Review

Circulating Tumor Cells in Colorectal Cancer: Detection Methods and Clinical Significance

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Abstract. *Colorectal cancer is one of the most frequently diagnosed malignancies in both men and women. Although curative resection is the major treatment option, approximately half of all patients eventually develop distant metastases. Thus, the need for early detection of occult metastases has led to extensive investigation with regard to the detection of disseminated tumor cells in biological fluids, including peripheral blood or bone marrow of cancer patients. In this review, we summarize the methods currently implemented for disseminated tumor cell detection in colorectal cancer. In addition, we discuss the pitfalls of each method and the future perspectives in the development of an easily applied, quick and inexpensive method which will enable the reliable detection of circulating tumor cells with optimal sensitivity and specificity.*

Colorectal cancer (CRC) is one of the most common forms of cancer, in regard to both incidence and mortality. In the Western world, CRC is the second most common malignancy diagnosed in women, after breast cancer, and the third most common in men, after prostate and lung cancer, accounting for 13.1% and 12.8% of all forms of cancer, respectively. In 2006, 412,900 CRC cases were newly diagnosed in Europe, whereas approximately 207,400 deaths from CRC were certified (1).

For the time being, surgical resection of the tumor remains the prominent choice for treatment followed by adjuvant chemotherapy. Despite surgery, roughly 45% of patients ultimately die of distant metastases; 5-year overall survival decreases from approximately 90% for stage I patients to about 8% for stage IV (2-4). Staging of CRC is based on the Tumor, Node and Metastasis (TNM) staging system, providing information on the spread of cancer, with reference to tumor size or penetration to the bowel wall (T), as well as regional lymph node (N) and distant (M) metastasis. Apart from predicting cancer prognosis, the TNM system is also widely used in the course of treatment choice (5). Nevertheless, although frequently updated, the TNM system often fails to discriminate among tumors of intermediate stages, or even morphologically similar tumors with different clinical behavior (4).

The stage of the disease at the time of diagnosis is crucial to survival; unfortunately, in a large number of cases, CRC is diagnosed in advanced stages. It is obvious that early detection is critical, however the available methods for screening encounter several difficulties in meeting that expectation (6). It is therefore of great importance that new and improved diagnostic and screening methods are applied. Prognosis, recurrence and response to therapy can be challenging for that purpose, given the fact that 90% of cancer-related deaths result from metastases (7). In CRC, metastasis occurs as a result of hematogenous and lymphatic spread: tumor cells shed from the primary tumor and migrate to distant sites, eventually leading to micrometastases. Detection of disseminated tumor cells could provide a very

Abbreviations: CRC: colorectal cancer, CTC: circulating tumor cells, EMT: epithelial to mesenchymal transition, CTM: circulating tumor microemboli, PCR: polymerase chain reaction, RT-PCR: reverse-transcription polymerase chain reaction, qRT-PCR: quantitative polymerase chain reaction, ICC: immunocytochemistry, CEA: carcinoembryonic antigen, CK: cytokeratin, GCC: guanylyl cyclase C, EGFR: epidermal growth factor receptor, hTERT: human telomerase reverse transcriptase.

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promising solution to the aforementioned hindrances, also providing a potential for changing the treatment approach in cancer patients (8).

The Concept of Circulating Tumor Cells (CTCs) in Cancer Metastasis

As mentioned above, disseminated malignancy is the main cause of cancer death, including that from colorectal cancer. Metastases are eventually developed in a large percentage of surgically treated patients and this phenomenon is attributed to the dissemination of tumor cells from the primary tumor site before surgery. It has been estimated that approximately 1×10^6 tumor cells per gram of tissue are shed daily into the bloodstream (9), with few of them, nonetheless, having metastatic potential (10). Although the notion of detecting malignant cells in blood is not novel, recent studies have confirmed the malignant nature of circulating tumor cells (CTCs), and their genetic association with the primary tumor (11, 12). The general model of tumor cell dissemination and metastasis includes the following steps: tumor growth and angiogenesis, local invasion and epithelial to mesenchymal transition (EMT), intravasation, dissemination, arrest in organs, extravasation, proliferation and formation of metastases.

As a tumor grows, the depletion of oxygen supplies results in angiogenesis induction (13). Epithelial tumor cells breach the basal membrane as a result of EMT, during which tumor cells progressively acquire mesenchymal characteristics, resulting in reduced cell to cell adhesion (mainly due to down-regulation of E-cadherin), reduced cell to extracellular matrix adhesion (mediated by integrins) and increased motility, thus allowing them to enter blood vessels (14). EMT is induced by the transcription factor Twist (15). Once they reach distant organs, tumor cells extravasate and undergo mesenchymal to epithelial transition (15, 16), where they either form metastases, or remain dormant, often for a long period of time, lasting up to 22 years (17).

