

Mini-review

Circulating tumor cells (CTC) detection: Clinical impact and future directions

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Abstract

Recent molecular and clinical studies have shown that invasion may occur very early in tumor development, thus emphasizing the potential importance of specific and sensitive detection of circulating tumor cells (CTC) and circulating tumor microemboli (CTM). The technical challenge in this field consists of finding “rare” tumor cells (just a few CTCs mixed with the approximately 10 million leukocytes and 5 billion erythrocytes in 1 ml of blood) and being able to distinguish them from epithelial non-tumor cells and leukocytes.

Many recent studies have discussed the clinical impact of detecting CTC/CTM. Although conflicting results have been obtained, these studies suggest the vast potential of CTC/CTM detection in cancer prognosis and follow up. However, the variable technical approaches which were used, as well as the number of millilitres of blood analyzed, the quality of sensitivity and specificity tests, the number of patients versus controls and the data interpretation make it very difficult to draw firm conclusions.

A particularly important recent finding is that invasive tumor cells tend to lose their epithelial antigens by the epithelial to mesenchymal transition (EMT) process. Furthermore, it is known that non-tumor epithelial cells can also be present in blood. Thus, it appears that a reliable diagnostic identification of CTC and CTM cannot be based on the expression of epithelial-specific transcripts or antigens.

Cytopathological examination of CTC/CTM, sensitively enriched from blood, represents a potentially useful alternative and can now be employed in routine analyses as a specific diagnostic assay, and be tested in large, blind, multicenter clinical trials. This basic approach can be complemented by immunological and molecular studies for further characterization of CTC/CTM and of their malignant potential.

This review is aimed at helping oncologists critically evaluate past and future research work in this field. The interest in development and assessment of this noninvasive marker should lead to more effective and better tailored anticancer treatments for individual patients, thus resulting in their improved life expectancy.

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1. Introduction

The spontaneous circulation of tumor cells and/or tumor microemboli is the hallmark of the “invasive behaviour” of a proportion of cancer cells. Their detection is expected to provide a powerful tool for cancer prognosis, diagnosis of minimal residual disease, assessment of tumor sensitivity to anticancer drugs, and personalization of anticancer therapy [1]. A highly sensitive and specific identification of circulating tumor cells (CTC) and circulating tumor microemboli (CTM) could also help, in the future, in early diagnosis of invasive cancers.

In order to understand the limits and potential impact of this new field in oncology, we have summarized (1) the mechanisms regulating the development of metastases and (2) the technical issues determining specificity and sensitivity of CTC detection. We have then discussed the clinical impact of these studies. This mini-review, which is not meant to be exhaustive, is specifically focused on non-invasive detection of CTC/CTM derived from solid cancers and on its potential biological significance as a tumor marker. It aims to attract the interest of oncologists to this new exciting research field and help them critically review the wide variety of reported methods and sometimes conflicting results.

2. Invasion and development of metastasis

Invasion, a military term meaning territorial occupation, also defines a key cellular process for life and death [2]. On the one hand, trophoblastic cell invasion is needed for successful embryo implantation and morphogenesis [2], yet on the other hand, acquisition of the invasive capacity by transformed cells and subsequent formation of metastases lead to approximately 90% of all deaths in cancer patients [3].

Although metastasis is the most important event leading to cancer death, its mechanism is still poorly understood [4]. We review here the outlines of this process, highlighting the new findings potentially relevant for CTC/CTM appraisal and detection.

2.1. Molecular mechanisms of invasion

The process leading to tumorigenesis and metastases involves an active molecular crosstalk within the tumor microenvironment [4,5], the role of tumor-associated proteins, like urokinase-type plasminogen activator (uPA) and fibrinogen and com-

plex signalling, like Akt1/PKB which modulate both invasion and apoptosis. It includes the following schematic steps: tumor cells growth, angiogenesis, tumor cell detachment, epithelial to mesenchymal transition (EMT), motility, intravasation, survival in vessels and embolization, possible extravasation, mesenchymal to epithelial transition (MET), formation of micrometastases and growth of macrometastases (Fig. 1).

In early tumor expansion, as in embryonic development, growing cells rapidly outstrip the supply of nutrients and oxygen. Actually, virtually all cells are obliged to reside within 100 μm of a capillary blood vessel [6]. HIF, which mediates the transcriptional response to hypoxia, is a strong promoter of tumor growth and invasion and controls angiogenesis via two key angiogenic factors (VEGF-A and angiopoietin-2) which also affect energy metabolism, pH, necrosis versus cell survival, and cell migration [6,7].

Necrosis leads to the release of inflammatory mediators such as cytokines and chemokines which recruit, among other cells, leukocytes and macrophages. These, in turn, stimulate angiogenesis, extracellular matrix breakdown, tumor motility and release of nitric oxide synthetase (NOS), giving rise to nitric oxide (NO) [8]. NO produces free radicals, which have a mutagenic and cytotoxic effect. Local production of basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF) and transforming growth factor beta (TGF-beta) mediate the control of tumor cell survival/apoptosis balance and of E-cadherin downregulation leading to reduced cell adhesion and increased tumor cell invasiveness [7].

Once the vascular supply has been re-established by angiogenesis, tumor cells may have acquired enough genetic mutations to be able to detach and invade blood vessels. Indeed, in murine cancer models, hypoxia has been shown to induce an increase of metastatic potential [9]. Moreover, the presence of hypoxia and iNOS activity within human tumors has been associated with increased invasiveness, vascular density and metastasis formation [7]. Furthermore, recent data show that hypoxia, acting through LOX induction and Snail activation, leads to E-cadherin repression [7], a crucial feature of the “epithelial to mesenchymal transition” (EMT) [10,11].

EMT is a phenotype switch which characterizes carcinoma invasion and metastasis. Epithelial cells, which play a major structural and functional role

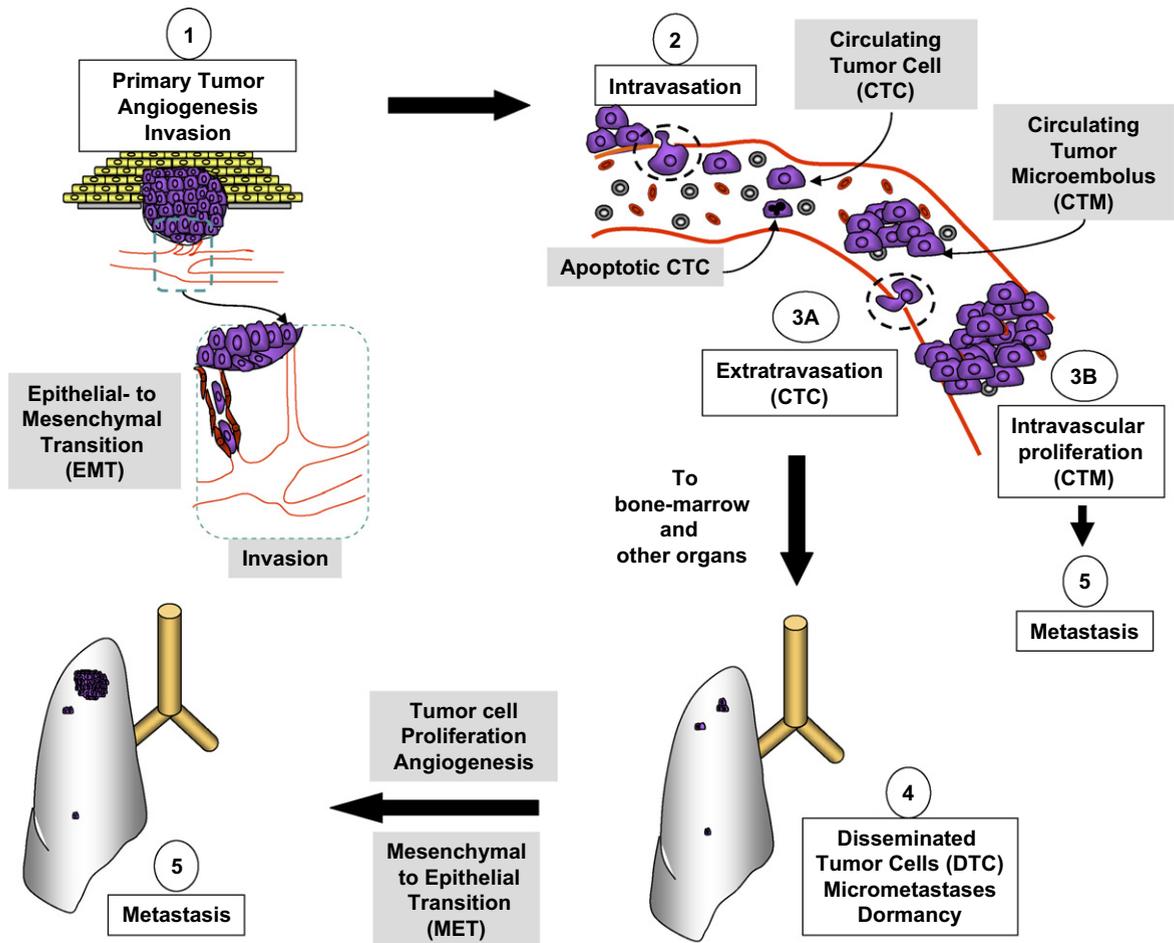


Fig. 1. Main steps leading to development of metastases. (1) Growing tumor cells outstrip oxygen supply and activate angiogenesis. Downregulation of E-cadherin leads to reduced cell adhesion and early invasiveness of tumor cells resistant to apoptosis. Invading tumor cells undergo the phenotype switch “epithelial to mesenchymal transition (EMT)”: they progressively lose epithelial antigens, acquire mesenchymal antigens, and motile propensities (like fibroblasts). (2) After entering blood vessels (intravasation), circulating tumor cells (CTC) undergo apoptosis or circulate as isolated CTC. They are out of cell cycle and do not proliferate. (3A) CTC extravasate to distant organs. (4) After extravasation, CTC remain as dormant solitary cells (disseminated tumor cells, DTC), or undergo limited proliferation (Micrometastases). (5) Unrestrained CTC proliferation gives rise to metastases, via phenotype reversion MET (mesenchymal to epithelial transition) and angiogenesis. (3B–5) Circulating tumor microemboli (CTM) represent “collective migration” of tumor cells. They have a high metastatic potential as they are resistant to apoptosis and keep proliferative capacities. CTM cannot extravasate, but arrest in capillaries and proliferate, rupturing the capillary walls and giving rise to metastases. Thus, CTM may mediate a shortcut to metastases.

in organs, are mutually and extensively adherent by cell-to-cell and cell-to-extracellular matrix (ECM) junctions. Cell-to-cell adhesion molecules include cadherins, while cell-to-ECM adhesion involves integrins. Cadherins and integrins rely on the “cytoskeleton”, which is a rigid structure formed by actin and cytokeratin filaments. Thus, in an intact epithelium, cells have a rigid structure and are immobile. Conversely, mesenchymal cells (like fibroblasts and leukocytes), have a much more relaxed organization, a far lower level of cell junctions and cytokeratin molecules, and show motile propensities.

