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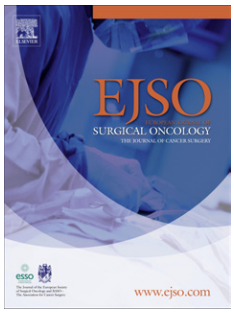
Accepted Manuscript



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PII: S0748-7983(11)00045-X

DOI: 10.1016/j.ejso.2011.01.025

Reference: YEJSO 3120

To appear in: *European Journal of Surgical Oncology*

Received Date: 11 November 2010

Revised Date: 14 January 2011

Accepted Date: 24 January 2011

Please cite this article as: Thorsteinsson M, Jess P. The clinical significance of circulating tumor cells in non-metastatic colorectal cancer - a review, European Journal of Surgical Oncology (2011), doi: 10.1016/j.ejso.2011.01.025

The clinical significance of circulating tumor cells in non-metastatic colorectal cancer - a review

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Word count: Text 2847 words and abstract 246 words.

Abstract

Background: Finding a clinical tool to improve the risk stratification and identifying those colorectal cancer patients with an increased risk of recurrence is of great importance. The presence of circulating tumor cells (CTC) in peripheral blood can be a strong marker of poor prognosis in patients with metastatic disease, but the prognostic role of CTC in non-metastatic colorectal cancer is less clear. The aim of this review is to examine the possible clinical significance of circulating tumor cells in non-metastatic colorectal cancer (TNM-stage I-III) with the primary focus on detection methods and prognosis.

Methods: The PubMed and Cochrane database and reference lists of relevant articles were searched for scientific literature published in English from January 2000 – June 2010. We included studies with non-metastatic colorectal cancer (TNM-stage I-III) and CTC detected pre- and/or postoperatively in peripheral blood.

Results: Nine studies qualified for further analyses. Detection rates of CTC in peripheral blood of patients with non-metastatic colorectal cancer varied from 4-57%. Seven studies applied RT-PCR and two studies used immunocytochemical methods. Seven studies found the presence of CTC to be a prognostic marker of poor disease-free survival.

Conclusion: The presence of CTC in peripheral blood is a potential marker of poor disease-free survival in patients with non-metastastic colorectal cancer. The low abundance of CTC in non-metastatic colorectal cancer requires very sensitive and specific detection methods. An international consensus on choice of detection method and markers , is warranted before incorporating CTC into risk stratification in the clinical setting.

Keywords: Colorectal cancer; Circulating tumor cells; Detection methods; Prognosis; Review.

Introduction

Colorectal cancer is the most common cancer and the second most common cause of cancer-related death in Europe, with 436000 new cases and 212000 deaths in 2008 [1]. The general treatment of non-metastatic colorectal cancer is intended curative resections, adjuvant chemotherapy depending on tumor stage, and frequent follow-up. Despite this intensive treatment program it is estimated that approximately 30% develop metastases and eventually die of metastatic disease after intended curative resection [2-4]. An early identification of patients at risk of developing metastatic disease after surgery may therefore be of great importance for improving clinical outcome.

In general the choice of treatment and prognosis are directed by the Tumor, Node and Metastasis (TNM) staging system, examining the extent of tumor invasion through the bowel wall (T), the presence of metastases or micrometastases in regional lymph nodes (N) and distant metastases (M) [5, 6]. The TNM-stage provides information on prognosis with approximately 93% 5-year stage-specific survival rate for stage I, 72-84% for stage II, 44-83% for stage III and 8% for stage IV colon cancer [7, 8].

Currently all stage III and a few selected stage II colorectal cancer patients receive adjuvant chemotherapy, typically based on 5-fluorouracil, leucovorin and oxaliplatin, to improve disease-free survival and overall survival especially in stage III patients [4, 9-11]. The finding of a clinical tool to improve the risk stratification and identifying those colorectal cancer patients with an increased risk of recurrence has been a field of interest for many researchers. By using new sensitive immunocytochemical and molecular assays it is possible to detect and isolate circulating tumor cells (CTC) from peripheral blood [12]. CTC can be defined as specifically identified tumor cells detected in blood or lymphatic vessels, which may represent the earliest sign of dissemination [13].

The presence of CTC has proven to be a strong predictor of short-term progression-free survival and overall survival in patients with metastatic colorectal cancer [14, 15]. A recently published meta-analysis has also demonstrated that the detection of CTC indicates a poor prognosis in patients with colorectal cancer [16]. Whereas most large-scale data deal with patients with metastatic colorectal cancer, there is only limited data on the significance of CTC in patients with non-metastatic colorectal cancer. Hence Peach et al. reviewed the prognostic value of postoperative detection of CTC in non-metastatic colorectal cancer [17]. The authors concluded that the presence of CTC in peripheral blood was an independent predictor of recurrence.