In order to explain the prognostic effect of lymph node metastases on survival or development of distant metastases in cancer patients, Pantel and Brakenhoff suggested a possible complementary pathway that incorporates lymphatic dissemination during the early stages of the disease and the formation of lymph node metastases. As the disease progresses, tumor cells disseminate from the lymph nodes and form distant metastases (18). On some occasions, tumor cells can enter the circulation as multicellular aggregates or clusters of epithelial-like cells. This process is referred to as 'collective' or 'cohort' migration and the clusters are also known as circulating tumor microemboli (CTM). CTM are thought to have high metastatic potential; they exit the circulation without extravasation, by proliferating inside the vessels and attaching to the walls of the vessels, resulting in their destruction and subsequently in micro- and macrometastases (16, 19). The presence of CTCs, although necessary, does not suffice for metastatic formation. This phenomenon is referred to as 'metastatic inefficiency' and might be attributed to CTC incompetence in developing metastases probably because solitary extravasated cells do not succeed in initiating tumor growth, or because of the failure of the micrometastases formed at the target site to give rise to macrometastases (20).

CTCs tend to metastasize to certain organ sites that are specific for cells derived from specific tumors. This preference was first reported in the 19th century, when Stephen Paget published the 'seed and soil' hypothesis, depicting the nonrandom pattern of metastases (21). According to the findings of Paget, metastases are not formed randomly, but are based on the affinity of certain tumor cells (the 'seeds') to the environment of the target organs (the 'soil'). Indeed, it is currently accepted that certain tumor cells selectively metastasize to specific organs (22-26); colorectal cancer cells for instance mainly disseminate to the liver, lungs and bone marrow.

A current definition of the 'seed and soil' hypothesis comprises three principles. Firstly, neoplasms contain genetically diverse tumor cell subpopulations, each with different metastatic potential. Secondly, of the biological variety of tumor cells in a neoplasm, metastases will be formed by those which will succeed in completing all the steps in the metastatic process. Therefore, metastases can have a clonal origin, meaning that each can occur by proliferation of a single cell. Thirdly, the specific choice of the 'soil' is mostly attributed to interactions between tumor cells and the organ microenvironment, in terms of specific recognition of endothelial cell antigens and response to local growth factors (27).

In addition, the recently developed perception of circulating cancer stem cells has proposed that cancer stem cells are present in the circulation and are capable of developing metastases with specific biotypic behavior, based on their interaction with particular host tissue microenvironment (28, 29) which can modify their biology (30).

Cell Enrichment Techniques used for the Detection of CTCs

The detection of CTCs in peripheral blood or bone marrow samples requires their enrichment from the initial specimen. The techniques used for this purpose involve CTC enrichment on the grounds of cell density, selection of CTCs based on expression of epithelial antigens and removal of nonepithelial cells (31).

Density gradient centrifugation using Ficoll-Hypaque is a commonly used method that allows the enrichment of disseminated tumor cells in the mononuclear blood cell fraction. OncoQuick® is a rather newly developed variation that is characterized by the addition of a porous barrier in the centrifugation tube, thus reducing the possibility of tumor cell loss due to migration to adjacent cell layers. OncoQuick® has

been reported to be superior to Ficoll-Hypaque due to increased depletion of mononuclear blood cells (32).

Erythrocyte lysis is also used for the recovery of CTCs. Whole blood samples are treated with a special isoosmotic ammonium chloride solution called erythrocyte lysis buffer (ELB) that contains ammonium chloride (NH_4Cl), potassium bicarbonate ($KHCO_3$) and tetrasodium ethylene-diaminetetraacetic acid (EDTA). After centrifugation, tumor cells remain in the pellet. Although the use of ELB does not allow specific isolation of tumor cells, it can be easily implemented in clinical practice due to convenience in application and low cost.

CTCs can also be isolated by filtration, based on their size. After erythrocyte lysis, blood cells are filtered using filters with pores of 8 μm in diameter (33). Cells larger than 11 μm, including tumor cells, are retained. Despite its ease of use, this method lacks sensitivity and specificity (34).

One of the mostly used methods for CTC enrichment is immunomagnetic cell enrichment, involving separation using either magnetic beads or a ferrofluid based system. In the first case, antibodies coupled with magnetic beads are targeted against specific molecules, allowing separation by using a magnet. The antibodies in these assays can be used either for positive or for negative selection. Positive selection involves antibodies specific for epithelial cell antigens such as CEA 125 and Ber-EP4, while negative selection is usually targeted against leukocytes using anti-CD45 antibodies, leading to their depletion. The ferrofluid-based system makes use of anti-epithelial cell adhesion molecule (anti-EpCAM) antibodies in EpCAM-bound ferrofluid. The selection also takes place *via* magnetic separation. Immunomagnetic enrichment offers the advantage of recovering live cells which, in combination with microscopic visualization and quantification of the enriched cells, can lead to dependable assumptions. Nonetheless, the problem of nonexistence of highly specific tumor markers remains, leading to false-positive results because of antibody binding to nonmalignant cells. Additionally, during the enrichment process there is a significant loss of CTCs; this loss could be of great importance, given the fact that tumor cells represent less than 0.1% of the enriched population (35). In general there are conflicting results concerning the superiority of immunomagnetic enrichment over traditional methods such as the aforementioned Ficoll-Hypaque, since although considered to provide greater specificity, immunomagnetic enrichment has been shown to be hampered by poor sensitivity (36).

Polymerase Chain Reaction-based Protocols for Detecting Circulating Colorectal Cancer Cells

Polymerase chain reaction (PCR) is a method that allows the *in vitro* amplification of a DNA sequence, using oligonucleotide primers that are specific for it. The reaction takes place in a thermocycler, where the sequence of interest is amplified exponentially with the use of a thermostable DNA polymerase. In reverse transcription PCR (RT-PCR), a sequence of RNA is used as a template and therefore an extra step of reverse transcription of RNA to cDNA precedes DNA amplification.