Epithelial to mesenchymal transition, which is instrumental for rapid morphogenetic changes in embryos and tumor cell invasion, is induced by the transcriptional factor Twist [12] and is characterized by degradation of cell-to-cell adhesion, decrease in E-cadherin expression, decrease of epithelial (e.g., cytokeratin), and increase of mesenchymal (e.g., vimentin) markers. In fact, down-regulated expression of cytokeratins has been shown to characterize malignant progression of human breast cancers [13,14]. It is interesting to note that, during EMT, Twist may need to activate anti-

apoptotic programs in order to allow epithelial cells to convert to a mesenchymal fate while avoiding anoikis (apoptosis induced by disruption of cell attachment and cell–matrix interactions) [12]. Mesenchymal-like tumor cells exhibit a highly motile phenotype and can readily intravasate and extravasate by traversing basement membranes, interstitial spaces and endothelial barriers.

Once the target organ is reached, mesenchymal-like circulating tumor cells may need to reverse to epithelial-like tumor cells via mesenchymal to epithelial transition (MET) in order to regain the ability to proliferate [10,12] (Fig. 1). It has to be noted that, since cell transition to an aggressive malignant phenotype is not an “all or nothing” event, but rather manifests a broad spectrum of phenotypic changes, epithelial-specific and mesenchymal-specific antigens may be expressed at variable degree in invasive cells [10].

The importance of the “collective cells migration” has been recently stressed [10,15]. In fact, tumor cells can also invade as multicellular aggregates or clusters (a process known as “collective” or “cohort” migration). Multicellular aggregates of epithelial-like tumor cells, also called circulating tumor microemboli (CTM) (Fig. 2), are thought to have potential advantages for survival, proliferation and establishment of micrometastatic lesions in distant organs [10]. Actually, it has been shown that CTM may give rise to metastasis without extravasation, by attaching to vessel walls of arterioles and capillaries, and proceeding to cell proliferation within the vasculature, rupture of capillary walls and formation of micro- or macro metastases [16] (Fig. 1). Thus, it is generally accepted that the presence of CTM in blood is a marker of highly metastatic potential [10,16].

The mechanisms involved in the preferential choice of a target organ for metastatic tumor cells proliferation (“seed and soil” theory) are still not completely understood. Organ-specific attractant molecules (chemokines) can stimulate migrating tumor cells to invade the walls of blood vessels and enter specific organs. Tumor-endothelial interaction, appropriate adhesion molecules expressed by endothelial cells in distant organs and local growth factors can drive metastatic tumor cell proliferation [17].

2.2. Invasion may be an early process during tumorigenesis

While conventional theories assume that invasion and metastases are late events, convergent results

have led to the present knowledge that invasion can be early and sometimes clinically dormant [3,18,19]. Tumor-induced neovascularization occurs in parallel with the transition to invasion and provides a vascular entry portal for dissemination which may precede evident primary tumor outgrowth by many years [18]. As mentioned, tumor hypoxia, which occurs at the very beginning of tumor development, can be the initiating factor that sets the tumor on the road to metastases, so that tumor cells spread may start very early instead of being a “late” phenomenon due to accumulation of genetic mutations over time [19].

Indeed: (1) Clinical data concerning breast cancer indicate that tumor cells spread may start years before diagnosis [18], and the probability of tumor cells spreading from small melanoma and breast cancers has been reported to be high [20]. Among patients with colon cancers, a significant proportion (20–30%) has macrometastases at diagnosis, confirming the view that some tumors may spread tumor cells at a very early step of development. (2) Tumor cells spread into blood vessels may be linked to tumor cell density and thus to tumor cell growth [21]. This model suggests that the cellular determinants for invasion are present before angiogenesis and that following development of new vessels can provide the final requirement for tumor cell spread. (3) Recent molecular studies have shown that the capacity to metastasize may be pre-ordained by the spectrum of mutations acquired early in tumorigenesis, which means that some cancers start out “on the wrong foot” [22]. In fact, it has been demonstrated that cancer cells in the primary tumor may harbor a gene-expression signature matching that observed in the metastatic colony and that this signature can be used to predict, with high accuracy, whether the tumor will remain localized or whether the patient will experience metastases and disease relapse [23].

2.3. Formation of metastases is a highly inefficient process

From model systems, it has been estimated that around 1×10^6 tumor cells per gram of tumor tissue can be introduced daily into the bloodstream [24]. Epithelial cancer cells have very low survival rates in circulation [25]. The fate of CTC includes a rapid phase of intravascular cancer cell disappearance which is completed in less than 5 min and accounts for 85% of the circulating cancer cells [26,27]. This

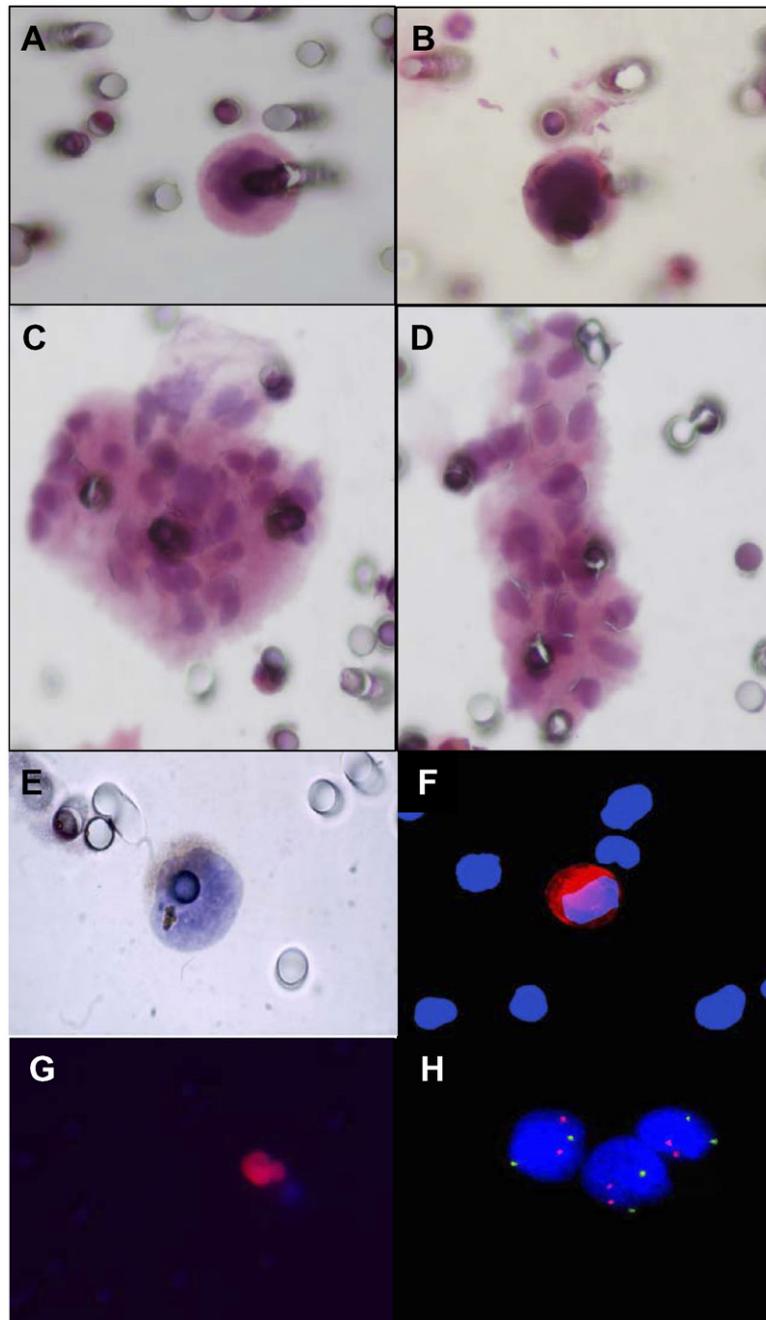


Fig. 2. Detection and characterization of circulating tumor cells (CTC) and circulating tumor microemboli (CTM) enriched by ISET. CTC (A and B) from a patient with prostate cancer and CTM (C and D) from a patient with kidney cancer, are enriched by filtration in a highly sensitive manner. The morphology is conserved so that they can be distinguished from non-tumorous epithelial and from non-epithelial cells. Pores have a calibrated size of 8- μ m and thus pass the vast majority of lymphocytes and neutrophils whose diameter is 8–11 μ m and retain fixed cells larger than 11 μ m. CTC (A and B) here have a diameter of around 40–42 μ m. They are characterized by high nucleus/cytoplasmic ratio (0.83), nuclear irregular shape and non-homogeneous texture (hematoxylin & eosin staining, 83 \times). CTM (C and D) are large tumor cells aggregates. They are known to be associated to a high risk of metastases (hematoxylin & eosin staining, 83 \times). (E): CTC from liver cancer labelled by immunocytochemistry with anti- α -fetoprotein antibody (Hematoxylin counterstaining, 100 \times). (F) CTC from cell line (HeLa) labelled by immunofluorescence with anti-cytokeratin antibody (KL1, 40 \times). (G) apoptotic CTC from prostate cancer isolated by ISET from peripheral blood and characterized by TUNEL (63 \times). H: HeLa cells characterized by FISH with probes specific to chromosome 8 and 1 (100 \times).

process has been related to “anoikis” (see above for definition). Many cancer cell types with increased metastatic potential are resistant to anoikis compared to the parental cells, a tumor cell behaviour related to the expression of apoptosis inhibitors [26].

