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Circulating tumour cells in colorectal cancer

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ABSTRACT

Background: The detection of circulating tumour cells (CTC) in blood sample in patients with early or advanced colorectal cancer has a potential prognostic value.
Methods: The challenge of CTC detection is related to the requirement of high sensitivity combined with high specificity method. CTCs detection can be distinguished between indirect and direct methods. The former ones are based on the recognition of tissue-, organ- or tumour-specific markers by immuno-histochemistry (indirect immuno-mediated methods) or (real-time) RT-PCR (indirect molecular methods), whilst the latter are related to CTCs selection based on the physical properties of density and sizes. Ongoing and future isolation by size of epithelial tumour cells (ISET) developments concerning automated image analysis on the filter and transmission of high definition images through the web for 'on line' cytopathological consultations are aimed to speed up the work of cytopathologists on CTC/ circulating tumour microemboli (CTM) detection.
Conclusions: CTC detection in colorectal cancer (CRC) correlates with pathological stage and clinical outcome in particular in those patients with advanced disease. CRC CTC level before and after CT are an independent prognostic factor for progression-free and overall survival. The positive prognostic value of complete clearance CTC after surgery may be useful to select patients for adjuvant chemotherapy.

1. Introduction

Colorectal cancer (CRC) is the fourth most common malignancy in the European Community and the second most frequent cause of cancer-related death. Every year, about 213,000 cases of CRC will be diagnosed in Europe, and 110,000 people will die because of this.[1] Approximately 70% of these cancers will arise in the colon, whereas 30% will occur in the rectum. Significant progress in adjuvant chemotherapy (CT) for stage III CRC has been achieved with the addition of oxaliplatin to infusional 5-fluorouracil and leucovorin. In stage II CRC the role of adjuvant CT is still controversial. New prognostic factors are necessary to select patients that will benefit from adjuvant CT in this subgroup of patients.

Over the past decade, many advances as in the understanding the metastatic CRC have reported, and the introduction of biologic therapies has increased the overall survival (OS) over 25 months. Consequently, physicians have multiple potential treatment options for patients with metastatic CRC. The selection of patients through clinical and biological prognostic factors to identify the best treatment for each patient represents the principal end-point.

Preliminary data suggest that the level of circulating tumour cells (CTC) detected in blood sample collected in patients with early or advanced disease are a potential

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prognostic factor. CTC count in the future could provide an evidence of early treatment success or failure to guide treatment decisions. The detection of CTC has been proposed as a method to assess the response to treatment of metastatic breast cancer (MBC). The detection of tumour cells may have clinical utility in the risk stratification in early breast cancer, in the early detection of relapse and in the monitoring of the response to treatment.[2] Cristofanilli et al. conducted a prospective study of 177 women with MBC compared to 345 women without breast cancer to confirm the correlation between the level of detection of peripheral blood tumour cells and the presence of metastatic breast disease.[3] Patients were tested prior to receiving breast cancer treatment and at the first follow-up after the beginning of the treatment. Five or more cells per 7.5 ml of blood were associated with poor prognosis.

At the initial sampling, 50% of the women with MBC had more than five CTC per sample, with an average progression-free survival (PFS) of less than 3 months and an OS of 10 months. Women who had less than five CTC per sample had a PFS of 7 months and an OS of 18 months. Ten patients died prior to the first follow-up; their cancer cell counts ranged from 9 to 23,618 per sample. Only 30% of the women with MBC had five or more cells per sample, with an average PFS of 2.1 months and OS of 8.2 months, compared to women who had less than five CTC per sample, who had a PFS of 7 months and an OS of greater than 18 months. The number of cells per sample at follow-up was indicative of prognosis. Multivariate analysis of clinical factors demonstrated that whilst clinical factors (including time to metastasis, HER2/neu status and type of therapy) still correlated to outcomes, the strongest predictors of PFS and OS were the levels of CTCs at baseline and at the first follow-up visit. Stathopoulou et al. described a significant negative prognosis after detecting CTC by a cytocheratin 19 (CK19) reverse transcriptase-polymerase chain reaction (RT-PCR) method in 128 breast cancer patients (stage I/II).[4] Patients, having CTC in blood after the removal of the primary tumour and before receiving adjuvant therapy had a significant lower survival time (risk factor 8.5) and a reduced disease-free interval (DFI) (risk factor 5.1). It should be noted that this was a patient group with a relatively favourable prognosis. Therefore, the identification of CTC in these patients represents an important additional factor for deciding about therapy.