PCR-based methods are widely used for the detection of CTCs, targeting both DNA and RNA markers. DNA is generally stable and independent of the transcription mechanisms of the cell; however, the stability of DNA can be a disadvantage, since it can be detected in blood due to release from dying cancer cells, meaning that the presence of DNA does not necessarily reflect the existence of viable tumor cells in the circulation (37). DNA markers are used based on specific genetic abnormalities that occur in certain types of cancer, although it has been reported that, at least in some cases, disseminated tumor cells are not necessarily clonal with the primary tumor (38). In general, few chromosomal alterations specifically characterize certain types of cancer, or even are frequent enough to serve as molecular markers. The most frequently encountered genomic alterations in CRC, commonly used for the detection of CTCs in the lymph vessels or nodes of CRC patients, include mutations in *K-Ras* and *p53* genes, sometimes investigated in combination with mRNA markers (see below) (39). The results concerning the significance of the aforementioned mutations in cancer prognosis and follow-up are contradictory (40, 41); studies investigating mutations of *K-Ras* and *p53* genes in CRC are shown in Table I.

The detection of occult tumor cells also engages targeting of tumor-specific mRNA, meaning mRNA that encodes for antigens that are specific either for the malignant phenotype or for the normal parental tissue. The use of mRNA markers is based on the notion that tumor cells continue to display the same pattern of antigen expression as their normal tissue of origin. Once released from malignant cells, mRNA is relatively unstable; therefore, once detected, mRNA markers are indicative of the presence of viable tumor cells in the sample examined. Furthermore, RT-PCR of tumor-specific mRNA is characterized by great sensitivity, in comparison to protein-based methods (42).

The success of an RT-PCR assay for the detection of occult tumor cells in the peripheral blood or the bone marrow of cancer patients relies on the balance between sensitivity and specificity in order to minimize the occurrence of false-positive or false-negative results (43, 44). The sensitivity of an RT-PCR reaction is determined in spiking experiments and usually ranges from the detection of 1 to 10 tumor cells among 10^6-10^7 blood mononuclear cells (45-47).

The great sensitivity of RT-PCR, although important for its clinical use, is challenging when false-positive results are encountered. False-positives can result from the expression

Table I. *K-ras and p53 mutations as markers for colorectal cancer.*

Method	Gene	Sample	Number of CRC patients included	Healthy donors	Positive for gene alterations	Correlation	Reference
PCR RFLP, RT-PCR	*K-ras*	Liver, LN, BM	246		67% , higher rate in liver metastases		(39)
IME, MASA	*K-ras*	TDB	24		29.2%	Prognosis	(134)
MACS, nested MASA	*K-ras, p53*	TDB, tissue	23		87% in tissue, 45% in blood positive for *K-ras* and/or *p53*	Survival, tumor size, invasion	(135)
MASA	*K-ras, p53*	CRC & LN tissue	26		17/26 primary tumors, 9/17 positive in LN	LN invasion	(46)
PCR, MLA	*K-ras, p53*	Tissue, serum	44		16/44 in tissue & 3/16 serum for *K-ras,* 10/44 in tissue & 7/10 serum for *p53*		(136)
PCR RFLP	*K-ras*	CRC, mucosa & liver tissue	121		54/121 at surgery	Survival, hepatic metastases	(137)

CRC, Colorectal cancer; BM, bone marrow; LN, lymph nodes; TDB, tumor drainage blood; MASA, mutant allele specific amplification; IME, immunomagnetic enrichment; MACS, magnetic-activated cell separation; PCR-RLFP, polymerase chain reaction-restriction fragment length polymorphism; MLA, mismatch ligation assay.

of the markers examined by nontumor cells; thus, the selection of the mRNA markers examined is crucial so as to avoid nonspecific expression, if possible. Highly overexpressed markers in tumor tissue compared with normal cells are an excellent choice. False-positives also result from the activation of promoters by ubiquitous transcription factors (leakiness of promoters) and although this results in the production of a small number of transcripts (48), the enhanced sensitivity of PCR can lead to the production of false-positives (43). This problem can be overcome by properly adjusting the number of PCR cycles, given the fact that the greater the cycle number, the larger the number of copies produced. False-positive results may also arise as a consequence of introduction in the circulation of cells during blood sampling or surgical procedures. The first is important especially when epithelial markers such as cytokeratins are used and can perhaps be avoided if the first few milliliters of blood that are most likely to be contaminated by epidermal cells are discarded; it has been reported, however, that discarding the first of multiple blood samples does not significantly change the outcome of the method (49). It is of particular interest that during and after surgery, tumor cells may spread into the circulation, something that has led to the suggestion that PCR-based detection of circulating tumor cells should be performed 2 months after curative surgery (31). Moreover, false-positives can occur as a result of amplification of pseudogenes, or even of genomic DNA that potentially contaminates cDNA in the reaction (44, 50, 51). A potential solution can be the careful design of primers so as either the primers are located on either side of an intronic sequence, or one of them contains an intron which is deleted during RNA processing (52). Contamination during sample preparation may also be a source of false-positive results, which implies the need for negative control inclusion in the reaction; negative controls contain water instead of RNA or cDNA in the RT and the PCR reaction respectively.