Animal studies, in which tumor local invasion and intravasation are bypassed (since they are difficult to reproduce [12]) and tumor cells are directly introduced into the systemic circulation, have established that around 1/40 CTC give rise to micrometastases and only approximately 0.01% proliferate into macrometastasis [27].

This metastatic inefficiency is principally determined by: (1) susceptibility of CTC to apoptosis, (2) failure of solitary cells extravasated in distant organs to initiate growth and (3) failure of early micrometastases in distant organs to stimulate angiogenesis and continue growth into macroscopic tumors [27]. Both solitary cells and micrometastases may remain in “dormancy” for years [28], being cell cycle arrested and not undergoing apoptosis. The immune system [29] and angiogenesis [28] have been shown to play a role in tumor cell dormancy, although the mechanisms may be variable in different tumors and are not completely understood. Finally, it has been suggested that any factor that tips the balance between proliferation and apoptosis may result in tumor progression or regression.

In conclusion, the appraisal of updated mechanisms involved in the process of metastasis is fundamental in order to critically review the issue of CTC/CTM. Based on this new knowledge: (1) Epithelial antigens are expected to be down-regulated in the most invasive CTC because of the epithelial to mesenchymal transition process. Mesenchymal antigens can be expressed by invasive cells as well as by mesenchymal non-tumor cells, like leukocytes, which largely outnumber CTC in blood. Thus, a reliable assay to identify CTC cannot be based on antibodies specific to epithelial or to mesenchymal cells. (2) Circulating tumor microemboli are the expression of “collective migration”, a phenomenon linked to high metastatic potential and thus expected to be clinically important. (3) Invasion may occur early in tumor development, thus raising the issue of the potential application of highly sensitive and specific methods to identify CTC in cancer screening. (4) The increasing capacity to characterize CTC/CTM in term of gene mutations and expression profile is expected to complement CTC/CTM-specific detection and counting, continuously improving

the process of non-invasive identification of those patients who are at higher risk of relapse and metastases.

3. Terminology

According to the path followed by tumor cells to generate metastases (Fig. 1), we propose that the terms circulating tumor cells (CTC) and circulating tumor microemboli (CTM) (Fig. 2) be used to specifically identify tumor cells detected in blood or lymphatic vessels. CTM represents “collective migration” of tumor cells and, as previously pointed out (see above), carry a highly metastatic potential.

The terms disseminated tumor cells (DTC) and isolated tumor cells (ITC) are sometimes used to indicate both tumor cells in organs and circulating tumor cells in blood [30,31]. In order to avoid confusion, they should be used only to indicate tumor cells in organs (bone marrow, lymph nodes or other organs) [27].

The term micrometastases is also used sometimes to indicate CTC or DTC [32], while it should be used only to define tumor cells in organs which underwent limited proliferation [27,33]. Micrometastases size is generally under 0.2 cm in greatest dimension and their reliable diagnosis is only possible by histologic examination [33]. As discussed earlier, “micrometastasis” may derive both from extravasated CTC and from non-extravasated CTM after limited proliferation and rupture of vessels walls.

4. CTC detection

The challenge of CTC/CTM detection is related to the requirement of high sensitivity combined with high specificity. Since invasion can start very early during tumor development (see above), identification and counting of CTC when they are very rare (few CTC/CTM per 10 ml of blood, which means few CTC/CTM mixed with approximately 100 million leukocytes and 50 billion erythrocytes) could alert the oncologist about a developing tumor invasion process.

Specificity is also an absolute requirement in this field. In fact, a wrong identification of “non-tumor cells” (like epithelial non tumor cells, for instance) as “tumor cells”, could generate poor clinical and therapeutical choices having a negative impact on the quality and/or expectancy of life in patients with cancer.

Several recent reviews concerning detection of CTC are available [1,30,31,34–37]. Many different methods have been developed and some are commercially available (Fig. 3, Tables 1 and 2). Our aim here is to critically analyze the advantages and disadvantages of the different approaches and suggest criteria to identify reference methods expected to provide reliable clinical information.

4.1. Indirect methods

Indirect methods do not provide a diagnostic identification of CTC. They target epithelial cells and/or use organ-specific markers which identify cells from specific organs but do not demonstrate their tumorous nature.

4.1.1. Indirect immuno-mediated methods

Immuno-mediated detection is performed by immuno-labelling of cells enriched by different approaches including immunomagnetic separation [38], and physical methods (density gradient, filtration) (Tables 1, 2, and Fig. 3).

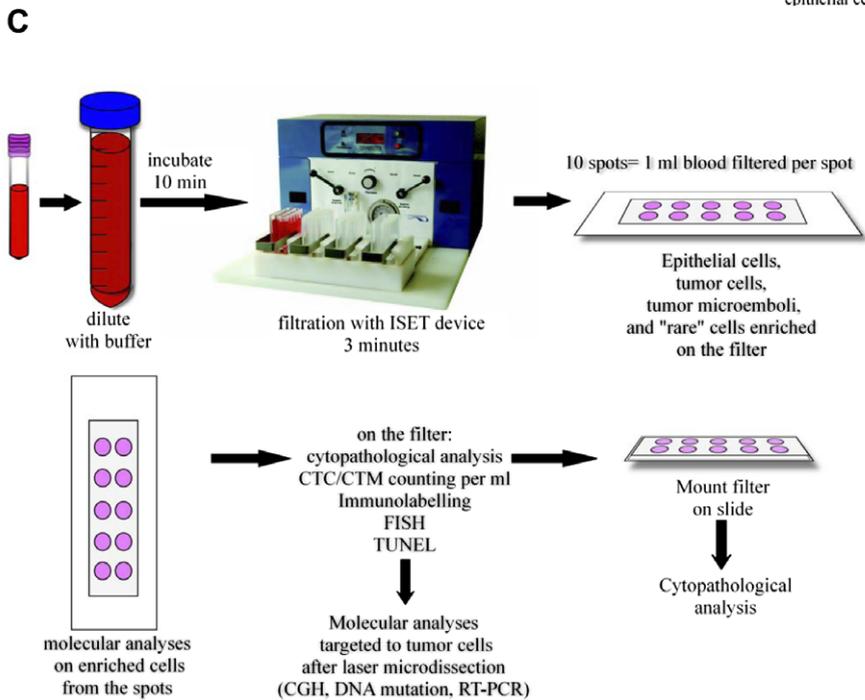
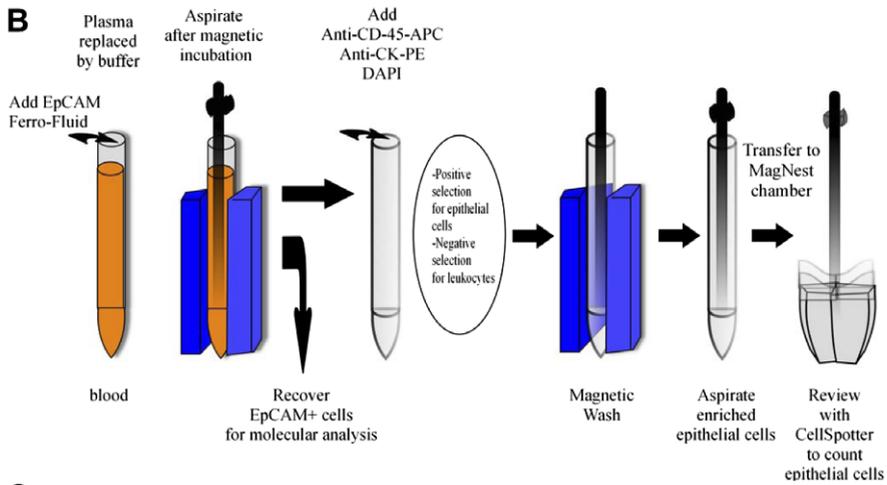
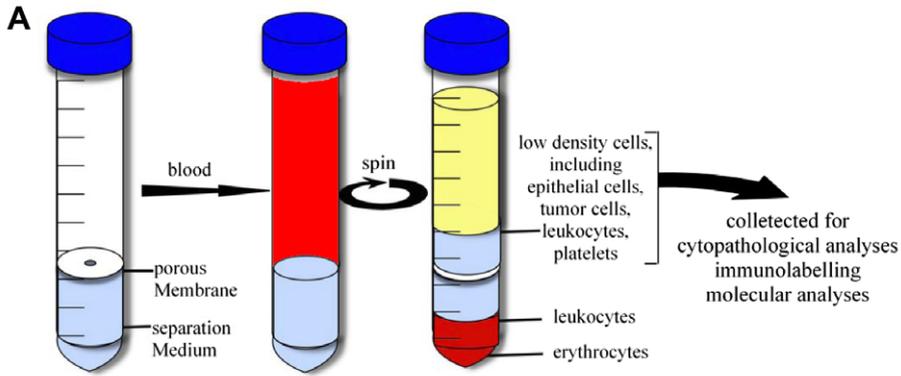
Enrichment of CTC obtained by commercially available immunomagnetic methods (MACS systems, macro-iron beads, magnetic beads, ferrofluid (colloidal iron)-based systems) ranges from 10^4 to 2×10^5 fold and avoids cell lysis, which characterizes the RT-PCR tests (see below), thus allowing the counting of target cells. However, since specific antigens characterizing CTC are not known at present (antigens expressed by all the tumor cells from a solid tumor type and not expressed by leukocytes nor by other circulating non tumor cells), authors have used antibodies specific to epithelial antigens to isolate CTC (EpCAM, BerEP4, Cytokeratins (CK)) (see Table 1). Epithelial-specific antibodies can label non-tumor epithelial cells by specific labelling and non-tumor non-epithelial cells by non-specific labelling, thus giving false positive results. The percentage of CK positive cells in normal controls ranges

from 0% to 20% [34,39]. Most of these cells are leukocytes. Antibodies against CK or other epithelial-specific antigens have been reported to bind both specifically and non-specifically to macrophages, plasma cells and nucleated hematopoietic cells precursors. The non-specific binding involves Fc receptor-bearing leukocytes and monocytes or illegitimate expression of epithelial antigens in normal hematopoietic cells [38]. However, some of these positive cells are morphologically difficult to distinguish from CTC. Variable numbers of epithelial cells [34] have been found in peripheral blood of subjects without malignancy, being related to benign epithelial proliferative diseases, inflammation, tissue trauma, semi-surgical and surgical interventions [39,40]. Organ-specific markers have been used (antibodies to mammoglobin, PSA, CEA and HER-2) to identify CTC. However, false negative results can occur since these antigens are not present in all tumor cells. Furthermore, some of these markers, mammoglobin and HER-2, are not entirely organ-specific [41]. Actually, no available antibodies are 100% tumor or tissue-specific [39].