Meropol et al., reported in 430 patients with advanced CRC that the number of CTC, before and after the beginning of therapy, is an effective, non invasive prognostic marker for OS and PFS.[5] They enroled in this preliminary trial patients with measurable metastatic CRC before CT (first, second or third line therapy). Peripheral blood was collected for CTC at baseline and again at 1–2 weeks, 3–5 weeks, 6–12 weeks and 13–20 weeks after the beginning of the treatment.

Based upon a systematic evaluation of correlation with imaging, they evaluated three CTC per 7.5 ml of blood as unfavourable. In this study, 53% of patients had no detectable CTC at baseline, and these patients had a better median OS (19 months) than patients with one or more CTC (12 months). Twenty-six percent of patients had three or more CTC at baseline. CTC and response rate (RR) were compared in the 320 evaluable patients. Clinical benefit (CB) [complete response (CR)+ partial response (PR) + stable desease (SD)] was obtained in 93% of patients with fewer than three CTC, compared to 7% of patients with three or more CTC. Thirty-eight patients (11.8%) had an unfavourable number of CTC and 20 of them (52.5%) had PD or died after the first assessment.

In patients that obtained a CB the median OS was 18.8 months in patients with fewer than three CTC and 7.1 months for patients with at least three CTC respectively.

Also the analysis of PFS showed a better result in patients with favourable number of CTC at baseline (7.9 months *versus* 4.5 months). In a multivariate analysis CTC was the best predictor of PFS and OS including other prognostic factors as therapy, age, and performance status. In patients with elevated CTC counts at baseline that obtained a reduction of CTC after CT the OS increase to 11.0 months respect the patients that did not obtained this reduction (3.7 months).

Meropol et al., are also investigating if CTC could serve as a source of tumour tissue for *in vivo* pharmacogenomic assay.

Sastre et al., reported in 97 patients with CRC stages I–IV that the CTC detection correlates with pathological stage, being significantly more frequent in those patients with advanced disease. Peripheral blood samples were collected for CTC from 4 to 12 weeks after surgery and immediately before adjuvant chemotherapy (if indicated) for patients with early-stages and before palliative chemotherapy in those with advanced disease. Quantification of CTC in 7.5 ml of blood was carried out with the semi-automated Cell Search System (Veridex). The authors used the cut-off of 2 CTCs for 7.5 ml of blood on the basis of previously reported date that established that 2 or more CTC were only present in malignant epithelial tumours.[6] A parallel group of 30 healthy volunteers were used as a control group.

Overall presence of 2 or more CTC was detected in 34 (36,2%) of 94 evaluated patients. The mean number of CTC isolated was 3.4 (range 0-61). Correlation was not found among positive CTC and location of primary tumour, increased carcinoembryonic antigen (CEA) level, increased LDH level and grade of differentiation.

Only stage correlated with positive CTC (20.7% in stage II, 24.1% in stage III and 60.7% in stage IV, $P = 0.005$). When a threshold of 3 or more CTC was considered as positive, again stage remained the only factor associated with CTC (12% in stage II, 16% in stage III and 64% in stage IV, $P = 0.0001$). There were no statistically significant differences between positive CTC found in high risk stage II patients compared with those in the low risk group (15.4% *versus* 25% $P = 0.663$). No CTC were found in the group of healthy volunteers.

The CTC detection cells search correlates with the stage, but not with other clinical and morphological variables in patients with CRC. Colon cancer tumour cells are detectable in all stages.

Allen-Mersh et al., assessed a potential role for RT-PCR based CTC identification to predict CRC recurrence. mRNA for CEA and cytocheratin (CK) 20 was identified by RT-PCR in blood from patients with CRC, before and after primary tumour resection.[7] Cancer recurrence was assessed up, followed-up and the accuracy of RT-PCR and primary tumour lymph-node positivity in predicting recurrence was estimated 196 patients were evaluated. RT-PCR positivity within 24 h of primary CRC resection is a strong predictor of CRC recurrence

and may be useful clinically. In fact haematogenous metastasis is an inefficient process, and most tumour cells apoptose within 48 h of entering the circulation. CTC appear early in the presence of an invasive solid tumour and disappear rapidly after complete tumour removal. The present result suggests that the estimation of tumour cells clearance from the circulation within the first 24 h after primary CRC removal could improve management decisions about adjuvant CT and intensity of follow-up.