RT-PCR is also hampered by false-negative results. RNA degradation and technical errors, *e.g.* during RNA extraction or RT-PCR, can lead to false-negatives. To avoid this problem, quality controls should be used. Such controls include housekeeping genes such as *glyceraldehyde-3-phosphate-dehydrogenase* (*GAPDH*) and *β-actin*, indicative of the sample quality. Intermittent shedding of tumor cells in the circulation or genomic instability of malignant cells can be a source of false-negative results since it is possible that at the time of examination, tumor cells do not express the anticipated markers; multiple sampling can provide a solution to this problem (53).

It is of great importance to mention the necessity for the use of multiple markers in the detection of occult tumor cells, as it can help resolve several problems encountered due to the nature of the experiments involved. Firstly, as a result of de-differentiation, the number of mRNA copies of a gene may be discrepant during the cell cycle. The employment of multiple markers can provide the ability to distinguish between actual variations in the expression of a marker and

false indications that could result from the aforementioned phenomenon (54). Secondly, in some cases, cancer therapy can lead to down-regulation of the gene of interest and thereby to false-negative results which can, however, be resolved by the examination of multiple markers (42). Thirdly, use of more than one marker can provide an alternative solution to the false-negative results that rise due to the intermittent shedding and the genomic instability of tumor cells, as mentioned before.

In conclusion, we suggest that meticulous design of a PCR reaction, in reference to primer design, cycling conditions and multiple marker use, could overcome the majority of the problems encountered where detection of disseminated tumor cells is concerned. In an ideal method, the marker panel would comprise at least one marker specific for cells of epithelial origin, one marker specific for metastatic cancer cells and one marker specific for CRC cells (55). In the absence, though, of universal CRC markers, the best choice would be the combination of at least one marker with great sensitivity, so as to allow the detection of rare tumor cells, and one very specific marker, in order to distinguish between disseminated tumor cells and normal cells of the circulation; further studies for the identification of more efficient markers are eagerly anticipated.

Quantification of PCR products using quantitative real-time RT-PCR enhances the reliability of RT-PCR assays, mainly in terms of specificity. qRT-PCR assays make use of fluorescent molecules, thus allowing screening of the transcripts produced in each PCR cycle. The detection of occult tumor cells by qRT-PCR relies on the definition of a cut-off value of marker transcripts, which serves as a threshold for positivity determination; measurements above this value of reference are considered as positive results, while those below this limit are considered to be false-positives.

Even though qRT-PCR is not particularly more sensitive than regular RT-PCR (56), the production of a nonlinear amplification curve when false-positive results are involved allows their immediate identification and removal (57). Nevertheless, as in conventional RT-PCR, special precautions are required so as the sensitivity and the specificity of qRT-PCR assays are preserved. These include cut-off value determination, sample preparation and RNA extraction optimization, primer selection, instrument calibration and carryover contamination avoidance (58). In fact, the quantitative potential of real-time RT-PCR combined with its technologically advanced characteristics is believed to be the future in the application of PCR-based assays to the detection of disseminated tumor cells. The use of real-time RT-PCR can provide answers to the problems encountered concerning the specificity of the chosen mRNA tumor markers and the reliability of the observed positive or negative PCR results in general.

Immunocytometry and Flow Cytometric Methods for the Detection of Circulating Colorectal Cancer Cells

Immunocytometric methods are based on the use of monoclonal antibodies against certain surface or epithelial antigens of circulating tumor cells and the subsequent visualization of the antibody-labeled tumor cells. Cytometric techniques were the first to be used for the detection of occult tumor cells in the peripheral blood and currently are the main methods for the detection of tumor cells in the bone marrow. The commonest antibodies used for that purpose involve those targeted against cytokeratins. Cytometry offers the advantage that the target cells can be morphologically examined, as, contrary to the PCR-based methods, no lysis of the cells is required. Immunocytochemical approaches also provide the ability to combine the morphological examination of stained cells with further examination by additional ICC staining or FISH (fluorescent *in situ* hybridization) for genetic mutations (59). On the other hand, the sensitivity of these approaches is controversial, given the low frequency of screened cells in peripheral blood. Additionally, false-positive results have been reported as a consequence of nonspecific antibody binding to cytokeratins expressed by normal blood cells (60), in percentages that range from less than 1% to more than 80% (61). Finally, it has been reported that routine application of immunocytometric assays could encounter difficulties because of the fact that they are labor intensive (62). Digital microscopy and fluorescence-activated flow cytometry (FACS) open up new horizons concerning the implementation of cytometric approaches by enabling automatic screening of samples based on nuclear and surface characteristics and by sorting the cells of interest according to certain parameters, allowing further *in vitro* analyses (37).

Specific Markers used for the Detection of Circulating Colorectal Cancer Cells

Carcinoembryonic Antigen (CEA)

CEA is a high molecular weight glycoprotein, first described by Gold and Freedman (1965) in human colon cancer tissue extracts (63). *CEA* gene is expressed in nearly 95% of all colorectal, gastric and pancreatic cancer (64). CEA is involved in cell adhesion (65, 66) and in tumor cell protection against apoptosis (67, 68), thus playing a role in CRC metastasis. Serum CEA protein levels are commonly used to follow the course of treatment in CRC patients; however, it has been reported that CEA serum levels are detectable in approximately half of CRC cases (69), while statistical analysis showed no correlation between CEA expression levels in tumor biopsies and the presence of serum CEA protein (70). Hence, considerable effort has been

made for the detection of *CEA* mRNA in blood specimens, with numerous studies reporting contradictory results concerning its detection in CRC patients, patients with benign diseases and healthy volunteers.