In the immuno-magnetic detection, whole blood or isolated (by density gradient) mononuclear cells are put in contact with magnetic particles (beads or ferrofluids)-bound antibodies. Labelled cells are then collected by applying a magnetic force while non labelled cells remain in the supernatant and are discarded. Since a large number of leukocytes still remain trapped with the target cells [42], some methods include a “negative” selection of leukocytes (e.g., with anti-CD 45) combined with a “positive” selection with antibodies specific to epithelial cells (EpCAM, Cytokeratins (CK)) (ex: CellSearch, Veridex) [38]. This procedure gets rid of the majority of leukocytes but still retains non-malignant epithelial cells and loses tumor cells which do not express epithelial antigens.

The CellSearch assay (Fig. 3 and Table 1) uses ferrofluids coated with epithelial cell-specific

Fig. 3. Examples of commercially available methods to isolate CTC. (A) Oncoquick. Tumor cells, epithelial cells, platelets and low density leukocytes are enriched from leukocytes and erythrocytes based on their particular density. They can then be collected for cytopathological analyses, immunolabelling and molecular studies. (B) CellSearch. Blood cells expressing epithelial antigens (EpCAM) are captured by EpCAM-bound ferrofluid. They can be recovered for molecular analysis or permeabilized and submitted to a negative selection with CD45 antibody (to capture leukocytes) and to a positive selection with antibodies specific to Cytokeratins 8, 18, 19 bound to ferrofluid. After washing, cells expressing epithelial antigens are transferred to the MagNest chamber and counted with CellSpotter. (C) ISET (Isolation by Size of Epithelial Tumor Cells). Cells larger than 11 μm , including tumor cells from carcinomas, are enriched from leukocytes (erythrocytes are lysed). Enriched cells are stained on the filter and CTC are precisely counted after cytopathological evaluation. Enriched cells can be studied by immunolabelling, FISH, TUNEL and molecular analysis. Molecular analysis can be targeted to the specifically identified CTC after laser microdissection. The filter can be mounted between slide and coverslip for routine microscope observation and storage.



EpCAM antibody (directed to a cell membrane antigen) to immunomagnetically enrich epithelial cells. Cells are then permeabilized, prefixed and labelled with the fluorescent nuclear dye DAPI, a fluorescent antibody to CD45 specific to leucocytes and fluorescent antibodies to intracellular cytokeratins (CK) 8, 18 and 19 specific to epithelial cells. Sample analysis is performed by the Cell-Spotter Analyzer, a four color semi-automated fluorescence microscope which identifies epithelial cells from being positive for the CK markers and negative for the CD45 marker. The advantage of the Cell-Search assay is that it is more sensitive than the Oncoquick method [43], semi-automated and reduces trapping of leucocytes with epithelial cells. It also allows cell counting. However, cell isolation and detection are performed with antibodies specific to epithelial cells (EpCAM, Cytokeratins 8, 18 and 19). As mentioned before, epithelial non-tumor cells can be spread in the peripheral blood, making difficult to determine, in a given patient having a certain number of circulating epithelial cells (CEpC), which is the actual number of tumor cells. This is particularly relevant when CTC counting is performed to assess the tumor response to the therapy, the risk of developing tumor recurrence and in cancer screening protocols. The finding that some CEpC identified in certain patients are characterized by aneuploidy [44] does not mean that any CEpC detected in any patient is actually a CTC. As discussed previously, the most malignant CTC lose epithelial antigens (by EMT), which means that assays targeting epithelial cells in blood are susceptible to missing the detection of the most invasive tumor cells. As a matter of fact, EpCAM has been found to be expressed in only 70% of 134 tumors with different histologic type [45]. In one study, Fehm et al. [44] found that cytokeratin-negative cells with aneusomy (tumor cells lacking epithelial antigens), in the blood of one patient with breast cancer, outnumbered cytokeratin-positive cells. Furthermore, CK 8, 18 and 19 were found to be lost in cell lines derived from disseminated tumor cells [13]. The loss of cytokeratins (CK) and the ectopic expression of vimentin, indicating EMT, has been demonstrated in 2,517 samples of breast cancer to be associated with a higher tumor grade and mitotic index, and with negative estrogen/progesterone receptor status [13]. Finally, CTM cannot be reliably detected by this approach, as multiple cell labelling and treatments with magnetic particles tend to dissociate tumor cells aggregates.

4.1.2. Indirect molecular methods

RT-PCR based methods analyse the expression of candidate genes specific to epithelial cells and/or to the normal tissues from which the tumor cells originate. [30,31,39,46]. The main advantage of this approach is its sensitivity which is considered to be higher than the reported sensitivity of immune-mediated detection and immunocytochemistry [30]. RT-PCR implies the following steps: (a) peripheral blood collection, (b) optional enrichment of nucleated cells by physical methods (density gradient) and/or by immune-mediated or immunomagnetic enrichment of epithelial cells, (c) RNA extraction, (d) complementary DNA (cDNA) synthesis, (e) marker gene cDNA amplification, (f) PCR product analysis (for instance, by gel electrophoresis). PCR methods can identify one target cell out of 10^6 – 10^7 normal cells which corresponds approximately to one cell in 0.1 ml–1 ml of blood. An important limitation of RT-PCR is that CTC are destroyed, making it impossible to count them or to analyse them individually. CTM are also undetectable as such by this approach. Another limitation is that the choice of the marker RNAs (the transcript(s) detected by the test and that should indicate the presence of tumor cells in blood) is difficult. The “ideal” marker would be a transcript expressed in all the tumor cells from a given tumor, but not expressed at all, not even by illegitimate transcription [47] (low level, non-specific transcription of certain genes, for instance, expression of albumin transcripts in lymphocytes [48]) in peripheral blood leukocytes (PBL) or in non-tumorous epithelial cells. Thus, a careful identification of the transcript and of its pattern of expression is very important. Finally, the high sensitivity of RT-PCR tests carries the risk of PCR products carry over which requires strict negative controls to validate the positive PCR signals.

Indeed, if the target gene is a typical gene expressed in epithelial cells (for instance, cytokeratins), the signal will be a false positive if the patient has circulating non-tumorous epithelial cells. CK19 mRNA has been found in the blood of 3.7% ($n = 54$) healthy donors as well as in 14.3% ($n = 28$) samples from patients with haematological malignancies and in variable proportions of control subjects (Tables 2 and 3). Detection of CK19 in healthy donors has been attributed to illegitimate transcription of the CK 19 gene in PBL [49] and/or to increased secretion of cytokines which can induce transcription of tissue-specific genes in PBL

Table 1
Characteristics of some available methods used to enrich CTC

Systems	Blood volume per test (ml)	Time for enrichment (min)	Principle of CTC enrichment	Method of detection	Type of enriched cells	Sensitivity threshold (cell per blood)	Reported detection of circulating tumor microemboli (CTM)
Oncoquick	15–35	45	Cellular density	Immunolabelling, possible cytopathological analyses	Low density cells	1 cell/4.5 μ l ^a	No
MACS	5–15	120	Capture of epithelial cells by immunobeads	Immunolabelling, molecular analyses	Cells expressing epithelial antigens	1 cell/0.3 ml ^b	No
CellSearch	7.5	40	Capture of epithelial cells (EpCAM positive) by ferrofluid	Negative selection by CD-45 (leukocytes) Positive selection by CK-8,18,19 (epithelial cells)	Cells expressing epithelial antigens	1 cell/0.5 ml ^c	No
ISET	10	15	Cellular size	Cytopathological analysis, immunolabelling	Cells larger than leukocytes: epithelial cells, tumor cells (and others “rare” cells) ^e	1 cell/ml ^d	Yes ^d

^a R. Gertler Recent Results Cancer Res. (2003).

^b C. Griwatz, J. Immunol. Methods (1995).

^c M. Kagan, J. Clin. Ligand Assay, (2002).

^d Vona et al. 2000.

^e “rare” circulating cells: trophoblastic, endothelial and stem cells.

Table 2
Sensibility and specificity of different methods used to detect CTCs

Type of enrichment method			Type of detection method ^a			Cancer type (cell line)	Sensitivity ^b	No. healthy subjects (% of positive)	No. patients with benign disease (% of positive)	References
Density gradient	Immuno- magnetic separation (antibodies)	Filtration	RT-PCR (transcript)	qRT-PCR (transcript)	Immuno-mediated detection (antibodies)					
Ficoll	—	—	—	nCK-20	—	Colon (HT 29) Breast (MCF-7)	1–10 cells/ 3 ml	150 (0)	ND	G. Giribaldi G, J Mol Diagn (2006)
Ficoll	—	—	CK20	—	—	Colon (HT 29)	1 cell/ml	60 (0)	9 (0)	M. Koch, Int. J. Colorectal Dis, 2006; J. Weitz, Clin Cancer Res, 1998
Ficoll	IMS (Ber- EP4/CD-45)	—	—	CEA	—	Colon (LS174T)	1 cell/ml	10 (0)	ND	J. Guo, J. Mol. Med. (2004)
Ficoll	—	Yes	—	—	ICC (CK-8)	Breast (MCF-7)	1 cell/ml	20 (0)	ND	H. J. Kahn, Breast Cancer Res. Treat (2004)
Ficoll	—	—	—	p1B, PS2, CK19, EGP2	—	Breast (MCF-7)	ND	96 (0)	ND	A.J. Bosma, Clin. Cancer Res. (2002)
Ficoll	—	—	Nested CK- 19	—	—	Breast (MCF-7)	1 cell/0.1 ml	54 (3.7)	ND	A. Stathopoulou, J. Clin. Oncol. (2002)
Ficoll	MACS (CD- 45, CK-7-8 or HEA)	—	—	—	ICC (pan-CK)	Renal (Caki- 1)	1/ml ^c	16 (0)	—	U. Bilkenroth, Int. J. Cancer (2001)
Ficoll	—	—	Nested PSA	—	—	Prostate (LNCaP)	1/ml	31 (0)	50 (0)	A. Mejean, J. Urol. (2000)
Ficoll	—	—	Nested AFP	—	—	Liver (Hep G2)	1/ml	37 (0)	65 (0)	Louha M, Hepatology, 1997
—	MACS (HEA-125)	—	Multiplex CEA, CGM2	—	—	Colon (HT29)	1 cell/0.1 ml	41 (0)	32 (0)	R. Douard, Clin. Chem. Lab. Med. (2005)
DG	MACS (CK)	—	—	—	ICC (HER-2)	Breast	ND	15 (0)	17 (0)	P. Wulfing, Clin. Cancer Res. (2006)