Hauchs et al., evaluated the clinical significance of CTC in breast cancer and CRC patients. Systematic changes in the expression profile of CTC in colorectal patients at different stages of disease were observed; EGFR was expressed in 90% of patients with CTC during primary disease whilst the expression level decreased to 15% in CTC of metastatic patients. The expression of CEA was low in CTC found after primary surgery (15%) and dominant in CTC of metastatic patients (80%). The molecular profiling of CTC may be used to identify therapeutic targets such as HER-2 or EGRF for personalised treatment.[8]

Hendlisz et al., at ASCO 2008 evaluated the impact of CT on the EGFR expression of CTC in comparison to the primary tumour EGFR status. Peripheral blood was collected from chemonaive patients with metastatic CRC, at baseline, before the second cycle and after four CT cycles. CTC were detected by using immuno-magnetic separation and immuno-fluorescent identification (cell search system, Veridex). The EGFR status in CTC was determined by immuno-fluorescent labelling. Fourteen (64%) patients had positive EGFR CTC at baseline, no correlation was found between the EGFR status of the primitive tumours and baseline CTC. Fourteen patients had baseline CTC EGFR positive, after four cycles of CT 13 patients had CTC EGRF positive, with median time to progression of 6.6 months (95% CI: 4–8.2), whilst 8 patients had baseline CTC EGFR negative, after 4 cycles of CT, 7 patients had CTC EGFR positive, with median TTP of 8.5 months (95% CI: 7.5–9.5), log rank test: $P = 0.04$. The study shows that CT administration induces changes in the EGFR status of CTC. EGFR negative tumours should be retested for EGFR status after CT, and the EGFR expression of CTC in metastatic CRC could be useful in predicting outcome under CT.[9]

Madajewicz et al., at ASCO 2008 presented a prospective two-centre study, to define a CTC gene expression signature that exhibits high detection specificity and sensitivity for CRC patients. A viable cell isolation system that utilises the invasiveness of tumour cells to a cell adhesion matrix was used to enrich CTC. The samples were examined for gene expression profiling using the Affimetrix microarray Hu 133A chip. The author identified a potentially specific CRC CTC signature, and the diagnostic value, the role as a prognostic and therapeutic marker will be evaluated in further studies.[10]

2. CTC detection and methods

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

Specificity is also an absolute requirement in this field. In fact, a wrong identification of 'non-tumour cells' (like epithelial non-tumour cells) as 'tumour 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 the detection of CTC are available.[11–15] Many different methods have been developed and some are commercially available. All of them possess advantages and disadvantages. One of the main aims of this manuscript is also to review the performance of some of the most reliable procedures for CTC detection in an appropriate clinical study.

A classification of methods for CTCs detection mainly distinguishes between cytometric and nucleic-acid-based techniques[16] on the basis of the methods used for CTCs detection. The cytometric approaches use immuno-cytochemical methods to identify single cells or groups of them, whilst the latter detect DNA or RNA sequences that are differentially represented in tumour and normal cells.[16] More recently a classification[17] which distinguish between indirect and direct methods was proposed. The former ones are based on the recognition of tissue-, organ- or tumour-specific markers by immuno-histochemistry (Indirect immuno-mediated methods) or (real-time) RT-PCR (Indirect molecular methods), whilst the latter are related to CTCs selection based on physical properties of density and sizes (Table 1). The major disadvantage of the indirect approach relies in the difficulty of demonstrating the cancerous nature of the cells or of their cell-related signal without any doubt.

3. Indirect molecular method

The main advantage of indirect molecular methods is sensitivity which is considered to be higher than the reported sensitivity of immuno-mediated detection and immunocytochemistry.[18] Currently two types of markers are used: epithelial markers and 'cancer specific' markers. The first should be expressed by all tumours of epithelial origin, but in particular conditions their expression can be activated also in blood nucleated cells.[19] In addition the expression of epithelial markers can be lost by tumour cells through the process called epithelial-to-mesenchymal transition (EMT) with obvious consequence on CTC number determination.[20,21] On the other hand, the list of cancer cell-specific markers is extensive and expanding rapidly[22], but due to the neoplastic heterogeneity there are currently no universally expressed markers in all tumour cells from a particular tumour type.[23] Various genetic markers including k-ras point mutations, CEA and cytokeratin 20 (CK20) have been used for detection of CTC in the peripheral blood of patients with CRC. The simultaneous use of multiple markers[24,25] reflects more accurately the presence of CTC into the blood stream. The high sensitivity of RT-PCR carries the risk of post-amplification car-