Among the possible explanations suggested for the unexpected positivity of non-cancerous specimens, some studies mention that CEA expression in control blood had used primers that were not specific for CEA and included sequences that were also common for CEA-associated proteins, such as CEACAM6 (non-specific cross-reacting antigen-NCA), expressed in skin and granulocytes (71). Furthermore, Hampton *et al*. using qRT-PCR for *CEA* mRNA observed elevated CEA levels in only 2 out of 32 (6%) CRC patients and demonstrated that white blood cells express a splice variant of CEA in which part of the exon is replaced by an intron sequence (72), a finding that highlights the need for careful primer selection.

In a large study, peripheral blood samples from 51 patients with histologically confirmed CRC, 18 patients with benign colorectal disease and 40 controls were analyzed by nested RT-PCR for CEA. PCR results were compared with those that emerged from the determination of serum marker levels and immunohistochemical analysis of samples taken from the same patients. A total of 69% of CRC patients were found to be positive for CEA by RT-PCR in the blood samples, whereas 35% were positive for serum CEA. As a possible explanation for this inconsistency, the authors suggested that CEA RT-PCR detects the presence of tumor cells in the blood, while serum CEA levels are associated with tumor mass. CEA PCR results correlated with disease stage, while no positive results were observed from noncancerous samples and only 3% of controls were marginally positive (71).

In another study, however, using real-time PCR in blood samples from 36 CRC patients and 10 healthy volunteers, CEA expression was reported in all control samples, although in 50% of them it was below the quantification limit. The authors noticed a significant difference in *CEA* expression levels in the advanced stages of the disease and concluded that quantification of *CEA* mRNA can be useful for predicting micrometastatic relapse (73). Guo *et al*. applied a combination of positive and negative immunomagnetic enrichment followed by real-time RT-PCR in order to maximize the specificity for *CEA* mRNA detection. With this method, they observed a highly significant correlation between relative *CEA* mRNA values and number of cancer cells, while CTC levels correlated with tumor diameter, presence of lymphatic and hepatic metastases, as well as with clinical course of the patients. None of the 10 normal blood samples were positive for *CEA* mRNA (74). In addition, other studies analyzed preoperative and postoperative blood samples by CEA RT-PCR combined with patient follow-up for a median of 42 and 36 months respectively, leading to the conclusion that the detection of CTCs in blood samples by CEA RT-PCR lacks prognostic significance (75, 76). However, these results were not recently confirmed by a large cohort of patients. In this study, blood samples taken 7-10 days postoperatively were analyzed by RT-PCR for CEA expression. After a median follow-up period of 52 months, it was concluded that the analysis of blood samples collected 7 days after curative surgery could provide useful information in patient prognosis (77). Studies investigating *CEA* mRNA expression in CRC are summarized in Table II.

Cytokeratin 20 (CK20)

CK20 is an intermediate filament protein and an important cytoskeletal keratin of the intestinal epithelium. *CK20* gene is expressed in cancer of the gastrointestinal tract, including CRC, as well as in normal intestinal cells; CK20 has been shown to be a prominent component of intestinal and gastric epithelium, urothelial umbrella cells, and Merkel cells of the epidermis (78). *CK20* mRNA detected in histopathologically negative lymph nodes from CRC patients has been found to have prognostic significance concerning tumor-related death (79). *CK20* mRNA is widely used as a diagnostic and prognostic marker for CRC and is generally considered to be a highly sensitive marker, although the results concerning its specificity vary.

For instance, using RT-PCR, Zhang *et al*. detected no CK20 expression in any of the 12 control samples included in their study, while the positive detection rate of 58 patient samples varied from 44.8% to 69%, correlating with disease stage (80). In concert with these results, RT-PCR did not detect CK20 expression in blood samples of 14 volunteers while *CK20* mRNA was detected in the blood of 42.1% of CRC patients (81); other studies also confirmed the high specificity of CK20 as a molecular marker in CRC (82, 83). On the other hand, Wyld *et al*. detected *CK20* mRNA transcripts in 1/12 control blood samples, whereas 48% of patient samples were positive for CK20 (84). Dandachi *et al*. detected *CK20* mRNA in 56% of CRC patients using real-time RT-PCR after immunomagnetic enrichment. The corresponding detection percentage in control samples was 22%, while 60% of patients with chronic inflammatory bowel disease were also positive; however, a significant difference in mRNA levels was observed between control and cancer patient blood samples (85). The studies investigating CK20 expression to date are summarized in Table III.