The aim of this article is to review data from the most recent studies on the significance of pre- and postoperatively detected CTC in non-metastatic colorectal cancer. The primary focus is on the different detection methods and their prognostic significance.

Methods

The PubMed database and the Cochrane database was searched for scientific literature published in English from January 2000 – June 2010. The search terms were “colorectal cancer”, “colon cancer”, “rectum cancer”, “circulating tumor cells”, “neoplastic cells, circulating”, “peripheral blood”, “detection methods”, “polymerase chain reaction”, “RT-PCR”, “immunological detection techniques”, and “prognosis”. A manual search of reference lists of the extracted articles was performed and the “related articles” function in PubMed was also utilized.

Four hundred and seventeen articles were identified with the above search terms. Forty-nine references were assessed in full. Following inclusion criteria for studies were set: (1) patients diagnosed with non-metastatic colorectal cancer (TNM-stage I-III),

(2) CTC detected in peripheral blood samples, (3) pre- and/or postoperative blood samples and (4) samples size of ≥99 patients. Nine studies qualified for further analyses (Table 1) [18-26].

Detection of circulating tumor cells

The detection of CTC can be very difficult with as few as 1 CTC in 100 million leukocytes [13, 27]. Therefore it typically requires an enrichment process based on either cell density, using gradient centrifugation (Ficoll-Hypaque) or immunomagnetic separation [28]. Methods for detecting CTC can be classified into immunological or molecular PCR-based techniques [27-29].

Immunological techniques

The immunocytochemical technique utilizes labeled monoclonal antibodies directed against epithelial or tumor-associated antigens and automated digital microscopy or flow cytometry to isolate and enumerate CTC [27, 28, 30]. This method allows identification of intact tumor cells for further characterization [31-33].

A semi-automated technique called CellSearch®, Circulating Tumor Cell Test (Veridex) is the only assay approved by the US Food and Drug Administration for detection of CTC in peripheral blood of patients with metastatic breast, colorectal and prostate cancer [14, 34, 35]. The assay consists of an immunomagnetic enrichment process where ferrofluids coated with antibodies against EpCAM (epithelial-cell adhesion molecule) are used to isolate epithelial cells. The isolated cells are fluorescently stained with nucleic acid dye 4,2-diamidino-2-phenylindole (DAPI), antibodies against cytokeratin 8, 18 and 19 and antibodies against leukocytes (CD45). Further identification and

enumeration of CTC is performed with a semiautomated fluorescence microscope. Finally a gallery of cellular images is reviewed by a trained specialist for the ultimate selection of CTC. Cells that are cytokeratin and DAPI positive, negative for CD45 and have a round to oval morphology are classified as CTC [36]. The test is considered to have a high specificity and reproducibility (Table 2) [36, 37]. Allard et al. demonstrated that CTC are present in peripheral blood of patient with various metastatic carcinomas, but very rarely present in healthy subjects or patients with nonmalignant diseases [36].

Another promising immunological approach to detect CTC is the “CTC chip”, a microfluidic platform separating CTC from whole blood by mediating the interaction of target CTC with antibody EpCAM-coated microposts under precisely controlled laminar flow conditions. The “CTC chip” identified CTC in 115 of 116 (99%) patients with metastatic lung, prostate, pancreatic, breast and colon cancer [38].

The sensitivity of approaches using EpCAM might be limited by the fact that CTC not expressing EpCAM will not be detected, leading to false-negative results. Rao et al. demonstrated that the expression of EpCAM was approximately 10-fold lower on CTC than on primary and metastatic tissues [39]. Moreover, recent data suggest that tumor cells can undergo epithelial-to-mesenchymal transition (EMT), a process of multiple biochemical changes enabling the cell to assume a mesenchymal cell phenotype. This includes altered epithelial protein expression, increased motility, and altered morphology [40, 41].

PCR-based techniques

CTC can also be detected indirectly by the use of a polymerase chain reaction (PCR) targeting tumor-specific DNA or RNA. Since RNA is unstable and disappears quickly from

the blood after cell death, the presence of RNA is due to the presence of viable tumor cells [28]. Therefore the most widely used PCR technique uses reverse transcriptase PCR ((RT)-PCR), where a sequence of mRNA is serving as a template for reverse transcription to cDNA and then amplified using an oligonucleotide primer [28, 42]. In colorectal cancer the RNA markers commonly used are CEA, CK19 and CK20 [43, 44].