	Ferrofluid (EpCAM) ^d				IF (CD45\CK8, CK18, CK19)	Breast (SKBR-3)	1 cell/0.5 ml ^e	145 (0.1)	200 (0.1)	M. Cristofanilli, N Engl J.Med. (2004); W. J. Allard, Clin. Cancer Res. (2004)
Lymphoprep	Dynabeads (Epithelial antibodies)		Nested CK-19, CEA			Colon	ND	25 (CEA = 4) (CK-1 = 20)	ND	J. M. Silva, Gut (2002)
Lymphoprep		—	Nested SCCA	—	—	Oesophageal (KYSE-273)	1cell /0.1 ml	19 (0)	3 (0)	J. Kaganoi, Br. J Surg. (2004)
			Nested PSMA			Prostate (LNCap)	1cell /ml	ND	71 (2.8)	N. Hara, Clin Cancer Res. (2002)
Ficoll	IMS (BerEP4)	—	Telomerase-PCR-ELISA			Breast (MCF-7)	1.23 /ml	9 (0)	ND	Soria J C, Clin Cancer Res (1999)
			ISET			Liver (HepG2)	1/ml	46 (0)	69 (0)	G. Vona, Hepatology (2004); G. Vona, Am. J. Pathol. (2000)
			Cytopathological analysis							
			ISET		ICC (KL1)	Breast (MCF-7)	1 cell/ml	40 (0)	ND	P. Pinzani, Hum. Pathol. (2006)
Oncoquick	—	—	—	—	ICC (CD45\CK8,18,19)	Breast (MCF-7)	1/72 microliters ^f	25 (8)	ND	V. Muller, Clin. Cancer Res. (2005)
Erythr lysis	—	—	CEA	—	—	Colon (HT 115)	1 cell/ml	70 (2.9)	ND	R.Q.Wharton , Clin Cancer (1999); S. Jonas, Gut (1996)
Erythr lysis	—	—	hMAM	—	—	Breast (SKBR5)	1cell/0.1–1 ml	27 (0)	41 (5)	O. Zach J. Clin. Oncol. (1999)
			membrane array assay			NSCLC (H358)	1cell/0.2 ml	100 (6)	47 (17)	C.C.Sheu, Int. J. Cancer (2006)
Ficoll	—	—	Semi quantitative AFP	—	—	Liver (HepG2)	1 cell/0.6 ml	ND	10 (0)	I.H. Wong, Clin. Cancer Res. (1999)

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Table 2 (continued)

Type of enrichment method			Type of detection method ^a			Cancer type (cell line)	Sensitivity ^b	No. healthy subjects (% of positive)	No. patients with benign disease (% of positive)	References
Density gradient	Immuno- magnetic separation (antibodies)	Filtration	RT-PCR (transcript)	qRT-PCR (transcript)	Immuno-mediated detection (antibodies)					
—	—	—	CEA	—	—	Colon (SW-480)	1 cell/0.2 ml	30(0)	ND	J.Y. Wang, World J. Surg. (2006)
Erythr Lysis	—	—	—	—	IF (HEA) (LSC)	Breast (MCF7)	1cell/ 2 µl	100(3)	25(0) ^h	K. Pachmann, Breast Cancer Res. (2005); K. Pachmann, Clin Chem. Lab. Med. (2005)

Ab, antibodies; AFP, a-fetoprotein; CEA, carcinoembryonic antigen adhesion molecule 5 (CEACAM5); CGM2, carcinoembryonic antigen adhesion molecule 7 (CEACAM7); CK, cytokeratin; Erythr Lysis, Erythrocyte lysis; HER-2, proto-oncogen product; HEA, human epithelial antigen; hMAM, mammaglobin; ICC, immunocytochemistry; IF, immunofluorescence; IMD, immunomediating detection; IMS, immunomediating separation; ISET, isolation by size of epithelial tumor cells; LCS, laser scanning cytometry; MACS, magnetic-activated cell separation; ND, non-determined; No., number; PSA, prostate-specific antigen; PSMA, prostate-specific membrane antigen; SCCA, squamous cell carcinoma antigen; NSCLC, non-small cell lung cancer; RT-PCR, reverse transcription-polymerase chain reaction, qRT-PCR, quantitative RT-PCR.

^a Original methods are in box.

^b Sensitivity threshold, presented as cell per maximum volume of blood still giving a signal.

^c Recovery rate 84%.

^d CellSearch.

^e Recovery rate 85%.

^f Recovery rate 70.6%.

^g Prostatitis.

^h Non-epithelial haematological malignancies.

Table 3
Studies reporting assessment of the clinical impact of CTC

Type of cancer	Method ^a	No. ml blood	No. patients ^b	Positive patients	No. controls ^c (% positivity)	Clinical impact ^e	References
Colon	Ficoll & qRT-PCR CK-20	10	99	22%	150 h.s. (0)	No association with metastasis	G. Giribaldi, J. Mol. Diagn. (2006)
Colon	MACS/HEA-125 & mRT-PCR/CEA/CGM2	10	45 Stage III–IV; 39 stage I–II	63% CEA and/or CGM; 238% CEA and CGM2	32 others (0); 41 h.s. (0)	Association with disseminated tumors	R. Douard, Clin. Chem. Lab. Med. (2005)
Colon	Erythr lysis & RT-PCR/ CEA, CK-20	14 ^d	100 (50M+)	48% CEA; 34% CK-20	70 (4.3)	No association with metastasis	R.Q. Wharton, Clin. Cancer, (1999)
Colon	nRT-PCR/ CGM2	10	121	48%	ND	No prognostic information	R. Douard, Surgery (2006)
Rectal	Ficoll & RT-PCR / CK-20	10	45 [median f-up 51]	38% before and after endorectal u-sound 24% only after endorectal u-sound	60 h.s. (0); 9 others (0)	Trend of worse prognosis if positive after endorectal u-sound	M. Koch, Int. J. Colorectal Dis. (2006)
Colorectal	RT-PCR/ CEA	4	72 [median f-up 28]	72.2%	30 h.s.(0)	Association with risk of postoperative metastasis	J.Y. Wang, World J. Surg. (2006)
Lung	Erythr lysis & IF/ HEA (LSC)	1	37	89%	100 h.s.(3)	Association with metastasis	K. Pachmann, Clin. Chem. Lab. Med. (2005)
Breast	Erythr lysis & IF/ HEA (LSC)	1	63	94% breast	100 h.s.(3)	Association with metastasis	K. Pachmann, Clin. Chem. Lab. Med. (2005)
Breast	Erythr lysis & RT-PCR/hMAM	5	114	25%	27 h.s. (0); 41 others (4.8)	Association with clinical stage	O. Zach, J. Clin. Oncol. (1999)
Breast	Ficoll & qRT-PCR/ CK-19	10	167 N- [median f-up 32]	21,6%	ND	Association with shorter PFS and OS	N. Xenidis, J. Clin. Oncol., (2006)
Breast	Ficoll & qRT-PCR/ CK-20	10	110	29%	150 h.s.2 (0)	Association with metastases	G.. Giribaldi, J. Mol. Diagn. (2006)
Breast	Ficoll + Filtration & ICC ⁵ /CK-8	5 ^d	131 (51 M+)	71% M+; 39% N–; 47% N+	20 h.s.	Association with disease stage, N+ and with metastasis	H.J. Kahn, Breast Cancer Res. Treat. (2004)
Breast	Ficoll & nRT-PCR/ CK-19	10	161 M- [median f-up 29]	27.3%	28 others (14.3); 54 h.s. (3.7)	Mild association with decreased PFS	N. Xenidis, Ann. Oncol. (2003)
Breast	Ficoll & qRT-PCR/ CK-19, p1B, PS2 and EGP2	1(6 × 10 ⁶ PBMCs)	94 M+ [median f-up 15]	31%	96 (0)	Association with shorter PFS and OS	B. Weigelt, Br. J. Cancer (2003); A.J. Bosma, Clin. Cancer Res., (2002)
Breast	DG + IMS & ICC/ HER-2	50	42 (7 M+)[median f-up 95]	100% M+ 48.6% M–	15 h.s. (0); 17 others (0)	Association with decreased PFS and OS	P. Wulfing, Clin. Cancer Res. (2006)
Breast	Ficoll & qRT-PCR/ CK-19	10	253 stage I–II; 239 stage III–IV	12% M–; 21% M +	ND	Association with CNS relapse	J. Souglakos, Breast Cancer Res. (2006)

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Table 3 (continued)