In conclusion, given the absence of the 'perfect' molecular marker, *CK20* together with *CEA* mRNA are the two markers most frequently used for CRC. The use of CK20 is in many cases hampered by its detection in nonmalignant samples, while background expression has been reported in granulocytes (86). Nevertheless, CK20 is a marker

Table II. *Results from studies detecting CTCs by examining the expression of CEA mRNA.*

Method	Sample	Number of patients included			Positive Results			Correlation	Reference
		CRC patients	Patients with benign diseases	Healthy volunteers	CRC Patients	Patients with benign diseases	Healthy volunteers		
Nested RT-PCR	PB	51	18	40	69%	-	5%	Stage	(71)
IME, multiplex PCR for CEACAM5 and CEACAM7	PB	84	32	41	63% for either marker, 38% for both	-	-		(138)
RT-PCR	PB	19		15	52.6%		-	Stage	(139)
Nested RT-PCR	Plasma	53		25	32%		4%	Serum CEA and CEA protein	(91)
Real-time PCR	PB	36		10	Significantly higher expression in Dukes' D		50% below detection limits	Stage	(73)
Real-time PCR	PB, PLF, MVB	39	7		15% before 26% after resection	-		Clinical outcome	(57)
Real-time PCR, Membrane array	PB, tissue	80		98	82.6% detection 82.5% overexpression		-	Stage & LN metastasis	(92)
Semi-quantitative RT-PCR, Southern blot	PB	33	-	26	88%	-	92%		(93)
Real-time PCR, IHC	PB	20 (with liver metastases)	20		5% preoperatively, 65% postoperatively	25%			(140)
Nested RT-PCR	PB	79	8	16	34%	25%	6%	Serum CEA, tumor size, LN or liver metastases	(141)
Real-time PCR	PB	129	13	45	86%	-	83.7%		(142)
Real-time PCR	PB, TDB	167	10	25	6/167 in PB samples, 19/167 in TDB samples			CEA and/or CK 20 positivity correlated with stage, depth of invasion, LN and liver metastases	(143)

CRC, Colorectal cancer; PB, peripheral blood; PLF, peritoneal lavage fluid; MVB, mesenteric venous blood; IME, immunomagnetic enrichment; LN, lymph nodes; CEACAM, carcinoembryonic antigen-related cell adhesion molecule.

characterized by great sensitivity, whereas its specificity can be improved by careful experimental design and its use in combination with other markers.

Cytokeratin 19 (CK19)

CK19 also belongs to the intermediate filament protein family and is expressed in cells of epithelial origin. It is found in the periderm, a layer that envelopes the developing epidermis. CK19 has been used as a molecular marker in a variety of studies using RT-PCR; a large number of publications report the expression of CK19 in the peripheral blood of prostate, breast and colorectal cancer patients. Nonetheless, there have been serious questions raised concerning its specificity; the observed false-positive results can result from amplification of two known CK19 pseudogenes (50, 87), illegitimate transcription in hematopoietic cells (88) or, as mentioned above, epithelial cell introduction into the circulation during

Table III. *Results from studies detecting CTCs by examining the expression of CK 20 mRNA.*

Method	Sample	Number of patients included			Positive Results			Correlation	Reference
		CRC patients	Patients with benign diseases	Healthy volunteers	CRC Patients	Patients with benign diseases	Healthy volunteers		
RT-PCR	PB	57		14	42.1%		-	LN metastasis, survival	(81)
RT-PCR	PB, BM	30	13	16	9/30 in PB 9/19 in BM	4/13	3/16		(144)
RT-PCR	PB, PVB, BM	58		12	52.6%			Stage, survival	(80)
RT-PCR	PB, MB	35		10	2/31 in PB 6/35 in MB		-	Survival	(83)
IME, Real-time PCR	PB	40	10	10	72.5% -82.5%	0/10-1/10	-	Stage, tumor diameter, LN& liver metastases	(145)
RT-PCR	PB, tissue	25		12	12/25 in PB, 8/9 CRC biopsies, 9/10 liver metastases		1/12		(84)
IME, Real-time PCR	PB	42 with localized, 40 with metastatic CRC	15	37	58% of localized, 55% of metastatic CRC	60%	22% , significantly lower levels from patients		(85)
RT-PCR	PB	72		30	52.8%		6.7%		(146)
Real-time PCR	PB, MB, PLF	39	7		10/39	-		Clinical outcome	(57)
Real-time PCR	PB	20	20		2/20 preoperatively, 5/20 postoperatively	6/20			(140)
RT-PCR	PB, BM, tissue	10	18 PB & 13BM	11	4/10 in PB 12/12 in primary & 5/5 in metastatic tissue	3/18 in PB 3/13 in BM	0/11 in PB		(147)
RT-PCR, Southern blot	PB	12		35	1/12		4/35		(96)
Real-time PCR	PB, tissue	129	13	45	88.4%		84.6% (of patients with benign diseases & healthy volunteers)		(142)
RT-PCR	PB, BM	142 PB samples, 127 BM samples			55.6% in PB and 33% in BM without adjuvant treatment, 40% in PB and 16.7% in BM with adjuvant treatment			Stage	(148)

Table III. *continued*

Table III. *continued*

Method	Sample	Number of patients included			Positive Results			Correlation	Reference
		CRC patients	Patients with benign diseases	Healthy volunteers	CRC Patients	Patients with benign diseases	Healthy volunteers		
Real-time PCR, Membrane array	PB, tissue	80		98	79.2% detection 78.8% overexpression			Stage & LN metastasis	(92)
RT-PCR, Southern blot	PB	35		22	14/22 with metastatic CRC, 1/13 with non-metastatic CRC		-		(149)
RT-PCR	PB	58 CRC, 7 with hepatic metastases	24	12	24/58 with CRC, 6/7 with hepatic metastases	-	-	Stage & timing of blood collection	(150)

CRC, Colorectal cancer; PB, peripheral blood; BM, bone marrow; PVB, portal vein blood; PLF, peritoneal lavage fluid; IME, immunomagnetic enrichment; LN, lymph nodes.

blood sampling (89). Furthermore, it has been suggested that increased secretion of cytokines can induce the transcription of tissue-specific genes in hematopoietic cells, as implied by the detection of *CK19* mRNA in hematological malignancies (90).

