli V, Giepmans BN. EpCAM: structure and function in health and disease. Biochim Biophys Acta 2013;1828:1989-2001.
17. Baeuerle PA, Gires O. EpCAM (CD326) finding its role in cancer. Br J Cancer 2007;96:417-23.
18. Munz M, Fellinger K, Hofmann T, et al. Glycosylation is crucial for stability of tumour and cancer stem cell antigen EpCAM. Front Biosci 2008;13:5195-201.
19. Ponta H, Sherman L, Herrlich PA. CD44: from adhesion molecules to signalling regulators. Nat Rev Mol Cell Biol 2003;4:33-45.
20. Schmidt DS, Klingbeil P, Schnölzer M, et al. CD44 variant isoforms associate with tetraspanins and EpCAM. Exp Cell Res 2004;297:329-47.
21. Kuhn S, Koch M, Nübel T, et al. A complex of EpCAM, claudin-7, CD44 variant isoforms, and tetraspanins promotes colorectal cancer progression. Mol Cancer Res 2007;5:553-67.
22. Nübel T, Preobraschenski J, Tuncay H, et al. Claudin-7 regulates EpCAM-mediated functions in tumor progression. Mol Cancer Res 2009;7:285-99.
23. Schnell U, Kuipers J, Giepmans BN. EpCAM proteolysis: new fragments with distinct functions? Biosci Rep 2013;33:e00030.
24. Antolovic D, Galindo L, Carstens A, et al. Heterogeneous detection of circulating tumor cells in patients with colorectal cancer by immunomagnetic enrichment using different EpCAM-specific antibodies. BMC Biotechnol 2010;10:35.
25. Allard WJ, Matera J, Miller MC, 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 2004;10:6897-904.
26. Cristofanilli M, Hayes DF, Budd GT, et al. Circulating tumor cells: a novel prognostic factor for newly diagnosed metastatic breast cancer. J Clin Oncol 2005;23:1420-30.
27. Budd GT, Cristofanilli M, Ellis MJ, et al. Circulating tumor cells versus imaging--predicting overall survival in metastatic breast cancer. Clin Cancer Res 2006;12:6403-9.
28. Hayes DF, Cristofanilli M, Budd GT, 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 2006;12:4218-24.
29. Scher HI, Jia X, de Bono JS, et al. Circulating tumour cells as prognostic markers in progressive, castration-resistant prostate cancer: a reanalysis of IMMC38 trial data. Lancet Oncol 2009;10:233-9.
30. Cohen SJ, Punt CJ, Iannotti N, et al. Prognostic

significance of circulating tumor cells in patients with metastatic colorectal cancer. Ann Oncol 2009;20:1223-9.
31. Krebs MG, Hou JM, Sloane R, et al. Analysis of circulating tumor cells in patients with non-small cell lung cancer using epithelial marker-dependent and -independent approaches. J Thorac Oncol 2012;7:306-15.
32. Hiltermann TJ, Pore MM, van den Berg A, et al. Circulating tumor cells in small-cell lung cancer: a predictive and prognostic factor. Ann Oncol 2012;23:2937-42.
33. Hiraiwa K, Takeuchi H, Hasegawa H, et al. Clinical significance of circulating tumor cells in blood from patients with gastrointestinal cancers. Ann Surg Oncol 2008;15:3092-100.
34. Kurihara T, Itoi T, Sofuni A, et al. Detection of circulating tumor cells in patients with pancreatic cancer: a preliminary result. J Hepatobiliary Pancreat Surg 2008;15:189-95.
35. Poveda A, Kaye SB, McCormack R, et al. Circulating tumor cells predict progression free survival and overall survival in patients with relapsed/recurrent advanced ovarian cancer. Gynecol Oncol 2011;122:567-72.
36. Rink M, Chun FK, Minner S, et al. Detection of circulating tumour cells in peripheral blood of patients with advanced non-metastatic bladder cancer. BJU Int 2011;107:1668-75.
37. Gazzaniga P, Gradilone A, de Berardinis E, et al. Prognostic value of circulating tumor cells in nonmuscle invasive bladder cancer: a CellSearch analysis. Ann Oncol 2012;23:2352-6.
38. Gazzaniga P, de Berardinis E, Raimondi C, et al. Circulating tumor cells detection has independent prognostic impact in high-risk non-muscle invasive bladder cancer. Int J Cancer 2014;135:1978-82.
39. Coumans FA, Doggen CJ, Attard G, et al. All circulating EpCAM+CK+CD45- objects predict overall survival in castration-resistant prostate cancer. Ann Oncol 2010;21:1851-7.
40. Gradilone A, Iacovelli R, Cortesi E, et al. Circulating tumor cells and "suspicious objects" evaluated through CellSearch® in metastatic renal cell carcinoma. Anticancer Res 2011;31:4219-21.