Table 1 – In vitro methods for circulating tumour cell detection

Method classification		
Molecular methods	RNA	Epithelial marker
		Tumour-specific marker
		Multi-marker
	DNA	Mutation analysis
		Epigenetic markers
		LOH
		FISH
		CGH
		Array
Immuno-mediated methods	Marker proteins	Antibodies and immunomagnetic separation (CellSearch™, CTC-chip)
		Density gradient and immuno-labelling
		Flow cytometry
Isolation based on physical and morphological features		Density gradient (Oncoquick)
		Filtration (ISET, chip)
		Blood smear

ry over which requires strict negative controls to validate the positive PCR signals.

Quantitative RT-PCR (q-PCR) assays have been used to improve both sensitivity and specificity of conventional RT-PCR methods by means of fluorescent probes that specifically hybridise to the amplified sequence. The q-PCR assays main advantage relies just in the quantitative approach which allows to define a cut-off value of a given transcript marker, compared to a reference marker expressed in any cell. However, the proportion of tumour 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.[26] Technical problems related to qPCR assay reliability have been discussed elsewhere[27–30] and are beyond the aim of this manuscript. Cell enrichment by immuno-magnetic techniques was also used prior to RNA extraction and qPCR to improve assay specificity.[31] An alternative molecular approach to qPCR was also reported[32] by using oligonucleotide probes and alkaline phosphatase detection for simultaneous evaluation of mRNA CTC target genes in a high-throughput colorimetric membrane-array.

4. Indirect immuno-mediated method

Immuno-mediated detection is performed by immuno-labelling of cells enriched by immuno-magnetic separation.[33] Enrichment of CTC can be obtained by commercially available immuno-magnetic methods which provides a high enrichment and thus leads to increased sensitivity of detection. They also avoid cell lysis, which characterises the RT-PCR tests and thus allows the counting of target cells. Some aspects should be considered when using these methods for detection of CTC. For instance, since specific antigens characterising CTC are not known at present (antigens expressed by the tumour cells from a solid tumour type and not expressed by leucocytes), the authors have used antigens specific for epithelial cells to isolate CTC [EpCAM, BerEP4, CK].[34–37] Epithelial-specific antibodies can label non-tumour epithelial cells by specific labelling and non-tumour non-epithelial cells by non-specific labelling, thus giving false positive results. Small numbers of epithelial cells[38,39] 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. Organ-specific markers have been used (antibodies to mammoglobulin, PSA, CEA and HER-2)[40,41] to identify CTC. However, false negative results can occur since these antigens are not present in all tumour cells. Furthermore, some of these markers, mammoglobin and HER-2, are not entirely organ specific.[42,43] Actually, no available antibody is 100% tumour or tissue-specific.

In the immuno-magnetic detection, whole blood or mononuclear cells isolated by density gradient are put in contact with magnetic particles (beads or ferrofluids)-bound antibodies. Labelled cells are then collected by applying a magnetic force whilst unlabelled cells remain in the supernatant and are discarded. Since a large number of leucocytes still remain trapped with the target cells, some methods include a 'negative' selection of leucocytes (i.e. with anti-CD 45) combined with a 'positive' selection with antibodies to epithelial cells (EpCAM, CK) (i.e.: CellSearch™, Veridex™). This procedure gets rid of the majority of leucocytes but could detect non-malignant epithelial cells and does not detect tumour cells which do not express epithelial antigens. The CellSearch assay[44] uses ferrofluids coated with EpCAM antibody (directed to a cell adhesion molecule commonly expressed on normal and malignant epithelial cells) to enrich epithelial cells. Cells are then permeabilised, prefixed and labelled with the fluorescent nuclear dye DAPI, a fluorescent antibody to CD45 specific to leucocytes and fluorescent antibodies to intracellular CK 8, 18 and 19. Sample analysis is performed by the Cell-Spotter Analyzer, a four colour semi-automated fluorescence microscope which identifies epithelial cells from being positive for the CK markers and negative for the CD45 marker. CellSearch assay is more sensitive than the Oncoquick method[45], it is semi-automated and reduces trapping of leucocytes with epithelial cells. Finally, it also allows cell counting. However, cell isolation and detection are performed with antibodies specific to epithelial cells (EpCAM, CK 8, 18 and 19). Actually, it is well known that epithelial non-tumour cells can be spread in the peripheral blood (by inflammation, cytokine stimulating therapies, semi-surgical and surgical interventions) making difficult to determine the actual number of