Most of the studies investigating CK19 expression in CRC do so by combining its use with other markers, most commonly with CEA and CK20 (82, 83, 91-96). The results from these studies are conflicting, as in some no expression in reference samples was observed (82, 83) and in others the specificity was lower as CK19 is also present in benign disease and healthy donor blood (91-96).

Guanylyl Cyclase C (GCC)

GCC is a brush border membrane receptor, selectively expressed by intestinal epithelial cells from the duodenum to the rectum, including normal intestinal and colorectal cancer cells, but not by normal extraintestinal tissues (97). GCC expression has been observed in normal intestinal cells as well as in all cases of benign diseases and cancer of the gastrointestinal tract, including liver metastases, but not in normal esophageal cells, or cells of the stomach (98). GCC binds the endogenous peptides guanylin and uroguanylin, as well as the bacterially derived heat-stable enterotoxin (ST) (99-101); binding of the ligand to the receptor results in intracellular cGMP elevation, chloride and eventually water secretion (102, 103). The physiological role of GCC remains unclear; it is known that binding of ST causes diarrhea, while it is hypothesized that binding of guanylin and uroguanylin plays a role in the regulation of fluid and electrolyte homeostasis in the intestine (104). Additionally, it has been found that GCC through binding to ST and uroguanylin is a tumor suppressor, as its binding to these ligands results in inhibition of enterocyte and colorectal cancer cell proliferation (105, 106).

GCC mRNA has been detected in all cases of CRC examined. It has been reported that *GCC* mRNA can indeed show histologically undetectable occult lymph node metastases of CRC and lymph node positivity for *GCC* mRNA is associated with recurrence and mortality (107, 108). Carrithers *et al*., applying RT-PCR and northern blot found that GCC was expressed in the blood of some Dukes' B and all Dukes' C and D patients, while no expression was observed in control or noncancerous blood (97). Bustin *et al*., using real-time RT-PCR, examined the expression of GCC, CK20 and CK19 in blood samples taken from 27 CRC patients and 21 healthy volunteers; GCC was expressed in 80% of patient samples and in only 1 sample from healthy volunteers, in contrast to the other two markers that were largely expressed in control samples (109). It has been reported that GCC is illegitimately expressed in peripheral blood $CD34^+$ progenitor cells, a problem that has been observed for other markers as well, including CK20 and CEA; however, the authors showed that depletion of $CD34^+$ cells, or a decrease of the amount of RNA analyzed to ≤0.8 μg can eliminate false-positives (110). In general, GCC is considered to be a very promising marker for CRC staging; the specificity exhibited in *GCC* mRNA expression could provide the answer to the frequently observed problem of false-positives (109, 110).

Epidermal Growth Factor Receptor (EGFR)

EGFR is generally known to exert control over normal cell growth and cancer pathogenesis in humans. It is normally expressed in a variety of cell types, including epithelial and mesenchymal cells. Deregulation of the EGFR/EGF system seems to be involved in stomach cancer development through induction of uncontrolled proliferation of the gastric mucosa cells. EGFR is used as a tumor marker in various types of cancer, including breast, stomach and colorectal. Most studies refer to EGFR as a specific marker with limited sensitivity. For instance, in a study using nested RT-PCR, 12.5% of CRC patient blood samples exhibited expression of EGFR, while none of the 23 healthy volunteer samples were positive (111). This high specificity observed is in agreement with the study of Giacomelli *et al*. who observed no EGFR expression in control samples, when 62% of patients were positive. The authors found a correlation of EGFR expression with the stage of the disease and tumor relapse (112). Gradilone *et al*. also detected no EGFR expression in control samples examined, whilst *EGFR* mRNA was expressed in 25% of patients (96). De Luca *et al*. found EGFR expression in 10% of healthy volunteers and in 73% of CRC patient samples; they also observed accordance between EGFR expression and cancer stage (94).

Human Telomerase Reverse Transcriptase (hTERT)

Telomerase is an enzyme responsible for the length conservation of chromosome telomeres and hence for the immortalization of cells. The catalytic core of telomerase is composed of an RNA subunit (hTR) and a protein subunit (hTERT) that catalyses the reaction for telomere synthesis (113). Although the hTR subunit is expressed independently of telomerase activation, hTERT expression is indicative of the fact that telomerase is active (114, 115). In general, there is controversy as to whether immortalized cells express hTERT. This disagreement lies in the distinction between two cell types: In the first category, which includes fibroblasts, hTERT is expressed as this expression is sufficient to immortalize the cells (116); the second category, however, requires that cell growth should be inhibited through other pathways so as the cells can become immortal. In the latter group, telomerase activity is undetectable and the telomere length is preserved through a telomerase-independent mechanism known as alternative lengthening of telomeres (117, 118).