41. Wendel M, Bazhenova L, Boshuizen R, et al. Fluid biopsy for circulating tumor cell identification in patients with early-and late-stage non-small cell lung cancer: a glimpse into lung cancer biology. Phys Biol 2012;9:016005.
42. Rossi E, Basso U, Celadin R, et al. M30 neoepitope expression in epithelial cancer: quantification of apoptosis in circulating tumor cells by CellSearch analysis. Clin Cancer Res 2010;16:5233-43.
43. Smerage JB, Budd GT, Doyle GV, et al. Monitoring apoptosis and Bcl-2 on circulating tumor cells in patients with metastatic breast cancer. Mol Oncol 2013;7:680-92.
44. Hou JM, Greystoke A, Lancashire L, et al. Evaluation of circulating tumor cells and serological cell death biomarkers in small cell lung cancer patients undergoing chemotherapy. Am J Pathol 2009;175:808-16.
45. van Dalum G, Lenferink A, Terstappen LW. Abstract 1459: Detection of EpCAM negative circulating tumor cells in CellSearch waste. AACR 104th Annual Meeting 2013. Washington DC, USA, 2013.
46. de Wit S, van Dalum G, Terstappen LW. Detection of circulating tumor cells. Scientifica (Cairo) 2014;2014:819362.
47. Bidard FC, Peeters DJ, Fehm T, et al. Clinical validity of circulating tumour cells in patients with metastatic breast cancer: a pooled analysis of individual patient data. Lancet Oncol 2014;15:406-14.
48. Smerage JB, Barlow WE, Hortobagyi GN, et al. Circulating tumor cells and response to chemotherapy in metastatic breast cancer: SWOG S0500. J Clin Oncol 2014;32:3483-9.
49. Müller V, Riethdorf S, Rack B, et al. Prognostic impact of circulating tumor cells assessed with the CellSearch System™ and AdnaTest Breast™ in metastatic breast cancer patients: the DETECT study. Breast Cancer Res 2012;14:R118.
50. Todenhöfer T, Hennenlotter J, Feyerabend S, et al. Preliminary experience on the use of the Adnatest® system for detection of circulating tumor cells in prostate cancer patients. Anticancer Res 2012;32:3507-13.
51. Agrawal B, Krantz MJ, Parker J, et al. Expression of MUC1 mucin on activated human T cells: implications for a role of MUC1 in normal immune regulation. Cancer Res 1998;58:4079-81.
52. Gradilone A, Petracca A, Nicolazzo C, et al. Prognostic significance of survivin-expressing circulating tumour cells in T1G3 bladder cancer. BJU Int 2010;106:710-5.
53. Lloyd JM, McIver CM, Stephenson SA, et al. Identification of early-stage colorectal cancer patients at risk of relapse post-resection by immunobead reverse transcription-PCR analysis of peritoneal lavage fluid for malignant cells. Clin Cancer Res 2006;12:417-23.
54. Hardingham JE, Kotasek D, Farmer B, et al. Immunobead-PCR: a technique for the detection of circulating tumor cells using immunomagnetic beads and the polymerase

chain reaction. Cancer Res 1993;53:3455-8.
55. Yang L, Lang JC, Balasubramanian P, et al. Optimization of an enrichment process for circulating tumor cells from the blood of head and neck cancer patients through depletion of normal cells. Biotechnol Bioeng 2009;102:521-34.
56. Shibata K, Mori M, Kitano S, et al. Detection of ras gene mutations in peripheral blood of carcinoma patients using CD45 immunomagnetic separation and nested mutant allele specific amplification. Int J Oncol 1998;12:1333-8.
57. Nagrath S, Sequist LV, Maheswaran S, et al. Isolation of rare circulating tumour cells in cancer patients by microchip technology. Nature 2007;450:1235-9.
58. Stott SL, Hsu CH, Tsukrov DI, et al. Isolation of circulating tumor cells using a microvortex-generating herringbone-chip. Proc Natl Acad Sci U S A 2010;107:18392-7.
59. Harb W, Fan A, Tran T, et al. Mutational Analysis of Circulating Tumor Cells Using a Novel Microfluidic Collection Device and qPCR Assay. Transl Oncol 2013;6:528-38.
60. Talasaz AH, Powell AA, Huber DE, et al. Isolating highly enriched populations of circulating epithelial cells and other rare cells from blood using a magnetic sweeper device. Proc Natl Acad Sci U S A 2009;106:3970-5.
61. Saucedo-Zeni N, Mewes S, Niestroj R, et al. A novel method for the in vivo isolation of circulating tumor cells from peripheral blood of cancer patients using a functionalized and structured medical wire. Int J Oncol 2012;41:1241-50.
62. Ozkumur E, Shah AM, Ciciliano JC, et al. Inertial focusing for tumor antigen-dependent and -independent sorting of rare circulating tumor cells. Sci Transl Med 2013;5:179ra47.