tumour cells, in a given patient having a certain number of circulating epithelial cells (CEpC). This is particularly relevant when CTC counting is performed to assess the tumour response to the therapy, the risk of developing tumour recurrence and in cancer screening protocols. The finding that some CEpC identified in patients are characterised by aneuploidy[46] does not mean that any CEpC detected in any patient is a CTC. As discussed previously, the most indifferentiated CTC lose epithelial antigens (by EMT), and assays targeting epithelial cells in blood are susceptible to missing the detection of the most invasive tumour cells. As a matter of fact, Ep-CAM has been found to be expressed in only 70% of 134 tumours with different histologic types.[47] Furthermore, CK 8, 18 and 19 were found to be lost in cell lines derived from disseminated tumour cells.[20] The loss of CK and the ectopic expression of vimentin, indicating EMT, has been demonstrated to be associated with a higher tumour grade and mitotic index, and with negative estrogen/progesterone receptor status in 2517 breast cancers.[20] Finally, CTM (circulating tumour microemboli) cannot be reliably detected by this approach, as multiple cell labelling and treatments with magnetic particles may induce dissociation of tumour cell aggregates. Recently, the development of a unique microfluidic platform (the 'CTC-chip') was developed for the efficient and selective separation of viable CTCs from peripheral whole blood samples, mediated by the interaction of target CTCs with antibody (EpCAM)-coated microposts under precisely controlled laminar flow conditions, and without requisite pre-labelling or processing of samples.[48] The CTC-chip successfully identified CTCs in the peripheral blood of patients with metastatic lung, prostate, pancreatic, breast and colon cancer in 115 of 116 (99%) samples, with a range of 5–1,281CTCs per ml and approximately 50% purity. In addition, CTCs were isolated in 7/7 patients with early-stage prostate cancer. This example of microfluidic rare-cell capture technology applied to cancer patients hold significant promises for identifying key biological determinants of blood-borne metastases and providing a robust platform aimed at early diagnosis and longitudinal monitoring of cancer.

A novel technique for the detection and *ex vivo* characterisation of single viable disseminated tumour cells derived from epithelial tumours, epithelial immuno-spot (EPISPOT), has been recently reported[49] based on the detection of specific secreted proteins by a modified protocol of enzyme-linked immuno-spot assay. By means of this new assay the authors demonstrated the viability of the CTC in patients affected by prostate and breast cancer and their heterogeneity with regard to the secretion of relevant proteins. This multiparameter technology reveals a unique fingerprint of proteins secreted by single viable CTC and, if validated in large-scale studies, can improve the understanding of the mechanisms underlying the metastatic process.

5. Direct methods

Direct methods are meant to provide a diagnostic identification of CTC obtained by cytopathological analysis of the isolated cells[7] and/or by the analysis of their genome providing clues to the cancer nature of the cells.[38,46]

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 characterisation.[46,50,51]

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 tumour biopsies and aspirates and of biological liquids (ascites, urine, cephalo-rachidien liquid). Cytopathological analysis could be used as a reference basic approach to recognise CTC/CTM, applying additional techniques (immuno-labelling, FISH, RNA/DNA analysis) to better characterise 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 as a routine manner, in order to find one CTC in one ml of blood, the analysis of 100 smears (10 μl per smear) must be performed.[17]

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), cytoplasmic and nuclear details become available to observation and thus allow cytopathological diagnosis of CTC/CTM.

Direct enrichment of epithelial cells by filtration has been first described by Vona et al. in 2000.[52] Isolation by size of Epithelial Tumour cells (ISET) (Metagenex, Paris France; http://www.metagenex.fr) is based on the observation that the vast majority of peripheral blood leucocytes (lymphocytes and neutrophils) are the smallest cells in the body, having a size ranging from 8 to 11 μm. They can thus be massively eliminated by blood filtration through polycarbonate membranes with calibrated pores of 8 μm.