Type of cancer	Method ^a	No. ml blood	No. patients ^b	Positive patients	No. controls ^c (% positivity)	Clinical impact ^e	References
Breast	IMS EpCAM & CD45, CK- 8,18,19 (CellSearch)	7.5	177 M+	61 % (cut off: >5 CTCs)	145 h.s. (0,1 ± 0.9) 200 others (0,1±0.2)	Association with shorter PFS and OS	M. Cristofanilli, N. Engl. J Med. (2004); M. Cristofanilli, J. Clin. Oncol. (2005); D.F. Hayes, Clin. Cancer Res. (2006)
Breast	Ficoll & cytospin ICC/CK-8,18,19	7-14 10 ⁶ MNCs	75 M- 39 M+ [median f-up 28]	16% M-; 41% M+	ND	Association with metastatic disease	J.Y. Pierga, Clin. Cancer Res. (2004)
Breast	Ficoll & nRT-PCR/CK-19	10	148 M-; 50 M+ [median f-up 28]	29.7% M-; 42% M+	59 others (8.5); 54 h.s. (3.7)	Independent prognostic value in M- patients	A. Stathopoulou, J. Clin. Oncol. (2002)
Breast	nRT-PCR Mammaglobin	20	101 M- [median f-up 24]	13.9%	30 h.s. (0); 40 others (0)	Association with shorter PFS	F. A. Vlems, M. Ntoulia, Clin. Biochem. (2006)
Breast	Oncoquick & ICC/CD45/CK	10	60 M-; 63 M+ [median f-up 28]	8.3% M-; 39.7% M+	25 h.s.(8)	Association with disease progression	V. Muller, Clin. Cancer Res. (2005)
Ovarian	Lymphoprep & IMD/MOC-31	40	90 [median f-up 25]	12%	ND	No association with poor prognosis	C. Marth, Cancer, (2002)
Kidney	DG & RT-PCR/MN/CA-9	8	37 (9 M+)	49%	5 others (0); 54 controls (1.8);	No association with tumor grade	J.M. McKiernan, Cancer, (1999)
Kidney	Ficoll + MACS (CD-45/CK-7-8 or HEA) & ICC/pan-CK (5,6,8,17,19)	8	59	32%	16 h.s. (0); 16 others (50)	Association with tumor grading	U. Bilkenroth, Int. J. Cancer (2001)
Squamous Oesophageal cancer	Lymphoprep & nRT-PCR/SCCA	10	70	33%	19 h.s.(0) 3; others (0)	Association with poor outcome	J. Kaganoi, Br. J. Surg. (2004)
Liver	Ficoll & nRT-PCR/AFP	15	84	33.3%	37 h.s. (0); 65 others (0)	Association with risk of metastasis	Louha, M. Hepatology (1997)
Liver	ISET & cytopathological analysis	3	44 M-	52%	38 h.s. (0); 69 others (0)	Association of CTC number with shorter survival	G. Vona, Hepatology (2004)
Prostate	IMS EpCAM & CD45/CK- 8,18,19 (Cell Search)	7.5	37 M+	62%	ND	Association with shorter survival	J.G.. Moreno, Urology (2005)
Prostate	Ficoll & RT-PCR/PSA	3	46 M-	22%	145 others (12)	No predictive value	N. Thiounn, Urology, (1997)
Prostate	Ficoll & nRT-PCR/PSA	15	99 (2 M+)	33%	31 h.s. (0); 50 others (0); 11 prostatitis (2);	Association with risk of tumor recurrence	A. Mejean, J. Urol. (2000)
Prostate	DG & RT-PCR/PSA	5	227	27%	65 others (0)	Association with pathological stage (multivariate analysis)	R.D. Ennis, Cancer (1997)

Pancreas	Ficoll & nRT-PCR/ CK20	20	154 (64M+) [median f-up 11]	33.8% (37,1%)	54 controls (16,7); 20 h.s. (0,5)	Association with pathological stage and with survival	E. Soeth , J. Cancer Res. Clin. Oncol. (2005)
Pancreas	DG & cytospin/ICC (CK7, CK19, CK20)	3	102 (31M+) [median f-up 27]	26% (39% M+)	60 controls (1.6); 6 h.s. (0)	Association with tumor stage No association with survival	K. Z'Graggen, Surgery (2001)
Gastric	DG & nRT-PCR/ CEA	5 from portal vein	57 M+ [median f-up 15]	36.8%	15 controls (0); 15 h.s. (0)	Iatrogenic diffusion of tumor cells Association with metastasis	F. Miyazono, Ann. Surg. (2001)
Colorectal	IMS/Ber-EP4 &RT- PCR/MUC1,MUC2 CK19,CK20	10	94 [median f-up 15]	20%	64 others (11); 20 h.s.(0)	Association with shorter PFS and OS	Hardingham, Int. J. Cancer (2000)
Colorectal	Ficoll & RT-PCR/ CEA	20	95 (20 M+) [median f-up 41]	M+ 60%; M– 36%	ND	No association with prognosis	X. Bessa , Gastroenterology (2001)
Colorectal	Ficoll & RT-PCR/ CEA	10	9 M+; 44 M- [median f-up 19]	M+ 67%; M– 36%	32 h.s.(0)	Association with shorter PFS	T. Taniguchi Cancer, (2000)
Colorectal	Ficoll & nRT-PCR/ (CEA,CK20)	10	52 (9)	CEA (38,4%); CK20(36,5%)	10 controls (0); 10 h.s.(0)	No association with tumor stage	K. Yamaguchi, Ann. Surg. (2000)

AFP, a-foetoprotein; CEA, carcinoembryonic antigen , CGM2, carcinoembryonic antigen adhesion molecule 7; CK, cytokeratin; CNS, central nervous system; CTCs, circulating tumor cells; DFS, disease free survival; DG, density gradient; Erythr Lysis, erythrocyte lysis; f-up, follow-up; HER-2/neu, proto-oncogen product; HEA, human epithelial antigen; hMAM, mammaglobin; h.s., healthy subject; ICC, immunocytochemistry; IF, immunofluorescence; IMD, immunomediated detection; IMS, immunomediated separation; IMS/Ber-EP4; epithelial enrich Ber-EP4-coated microsize immunobeads (DynaL Biotech, success, NY); ISET, isolation by size of epithelial tumor cells; LSC, laser scanning cytometer (Computer Corporation, Cambridge, MA, USA); M+, patients with metastasis; M–, patients without metastasis; N–, lymph node negative; mRT-PCR, multiple RT-PCR; MACS, magnetic cell sorting; M+, with metastases; M–, without metastases; median f-up, median follow-up; MUC1/MUC2, oncoprotein; nRT-PCR, nested RT-PCR; ND, nondetermined; No., number; N–, lymph node-negative; N+, lymph node positive; OS, overall survival; PBMC, peripheral blood mononuclear cells; PFS, progression free survival; PSA, prostate-specific antigen; qRT-PCR, quantitative RT-PCR; nRT-PCR, nested RT-PCR; SCCA, squamous cell carcinoma antigen; u-sound, ultra sound.

^a The symbol & is placed in between the enrichment and the detection method, its absence indicates that any enrichment method has been performed.

^b The number of patients with metastasis is in parenthesis (M+), median follow-up is in months.

^c h.s., healthy subjects; others, patients with other cancers or benign diseases.

^d Multiple sampling.

^e According to statistical analysis.

^f Tested after surgery.

[47,50]. Conflicting results have also been published for CK20. CK20 mRNA was detected in the blood of a variable number of healthy donors and controls (Table 3).

Organ-specific marker genes like PSA/KLK3, a prostate-specific antigen, are expressed in all prostatic cells, thus they can give false positive results if non-tumorous prostate cells are spread in blood by inflammation [51], invasive diagnostic procedures (e.g., biopsy) and/or surgery [40,52]. Mammoglobin mRNA, a breast-specific transcript, has reportedly been induced in non cancerous patients by several cytokines [53], while its expression has never been reported in healthy donors [30] (Tables 2 and 3). MUC-1, which is expressed in normal breast, bronchial, pancreatic, uterine, salivary, intestinal and other glandular cells, has been repeatedly shown to be expressed in leukocytes and blood samples from healthy donors and controls. In one study, it was reported to be expressed in 70% ($n = 40$) blood samples from normal subjects and 73% ($n = 15$) of patients with haematological malignancies [30].

Tumor-specific markers may also be expressed in non-tumorous cells. Alpha Fetoprotein, for instance, is expressed in non-tumorous liver-derived cells [52] and CEA transcripts have been detected in the blood of healthy donors and in patients with inflammatory bowel diseases [30]. EGFR has been shown to be expressed in 9% ($n = 22$) of subjects without tumor and in 10.5% ($n = 38$) of healthy donors [54,55]. HER-2 mRNA was found to be expressed in the blood of 10% ($n = 20$) of healthy women and in most blood samples from healthy donors [30]. Actually, EGFR, mammoglobin, small breast epithelial mucin and squamous-cell carcinoma antigen were shown to be expressed in proliferating, but not resting peripheral blood leukocytes [56]. Telomerase, a specific polymerase expressed in approximately 85% of malignant tumors, can be expressed in some non-neoplastic tissues and in some lymphoid cells [30]; however it has never been reported to be expressed in healthy donors and control samples, although tested in small number [30].

Quantitative RT-PCR (q-PCR) and nested real-time RT-PCR [31,46] assays have been used to improve the specificity of RT-PCR. Quantitative RT-PCR tests are based on the attempt to define a cut-off value of a given transcript marker, compared to a reference marker expressed in any cell, which would be indicative of the presence of tumor cells

in blood. The advantage of qRT-PCR tests over conventional RT-PCR assays is that they use internal probes (between primers) that specifically hybridize to the amplified sequence, increasing the PCR specificity. However the proportion of tumor cells in blood may be highly variable and the RT step introduces significant variability making it problematic to define a relevant quantitative “cut-off” point [46]. Schuster R et al. [57] for instance, were not able to set a definite cut-off value to differentiate, by quantitative real time PCR, mRNA from tumor cells and those from illegitimate transcription in PBL. Practically, since it is impossible to predict the number of normal and of tumor cells expressing the different types of transcripts, cut-off thresholds can be generally valid but not adapted to individual cases.

4.2. Direct methods

Direct methods are meant to provide a diagnostic identification of CTC. Given the important limitations of immune-labelling and RT-PCR assays, direct diagnosis of CTC/CTM can only be obtained by cytopathological analysis of the isolated cells [39] and/or by the analysis of their genome providing clues to the tumorous nature of the cell [34,44]. Cytopathological analysis can be carried out in a routine manner, provided that CTC enrichment does not damage cell morphology. In contrast, genome analyses (FISH, CGH, mutation analysis) have not been applied routinely, for technical reasons, to the detection of CTC, but rather to their characterization [42,44,58]. In fact, FISH probes generally do not label all the target cells (which is a limitation when testing rare cells), the interpretation of the signal can be difficult and some cells can be tumorous without a detectable aneuploidy. Comparative genomic hybridization (CGH) and mutation analyses are expensive and time consuming procedures which, to be informative, have to be directed, by laser microdissection, to individual cells [58]. Furthermore, very few “marker” mutations or translocations (present in any tumor cell of a given tumor type) are known to characterize solid tumors, in contrast with hematologic malignancies.