hTERT is expressed in normal tissues as well, including dividing lymphocytes and normal intestinal epithelium (119-122). As far as the use of hTERT as a molecular marker is concerned, there is general agreement about its detection in healthy donors. Still, when real-time PCR is applied, hTERT is clearly shown to be expressed at higher levels in patient samples compared to normal controls. Accordingly, there is conformity with reference to the suitability of hTERT as a tumor marker when quantitative analysis is implemented (123-126). Myung *et al*., using RT-PCR in biopsies from 34 patients with CRC, 21 patients with ulcerative colitis and 11 healthy volunteers, detected *hTERT* mRNA in 94% , 57% and 45% of samples, respectively (127). Lledo *et al*. examined 50 CRC and 50 normal plasma samples by real-time RT-PCR; hTERT mRNA was detected in all samples, although the median expression in patients was elevated compared to healthy controls (128). Niiyama *et al*., analyzing 140 CRC and 140 normal tissue specimens as well as 20 adenomas by real-time RT-PCR, observed higher levels of *hTERT* mRNA expression in carcinomas compared to adenomas or adjacent tissues (124). The aforementioned results are in complete agreement with a recent study applying real-time RT-PCR to 53 pairs of CRC and adjacent normal tissues as well as 9 adenomas (125).

Future Perspectives

The currently implemented staging system for colorectal cancer is the TNM system and although often updated it still fails to discriminate between stages, specifically between stages II and III. This drawback of the staging system can lead to employing adjuvant treatment for patients that do not need to be treated and *vice versa*, since although patients with stage III CRC receive therapy, this is not the rule for stage II patients (129). In addition, as the TNM system lacks credibility concerning the discrimination between stages II and III, it is probable that it fails to predict disease outcome within stages (4).

Although CRC is one of the most common types of cancer encountered, the use of currently available screening methods such as colonoscopy is not as common as would be anticipated. People presenting high risk for developing CRC, for instance people with personal or familial CRC history, are expected to undergo examination on a regular basis; still, several hindrances are encountered, with the most prominent being the patient discomfort for the procedure (6). The treatment of choice for CRC includes surgery and implementation of adjuvant therapy. However, as stated above, a large percentage of patients develop distant metastases after the resection of the primary tumor.

All the aforementioned obstacles emphasize the need for the introduction of a new staging system which can minimize the problems continually encountered; the use of molecular markers is very promising in this direction. Every assessable biochemical parameter can serve as a molecular marker, *e.g.* an oncogene, an enzyme or a hormone. Molecular markers are present and can be measured in tumor tissues or the lymph nodes and in occult tumor cells in peripheral blood, bone marrow and other body fluids

(130). For all the mentioned reasons, it is quite clear that a staging system based on molecular parameters shows great potential. The use of cancer markers would prove valuable in cancer prognosis; defining the outcome of a certain cancer case, in terms of recurrence and survival is a necessity in cancer medicine. Moreover, as stated earlier, defining administration of adjuvant therapy is essential; observation of individual patients' responses to therapy is also of great significance.

Identifying occult metastases can be an excellent weapon against cancer, since metastases can occur as an early event in cancer development, or as a postsurgical event. Ito *et al*., using real-time RT-PCR, found elevated levels of *CEA* mRNA in preoperative blood samples from 99 patients with CRC compared to postoperative samples from the same patients; they also observed a correlation between CEA positivity and reduced disease-free survival (131). Examining bone marrow and blood samples from patients undergoing hepatic metastases resection, Koch *et al*. found that detection of occult tumor cells in intraoperative blood and preoperative bone marrow samples can be an independent prognostic factor for tumor relapse (132). The use of molecular markers has provided the ability for predicting development of lymph node metastases in histopathologically negative lymph nodes, as described above (41, 79, 107, 108, 133).

Conclusion

Despite the controversial results of the studies dealing with the molecular detection of disseminated CRC cells, there is increasing interest in the use of molecular staging in CRC prognosis and treatment. The discrepancies observed in the studies emphasize the need for the establishment of a universally applied standardized method that would provide comparable results. For that purpose, care concerning several parameters that affect the efficiency of a method should be taken. The choice of molecular markers is one of the most prominent; given the fact that, at least in the case of CRC, there is no such thing as the perfect marker, the best choice would include a combination of sensitive and specific markers in a multimarker assay in order to achieve maximum clinical relevance. In addition, the use of quantitative methods and the implementation of enrichment techniques are proposed. As far as false-positive or -negative results are concerned, cautious sample preparation should reduce contamination; careful design of primers can help to avoid amplification of genomic sequences or pseudogenes; use of internal controls can provide assurance for RNA quality; time of performing PCR based protocols, vis-à-vis surgery and multisampling, can overcome intermittent shedding, thus defining the suitable timing of blood sampling which can solve the problem of cell dissemination during surgical intrusion. Future studies of large cohort size and long-term follow-up of the patients included are mandatory so as to evaluate the clinical relevance of the detection of circulating colorectal cancer cells, based on PCR protocols which will involve multiple tumor markers.

In conclusion, the use of molecular markers in cancer medicine is still evolving and it is clear that there is much yet to be clarified. It is evident that we stand at the beginning of a new era for cancer prognosis, disease staging and treatment choice. Further analyses will reveal the potential of molecular diagnostics and allow their routine use in clinical practice.

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