The assay is simple and should reduce the loss of tumour cells because it does not require multiple steps of isolation. In this procedure, EDTA peripheral blood is diluted with the ISET buffer (which fixates cells), 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 one ml of blood. This permits the precise counting of the number of CTC per ml of blood independently from the volume treated. Enriched cells can be marked with cytological staining (i.e. May-Grunwald Giemsa, Haematoxylin and Eosin), and/or characterised by immuno-labelling, FISH, or TUNEL assays in order to analyse their antigens, aneuploidy and the rate of apoptotic cells. Interestingly, CTM, which are thought to carry a high metastatic potential, are also sensitively enriched and can be reliably counted (Fig. 1). Molecular analyses can be carried out after laser CTC/CTM microdissection. In a previous study, we[53] used ISET followed by CK-19 staining of the enriched cells and demonstrated the feasibility of studying the HER2 DNA amplification in tumour cells microdissected after enrichment by ISET.

Alternatively, molecular analyses can be carried out by lysing all the enriched cells on the spots. Enrichment by direct

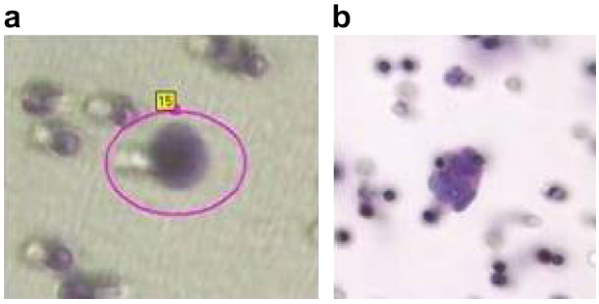

Fig. 1 – Circulating tumour cell (a) and circulating tumour microemboli (b) isolated by ISET from peripheral blood of a patient affected by colon cancer.

filtration is very sensitive since fixed cells larger than 11 μm cannot cross the 8-micron pores.[17] Furthermore, this direct method avoids multiple steps and cell damage, both contributing to enrichment sensitivity.

Finally, the design of Metablock has been planned specifically to avoid cell loss, and has been shown to isolate one single tumour cell added to 10 ml of blood by micropipetting (the only way to reliably count the tumour cells added to blood) in 80% of the tests. This high sensitivity is maintained when treating blood samples within 3–4 h after collection. It should be pointed out that CTC are fragile and can be lysed by blood storage, even if a conservative reagent is added.

The size of tumour cells from patients with solid cancers (breast, colon, lung, prostate, liver, pancreas, ovary and many others) tested up to now is not as small as that of lymphocytes and neutrophils. Meng et al.[54] reported that the mean diameter of tumour cells in blood from patients with breast cancer ranges from 29.8 μm (patients with cancer dormancy) to 33.9 (patients with metastatic cancer) and 32.0 μm (patients with primary tumour). Since the cell morphology is conserved, it is easy to distinguish epithelial non-tumour cells from tumour cells by cytological staining and cytopathological examination, complemented if required by immuno-labelling. A limited number of leucocytes are also retained on the filter, but they are very easy to recognise without any additional labelling.

Ongoing and future ISET developments concerning automated image analysis on the filter and transmission of high definition images through the web for "on line" cytopathological consultations are aimed to speed up the work of cytopathologists on CTC/CTM detection. Remarkably, images of CTC/CTM found in the patient blood (and often characteristic of individual patients) can be included in the patient file, along with the assessment of their number, to provide oncologists with a visual aspect of this new marker and its evolution during follow-up (modification of CTC morphology towards more malignant traits, appearance of CTM, appearance of apoptotic cells).

Interest on the filtration approach to CTCs isolation is demonstrated by recent paper studying extensively the filter structure and proposing new and more efficient support to optimise blood filtration.[55] SEM pictures were taken for LNCaP cells isolated on membrane filer (Fig. 2). Compared with commercially available polycarbonate filters (Fig. 2A), the new parylene filters (Fig. 2B and C) are more dense and without fused pores. The SEM fixation procedure (Fig. 2D) preserved the cell shape