Thus, there is a strong argument to be made that cytopathological analysis should be the reference diagnostic method, and be used to identify CTC and CTM, just as it is in other oncological diagnostic settings (PAP-test, cytopathological analysis of

tumor biopsies and aspirates of biological liquids (ascites, urine, cephalo-rachidien liquid)). Furthermore, cytopathological study of enriched cells from blood allows the identification of CTM, which are the expression of “collective migration” and carry a higher risk of development of metastases (see Section 2.1). Cytopathological analysis could be used as a reference basic approach to recognize CTC/CTM, applying additional techniques (immunolabelling, FISH, RNA/DNA analysis) to better characterize their malignant nature and their invasive potential.

In the past, the classical technique of blood smears has been applied to perform cytopathological analysis of CTC. However, this is not feasible in a routine manner, for in order to find one CTC in 1 ml of blood, the analysis of 100 smears (10 μ l per smear) must be performed. Automatic instruments, routinely used for PBL counting, analyze blood samples of 50 μ l, and are thus unsuitable for detecting “rare cells”.

Enrichment approaches aimed at isolating CTC independently from their antigens and avoiding damage to cell morphology are based on physical properties of CTC: density and size. After cytological staining (May-Grunwald Giemsa, Hematoxylin & Eosin, etc...), cytoplasmic and nuclear details become available to observation and thus allow cytopathological diagnosis of CTC/CTM.

Density gradient separation of mononucleated cells from blood (including CTC), is obtained by using Ficoll (Amersham, Upsala, Sweden), Lymphoprep (Nycomed, Oslo, Norway) or other similar density gradient liquids. Whole blood is directly layered on the density gradient. After centrifugation, from bottom to top are found: erythrocytes, neutrophils, density gradient, mononuclear cells (lymphocytes, monocytes, epithelial cells, tumor cells), and plasma which is the upper layer. Tumor cells can also migrate in the plasma fraction. However, whole blood rapidly starts to mix with the density gradient if it is not immediately centrifuged, preventing optimal cell separation. OncoQuick (Greiner, Frickenhausen, Germany) (Fig. 3 and Table 1) consists of 50 ml tubes containing the density gradient placed under a porous barrier. It has been designed to isolate mononuclear cells with low density cells and particles (low density leukocytes, epithelial cells, tumor cells, platelets) from neutrophils and lymphocytes. The tubes permit the layering of whole blood (15–35 mL) on the porous barrier, thus avoiding its mixing with the density gradient before centrifugation. In tumor cells spiking assays, although the

tumor cells recovery rate has been shown to be similar for Ficoll and Oncoquick, the last method obtains a greater enrichment of tumor cells from leukocytes, which simplifies further analyses [59] as cell staining, immunolabelling, and molecular studies. The limiting problem of OncoQuick is that rare CTC can be lost during the isolation step as they can migrate in the plasma fraction or are trapped among erythrocytes and neutrophils [34,59], so that the system has very low and variable sensitivity depending on tumor cell characteristics, centrifuge time, temperature etc... In spiking assays, Oncoquick sensitivity was lower than that of CellSearch [43].

Direct enrichment of epithelial cells by filtration has been first described by Vona et al. in 2000 [58,60]. Isolation by size of Epithelial Tumor cells (ISET)(Metagenex, Paris France; www.metagenex.fr) (Figs. 2, 3 and Table 1) is based on the observation that the vast majority of peripheral blood leukocytes (lymphocytes and neutrophils) are the smallest cells in the body, having a size ranging from 8 to 11 μ m. They can thus be massively eliminated by blood filtration through polycarbonate membrane with calibrated pores of 8 μ m.

The simplicity of the assay avoids losing rare cells in multiple steps of isolation. Peripheral blood is collected on EDTA, diluted with the ISET buffer (which fixates cells), let to stand 10 min, then loaded into the Metablock and filtrated by the ISET device (2–3 min). Filtration takes place through distinct spots on the filter according to the blood volume, so that every spot will show the retained “large” cells which were, before filtration, in 1 ml of blood. This permits the precise counting of the number of CTC per millilitre of blood independently from the blood volume treated. Enriched cells can be stained with cytological stainings (i.e., May-Grunwald Giemsa, Hematoxylin & Eosin, etc...), and/or characterized by immunolabelling, FISH, or TUNEL assays in order to analyse their antigens, aneuploidy and rate of apoptotic cells (Fig. 2). Interestingly, circulating tumor microemboli (CTM), which are thought to carry a high metastatic potential, are also sensitively enriched and can be reliably counted (Fig. 2). Molecular analyses, specifically focused on cytopathologically identified tumor cells, can be carried out after laser CTC/CTM microdissection [58]. Pinzani et al. [61] (Table 2) demonstrated the feasibility of studying the HER2 DNA amplification in tumor cells microdissected after enrichment by ISET. Enrichment by

direct filtration is very sensitive since fixed cells larger than 11 μm in at least one diameter cannot pass through the 8- μm pores. Furthermore, this direct method avoids multiple steps and cell damage, which both contribute to enrichment sensitivity. In repeated analyses, the system has been shown to isolate one single tumor cell added by micropipetting to 1 ml of blood [60].

Meng et al. [62] report that the mean diameter of tumor cells in blood from patients with breast cancer ranges from 29.8 to 33.9 μm . These cells are isolated by ISET without difficulty since the pore size is 8 microns. Moreover, ISET enriches smaller and larger cells with clear cytopathological features of CTC. Since the cell morphology is conserved, it is easy to distinguish epithelial non-tumor cells from tumor cells by cytological staining and cytopathological examination, complemented if required by immunolabelling. A limited number of leukocytes are also retained on the filter, but they are easy to recognize without any additional labelling [60]. Images of CTC/CTM can provide oncologists with a visual aspect of this new marker and its evolution during follow up (modification of CTC morphology toward more malignant traits, appearance of CTM, apoptotic cells, etc...) (Fig. 2).

Zabaglo et al. [63] used filtration of whole blood through polycarbonate membrane with 8-microns calibrated pores and found the recovery of 85–100% MCF7 and T47D breast cancer cells with about 0.1% leukocytes remaining on the filter (approximately 10,000 PBL per millilitre of blood). Thus, the enrichment power of this method is lower than that of ISET, which retains from 0.0002% to a maximum of 0.02% PBL (thus less than 2000 PBL per millilitre of blood) [60]. The approach of Zabaglo et al., which is less complex and less expensive than immunomagnetic methods, has been associated with automatic analysis of CK-positive cells. This method could still give false positive and false negative results, unless manual re-staining of CK positive cells by H&E and cytopathological analysis is performed. Comparison of filtration (Zabaglo et al. method), immunomagnetic separation and multimarker real-time RT-PCR showed that RT-PCR is more sensitive than the two other approaches in detecting circulating epithelial cells [64]; however, a stringent detection of circulating tumor cells by cytopathological analyses, required to specifically count the number of CTC/CTM, has not been assessed in this work.

Kahn et al (Table 2) isolated mononuclear cells by Ficoll, before fixing and filtering them several times through a polycarbonate filter with 8- μm diameter pores mounted onto a syringe. Recovered cells were then transferred to a glass slide and immunostained with anti-CK 8 antibody. This method demonstrated an average recovery of 63% of MCF7 cells in spiking assays. This low sensitivity is consistent with the multiple steps (including Ficoll and multiple filtrations) which cause tumor cell damage and loss.

5. When are we certain that tumor cells are in blood?

From a clinical point of view, we expect that informative and reliable results are obtained in proportion to the specificity and sensitivity of the assay designed to identify CTC. But when are we sure that CTC are in blood?

Due to the lack of truly reliable “marker” genes, RT-PCR analyses generally use epithelial- or organ-specific markers without any proof that the test reliably identifies tumor cells in blood. Even tumor-related markers (for instance, Alpha-fetoprotein, CEA, etc...) can be expressed in non tumor cells without providing the proof that tumor cells are reliably detected. So, for a large majority of these tests the result is still in terms of “probability”.

Identification of epithelial cells in blood by immune-mediated assays is also associated with the “probability” of identifying CTC and does not allow their precise identification and counting. In 1999, standardized immunocytochemical criteria for detection of cancer cells in bone marrow were reported [65] and tumor cells were defined according to “pathognomonic signs of epithelial tumor cell (TC)-nature, i.e. a clearly enlarged nucleus, high nucleus/cytoplasmic ratio and/or clusters of ≥ 2 immunopositive cells”. However, epithelial cells found in the bone-marrow are supposed to be extravasated cells, thus more susceptible to be tumor cells than epithelial cells found in blood, where they can be in transit after spreading from organs. Moreover, this standardization criteria had been proposed in 1999, when expression of epithelial antigens was seen as a fundamental and constant characteristic of tumor cells derived from carcinoma, while we know now that this is not true (see above, epithelial to mesenchymal transition). Also, the concept that “epithelial cells do not circulate, unless they become tumorous” was widely accepted and not yet challenged by later studies.

As mentioned, evidence has been provided, in the last years, that malignant tumor cells from carcinoma tend to lose their epithelial antigens (EMT) and that normal epithelial cells may be spread in the blood circulation.

So, when are we certain that tumor cells are in blood? The possibility of analyzing the nuclear and cytoplasmic characteristics in great detail provides a major diagnostic element. The cytopathologist currently identifies tumor cells in other oncological settings and can provide a cytopathological assessment of the cell nature. Expression of organ-specific markers (e.g., PSA in cells from prostate cancer) or epithelial-specific markers (e.g., CK) on circulating cells having a clear tumor cell morphology can assist the pathologist in the differential diagnosis with circulating micromegakaryocytes. However, as reported in the literature, “epithelial antigens are not always expressed on tumor cells”. Occurrence in blood of micromegakaryocytes is the sign of a pathological condition, as these immature cells are found in myeloproliferative syndromes like the myeloid splenomegaly, in the AML7 (acute megakaryoblastic myeloid leukemia) and rarely in other myelodysplastic syndromes. Micromegakaryocytes are thus exceptional in patients with solid cancer and can be identified by specific labeling with the CD61 and CD42 antibodies. Thus, cells found in blood and having a tumor-like feature can be specifically identified as CTC on the basis of their cytopathological analysis and be further characterized by immunolabelling and/or molecular analyses.