63. Vona G, Sabile A, Louha M, et al. Isolation by size of epithelial tumor cells : a new method for the immunomorphological and molecular characterization of circulatingtumor cells. Am J Pathol 2000;156:57-63.
64. Wong NS, Kahn HJ, Zhang L, et al. Prognostic significance of circulating tumour cells enumerated after filtration enrichment in early and metastatic breast cancer patients. Breast Cancer Res Treat 2006;99:63-9.
65. Desitter I, Guerrouahen BS, Benali-Furet N, et al. A new device for rapid isolation by size and characterization of rare circulating tumor cells. Anticancer Res 2011;31:427-41.
66. Ma YC, Wang L, Yu FL. Recent advances and prospects in the isolation by size of epithelial tumor cells (ISET) methodology. Technol Cancer Res Treat 2013;12:295-309.
67. Farace F, Massard C, Vimond N, et al. A direct comparison of CellSearch and ISET for circulating tumour-cell detection in patients with metastatic carcinomas. Br J Cancer 2011;105:847-53.
68. Adams DL, Zhu P, Makarova OV, et al. The systematic study of circulating tumor cell isolation using lithographic microfilters. RSC Adv 2014;9:4334-42.
69. Mach AJ, Kim JH, Arshi A, et al. Automated cellular sample preparation using a Centrifuge-on-a-Chip. Lab Chip 2011;11:2827-34.
70. Hur SC, Henderson-MacLennan NK, McCabe ER, et al. Deformability-based cell classification and enrichment using inertial microfluidics. Lab Chip 2011;11:912-20.
71. Sollier E, Go DE, Che J, et al. Size-selective collection of circulating tumor cells using Vortex technology. Lab Chip 2014;14:63-77.
72. Zheng S, Lin HK, Lu B, et al. 3D microfilter device for viable circulating tumor cell (CTC) enrichment from blood. Biomed Microdevices 2011;13:203-13.
73. Alix-Panabières C, Pantel K. Challenges in circulating tumour cell research. Nat Rev Cancer 2014;14:623-31.
74. Liu H, Zhang X, Li J, et al. The biological and clinical importance of epithelial-mesenchymal transition in circulating tumor cells. J Cancer Res Clin Oncol 2015;141:189-201.
75. Yu M, Bardia A, Wittner BS, et al. Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. Science 2013;339:580-4.
76. Huangyang P, Shang Y. Epigenetic regulation of epithelial to mesenchymal transition. Curr Cancer Drug Targets 2013;13:973-85.
77. Tsai JH, Yang J. Epithelial-mesenchymal plasticity in carcinoma metastasis. Genes Dev 2013;27:2192-206.
78. Strnad J, Hamilton AE, Beavers LS, et al. Molecular cloning and characterization of a human adenocarcinoma/epithelial cell surface antigen complementary DNA. Cancer Res 1989;49:314-7.
79. Gires O, Stoecklein NH. Dynamic EpCAM expression on circulating and disseminating tumor cells: causes and consequences. Cell Mol Life Sci 2014;71:4393-402.
80. Driemel C, Kremling H, Schumacher S, et al. Context-dependent adaption of EpCAM expression in early systemic esophageal cancer. Oncogene 2014;33:4904-15.
81. Dollé L, Theise ND, Schmelzer E, et al. EpCAM and the biology of hepatic stem/progenitor cells. Am J Physiol Gastrointest Liver Physiol 2015;308:G233-50.
82. Maetzel D, Denzel S, Mack B, et al. Nuclear signalling by tumour-associated antigen EpCAM. Nat Cell Biol

2009;11:162-71.
83. Chaves-Pérez A, Mack B, Maetzel D, et al. EpCAM regulates cell cycle progression via control of cyclin D1 expression. Oncogene 2013;32:641-50.
84. Ralhan R, Cao J, Lim T, et al. EpCAM nuclear localization identifies aggressive thyroid cancer and is a marker for poor prognosis. BMC Cancer 2010;10:331.
85. Ralhan R, He HC, So AK, et al. Nuclear and cytoplasmic accumulation of Ep-ICD is frequently detected in human epithelial cancers. PLoS One 2010;5:e14130.
86. Fong D, Seeber A, Terracciano L, et al. Expression of EpCAM(MF) and EpCAM(MT) variants in human carcinomas. J Clin Pathol 2014;67:408-14.
87. Munz M, Baeuerle PA, Gires O. The emerging role of EpCAM in cancer and stem cell signaling. Cancer Res 2009;69:5627-9.
88. Lin CW, Liao MY, Lin WW, et al. Epithelial cell adhesion molecule regulates tumor initiation and tumorigenesis via activating reprogramming factors and epithelial-mesenchymal transition gene expression in colon cancer. J Biol Chem 2012;287:39449-59.
89. Mani SA, Guo W, Liao MJ, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 2008;133:704-15.

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