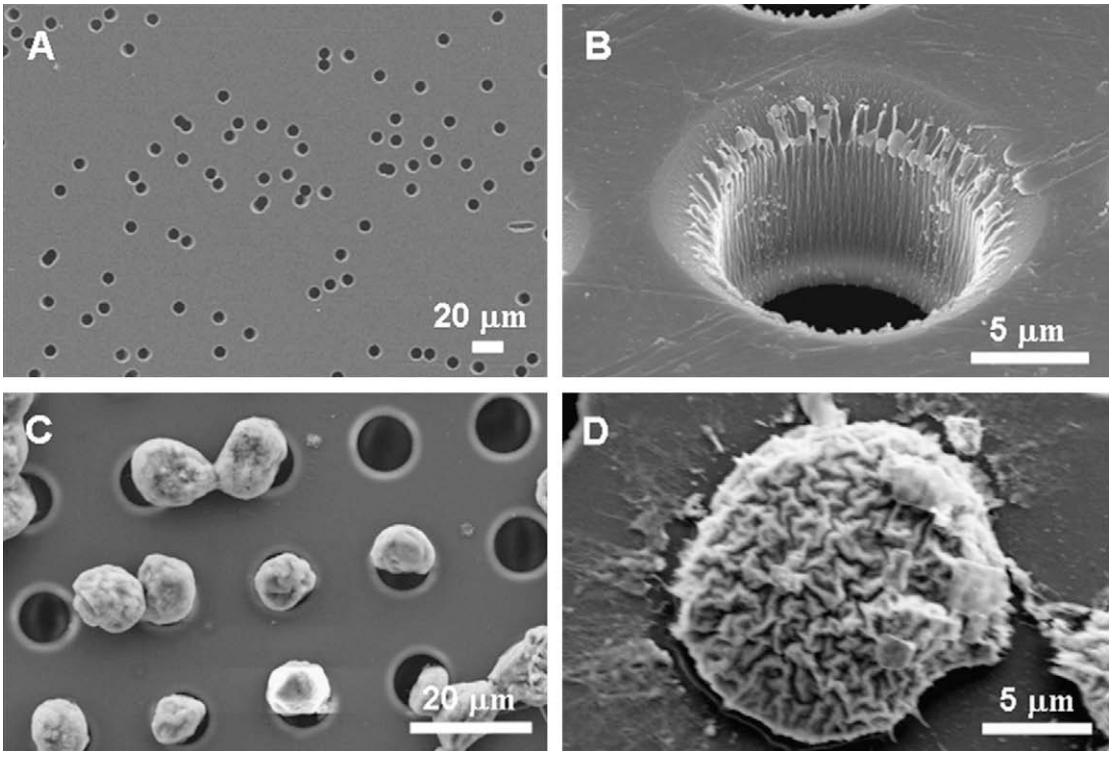

Fig. 2 – SEM pictures. (A) Commercial membrane filter with sparse and occasionally fused pores, (B) microfabricated parylene membrane filter, (C) parylene membrane filter with cells captured without SEM fixation treatment and (D) parylene membrane filter with cells captured after SEM fixation procedure [55].

better than those without fixation (Fig. 2C). The support is integrated in a micro-electro-mechanical system that make the filtration process simpler, faster, better and cheaper. The size difference between CTCs and human blood cells was investigated to achieve the CTC capture on filter with 90% recovery within 10 min, which is superior to current approaches.

A biochip aimed to separate cells based on their size and ability to deform has been developed;[56] even if its application was limited to the isolation of foetal cells from maternal circulation, which in concept resemble closely the situation of CTC in patients affected by cancer.

6. Conclusion

The importance of tumour cells disseminated into bone marrow as an independent prognostic marker has been widely confirmed. Recently, also the clinical significance of CTC in peripheral blood has been proven. The process is completely different, the detection of CTC in blood is a dynamic process, whereas the presence of tumour cells in bone marrow might be considered a static process. Tumour cells in bone marrow are usually dormant and have a very long survival time because they are not destroyed be the body's defence mechanisms.

The challenge of CTC detection is related to the requirement of high sensitivity combined with high specificity. Since invasion can start very early during tumour development, identification and counting of CTC when they are very rare (few CTC per 10 ml of blood, which means few CTC mixed with approximately 100 million leucocytes and 50 billion erythrocytes) could alert the oncologist about a developing tumour invasion process. Specificity is also an absolute requirement in this field.

The detection of CTC correlates with pathological stage and clinical outcome in particular in those patients with advanced disease. CTC levels before and after CT are an independent prognostic factor for PFS and OS. One of the major problem in this field is the reproducibility of the method. Sastre et al., demonstrated that CTC research in CRC is highly reproducible method that correlates with stage. RT-PCR and other methods have been developed to detect the presence of CTC, Mersh et al., suggested a positive prognostic value of complete clearance of CTC after surgery to determinate the patients who can benefit by chemotherapy. Future clinical studies will evaluate the clinical significance of CTC in colorectal cancer and in other tumours.

Conflict of interest statement

The authors disclose no financial and personal relationship with other people or organisations that could inappropriately influence their work.

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