We would like to stress the potential consequences of the confusing terminology related to technical approaches having low specificity: “The criteria for an object to be defined as a Circulating Tumor Cell include round to oval morphology, a visible nucleus (DAPI positive), positive staining for cytokeratins and negative staining for CD45” [38]. These criteria cannot distinguish between epithelial non tumor cells and tumor cells. The issue is particularly relevant when a CTC count is required. How can we be sure that we strictly count tumor cells if we target epithelial cells? A confused terminology would hinder rather than help scientific advances. The statement “CTCs are rarely present in patients with non-neoplastic diseases” [38], for instance, should stimulate researches to understand why CTC are present in patients without cancer and what is their fate and clinical relevance. However, the test applied in this setting may detect circulating epithelial cells, not tumor cells. Thus, we still have

to prove that tumor cells circulate in the blood of patients without a neoplastic disease. Another drawback of a confusing terminology relies on molecular analyses aimed at characterizing CTC. The application of gene expression profiling assays [42] to cells enriched by immuno-labelling which are defined as CTC but are, in fact, circulating epithelial cells (CEpC), needs appropriate interpretation of results. Markers identified by studying CEpC will not be able to distinguish circulating tumor cells from circulating epithelial non tumor cells. Since the most malignant tumor cells, as discussed before, lose their “epithelial-specific antigens” (EMT, see above), defining CTC those cells which express epithelial antigens may carry important interpretation bias.

6. Characterization of CTC

It is important to characterize CTC to obtain further proof of their malignant nature and to assess the invasive potential of individual CTCs. We know from animal studies that approximately 1 out of 10,000 CTC is able to found a metastasis [27]. Even though this figure may differ in the case of human pathology and will be dependent on tumor variability, it is clear that research must be performed to identify, among CTCs, those having the highest metastatic potential [36].

Genotyping of CTC can be performed by FISH (fluorescence in situ hybridization) [44,60,62,66] or by CGH (comparative genomic hybridization) directed to single tumor cells or pools of tumor cells [67]. Analyses of oncogene amplifications (ex HER2) can be performed by FISH and/or by quantitative PCR after laser microdissection of CTC [61]. Oncogene mutations can be recognized in cytopathologically validated CTC after laser microdissection [58]. Immunolabelling is an interesting approach to characterize the invasive potential of CTC by assessing the expression of tumor markers (for instance, HER-2, metalloproteinases, EGFR, uPAR, alpha-fetoprotein) on enriched cells. New markers are expected from gene-expression profiling studies of human tumors [68] to be used to explore the invasive potential of CTC and orient anti-cancer treatment.

However, assays aimed at characterizing CTC have to be developed using strict criteria of specificity and applied with appropriated controls. FISH results have to be interpreted carefully according to rigorous criteria [34] in parallel with results obtained on normal blood cells. Immunological

staining may have a certain rate of non-specific labeling.

It has been reported that a relevant number of epithelial cells detected in blood of patients with breast cancer can be identified as apoptotic cells by CK staining and TUNEL (TdT-uridine nick end labeling) analysis [69]. Detection of apoptotic cells is relevant; however, we have to take into account that the method used to prepare the cells for analysis may induce apoptotic cell death, in cells made fragile by blood storage with conservative agents, through multiple manipulations and contact with magnetic particles [70]. If cell enrichment is performed without damage to the cell morphology, typical cellular features characterizing cell apoptosis, such as cell shrinkage, nuclear condensation, pyknotic nucleus, plasma membrane blebbing and apoptotic bodies can be recognized at the cytomorphological analysis. In addition, the TUNEL assay can demonstrate the typical DNA breaks. Detection and counting circulating apoptotic cells may be highly relevant before and after anticancer therapy, in order to assess the pro-apoptotic effect of therapeutic programs. However, once modification of cell morphology has started as a result of apoptotic cell death, it is difficult to distinguish non-tumorous apoptotic cells from tumorous apoptotic cells.

7. Clinical impact of CTC detection

Despite the large number of studies focused on detection of CTC, we still do not have a clear view about the clinical impact of these tests. This is because a substantial number of studies do not meet essential criteria for quality assurance and many reported works seem to overestimate the importance of findings.

Variation of technical details likely causes variation in the final results (Tables 2 and 3). In some, but not in all studies, the first milliliters of collected blood are discarded, with the intention of eliminating epidermal cells that could generate false positive results if markers (RNA or antigens) specific to epithelial cells are being used. This step is not necessary if CTC are detected by cytopathological analysis as the morphology of epidermal cells is easy to distinguish from that of tumor cells.

In RT-PCR studies, previous enrichment of mononuclear cells is sometimes, but not always done, while it is known to be effective in reducing the background noise due to PBL. As discussed, RT-PCR tests cannot allow counting of CTCs and

give a positive/negative response which depends on the sensitivity of the test and on the number of ml of blood tested. However, this parameter is often not reported. Some authors report the amount of RNA tested in micrograms, or the number of mononuclear cells from which they extract the RNA, but these parameters do not specify the volume of blood tested. After mononuclear cell enrichment, some authors use very small amounts of cells to reduce the background, so reducing the sensitivity of the test. Others have proposed to decrease the sensitivity of the PCR test in order to reduce the background [71], which does not seem a good way to obtain specificity. In RT-PCR and quantitative RT-PCR tests, specificity relies on the relative proportion of tumor cells and blood cells, which number may be highly variable among different patients.

Conflicting results, concerning specificity and sensitivity, have been reported about several markers, both RNAs and antigens, making interpretation of results extremely difficult, even for researchers aware of the methodology. Actually, it has been shown that activated leukocytes, whose number is higher in patients with cancer than in controls, may express markers used to detect CTC [50,56]. Furthermore, it has been pointed out that false positive rates obtained with immunolabelling range from 22% to 61% and can be variable according to the antibody and the staining methodology [35], thus introducing a bias in the interpretation of results. This is particularly true when the technical approach to identify CTC uses several steps which can be performed differently in different laboratories. For this problem, commercial methods offering automated solutions may be useful, provided that the technical approach does not create bias on specificity and sensitivity.

Besides non-specific results that can originate from the use of primers and antibodies with limited specificity, a major source of bias in establishing the clinical impact of results concerns the time when the blood sample is collected. Very few studies report if blood samples have been obtained before any semi-surgical (biopsy) or surgical procedure. This is a very important point as epithelial cells can be spread in blood by iatrogenic procedures, thus increasing the number of epithelial cells detected by epithelial-specific markers. Cytopathological methods distinguish epithelial non-tumor from tumor cells, and thus allows specific counting of tumor cells. However, even when using cytopathological detection of CTC, it is important to know if the CTC

count represent the number of tumor cells which circulate spontaneously or if it also includes iatrogenic CTC spread. Determination of the time epithelial cells and tumor cells remain in the blood after iatrogenic spreading is important and difficult. With respect to tumor cells, it can be variable according to different tumor cells origin and characteristics. Animal studies have reported that CTC may remain for 3–4 weeks [72]. In humans, studies have been performed with methods unable to distinguish tumor cells from epithelial non-tumor cells [62,73], preventing a clear answer to the question.

Using a test which detects epithelial cells by anti-epithelial antibodies, Patchmann et al. [73] found a 1000-fold increase in epithelial cells during the first 3 days after surgery, with a large part of these cells disappearing after another 2–4 days. On the other hand, Meng et al. [62] found that the majority of epithelial cells disappear 1–2.5 h after surgery of the primary tumor. As these authors use the term “circulating tumor cells” to indicate cells detected using antibodies specific to epithelial antigens, they conclude that “CTC” have a very rapid turnover and that a remote tumor must be feeding these cells into the circulation. However, since they in fact detect circulating epithelial cells (CEpC), and that these cells are known to be massively spread by surgery, and eliminated thereafter, their results only indicate CEpC spreading without helping in clarifying the issue of circulating tumor cells turnover. Studies using cytopathological detection of tumor cells performed before and at different times after surgery will provide specific data on this issue.

A crucial and still unresolved issue regards the optimal number of assays (simple or duplicate or triplicate) required to demonstrate whether a blood sample is positive for CTC [1]. Some studies, using RT-PCR or immunolabelling have shown that the rate of positive patients increases when they are tested several times instead of only once. While this is plausible, we have to admit our complete ignorance about the factors which regulate the spontaneous circulation of CTC. Are they spread during 24 h at the same rate or can spreading be increased by factors such as physical exercise, digestion (for intestinal cancers), hyperventilation (for lung cancer) etc.? For these studies we need a diagnostic cytopathological detection of CTC and collection of specific data.

Despite all these difficulties and despite conflicting results, a general trend in the literature shows increasing rates of CTC in patients having more dif-

fuse cancers, higher risk of relapse and poor prognosis (see Table 3).

However, we still lack data helping oncologists to individualize treatment based on the individual risk of harboring CTC. A large clinical study performed on a cohort of 177 patients with metastatic breast cancer has been performed using immunomagnetic isolation, detection and counting of CEpC. Using a cut-off limit of 5 CEpC per 7.5 ml of blood, results show that patients under the cut-off have a significant longer overall survival and progression free survival as compared with patients whose CEpC number is over the cut-off limit [74,75] (Tables 2 and 3). The authors detected a range of CEpC from 0 to 23,618 [75]; thus, at the level of individual patients, should the oncologist treat with the same protocol patients with 6 CEpC and those with 23,000 CEpC, while treating differently patients with 5 CEpC? How can the oncologist decide to apply a more aggressive treatment if the actual number of CTC in blood is not reliably obtained? How the efficacy of the therapy can be assessed without precisely counting the number of CTC in blood?

The field of circulating tumor cells has experienced important advances during the last few years and patients themselves are becoming increasingly aware of the impact of these studies on their treatment and follow up. Nevertheless, many questions remain unanswered and need improved specific approaches. Criteria for translation of new prognostic/predictive markers into clinical routine have been defined. However, for detection of tumor cells in blood, a gold standard assay has not been identified yet.

8. Concluding remarks

In conclusion, this new field of oncology seems likely to bring valuable new information about tumor invasion, progression and response to therapy. The definition of a standardized, uniform, cytologic method to specifically and sensitively detect CTC/CTM is now crucial to perform large clinical trials focused on patients with different types of solid cancers at different clinical stage. These trials are expected to generate reliable results and provide guidelines to use the new marker in Clinical Oncology.

Technical progress, focusing routine molecular studies to rare circulating cells, combined with the discovery of new tumor markers, will bring new tools for CTC characterization. These advances

are expected to expand our knowledge of the invasion process and to generate new CTC data aimed at improving the patient's quality and expectancy of life.

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