The advantage of RT-PCR is a higher sensitivity than those of immunocytochemical techniques (Table 2) [45, 46]. Unfortunately the specificity of RT-PCR is hampered by high numbers of false-positive results due to contamination or target genes expressed in other non-malignant cells [47-49]. By using a RT-PCR assay Novaes et al. demonstrated that mononuclear cells from peripheral blood of healthy donors express CK19 [49]. Furthermore both CEA and CK20 transcripts are elevated in patients with inflammatory diseases [47, 48]. Another important limitation of PCR-based methods is that CTC can not be isolated for further analysis.

Results

Detection rates

There is a great variability in the rates of CTC detection in the different studies as shown in Table 1. By using the CellSearch method to detect CTC from blood samples taken 4 to 12 weeks after curative surgery, Maestro et al. found ≥2 CTC per 7.5 ml blood in 25 of 164 patients with localized colorectal cancer [18]. Other small studies, not included in this review, have detected CTC in 7-26% of non-metastatic colorectal cancer patients, also applying the CellSearch System [50, 51]. To improve the sensitivity of the CellSearch System Lalmahomed et al. increased the blood sample size from 7.5ml to 30ml in 15 patients with colorectal liver metastases [52]. The 30ml of blood where hereafter reduced

to a volume of 7.5ml of enriched blood by density gradient separation. The median number of detected CTC increased from 1 (range 0 to 4) in 7.5ml blood to 2 (range 0 to 9) in 30ml blood. It is possible that this procedure might increase the CTC counts in patients with non-metastatic colorectal cancer as well.

In a different immunocytochemical assay Wong et al. developed a gastrointestinal-specific anti-cytokeratin 20 antibody to detect CTC [19]. They were able to demonstrate CTC in 58 of 101 patients with stage I-III colorectal cancer preoperatively and furthermore detect a decrease in CTC in 51 of these 58 patients (88%) after surgery.

As previously mentioned, the sensitivity of the RT-RCR method is considered to be higher than that of the immunological approach. The majority of the included studies reflect this statement as CTC are detected in 22-57% postoperatively with RT-PCR [20-24]. Two studies reported markedly low detection rates very different form the others (Table 1) [25, 26].

Correlation to TNM-stage

The yield of CTC in colorectal cancer is frequently found to correlate to TNM-stage and is probably related to tumor burden. In non-metastatic colorectal cancer (TNM-stage I-III) the CTC counts and the percentage of patients with detectable CTC is lower than in the advanced stage (TNM-stage IV) [18, 50]. In four of the nine included studies CTC was significantly correlated to TNM-stage (see Table 1) [18, 19, 22, 25].

Conversely two studies found no significant correlation to TNM-stage [23, 24] and in the remaining three studies, no data was found on the association between CTC and TNM-stage [20, 21, 26].

Clinical value of CTC in colorectal cancer

The prognostic significance of CTC in non-metastatic colorectal cancer was evaluated in eight of the nine included studies (Table 1). Seven studies found the presence of CTC to be correlated with early recurrence, poor disease-free survival or overall survival [19-21, 23-25].

In two of the studies the blood samples were taken preoperatively. Wong et al. examined 101 patients with TNM-stage I-III colorectal cancer with an immunological method, detecting CTC with a gastrointestinal-specific CK20 [19]. Sixty-two of 101 patients were followed for a period of 24 months and the association between preoperative elevated CK20 and recurrence was found to be highly significant ($p < 0.001$) [19]. Moreover CTC were an independent prognostic factor of survival ($p = 0.005$) in a multivariate regression analysis including TNM-stage, lymph node status, age, sex, tumor stage and degree of differentiation. However, it should be mentioned that patients with TNM-stage IV was included in these survival data. In accordance, Iinuma et al. was also able to demonstrate poor disease-free survival for colorectal cancer patients with preoperatively elevated CTC using a RT-PCR based method [25].

The remaining five studies with positive results on prognosis were performed with RT-PCR method on blood samples taken postoperatively [20-24].

Using RT-PCR on blood samples from 147 patients with TNM-stage I-III colorectal cancer, Allen-Mersh et al. demonstrated that poor disease-free survival was associated with the occurrence of CEA or CK20 24 hours postoperatively ($p < 0.001$) [20]. Interestingly, the same authors did not find any difference in the probability of recurrence between patients positive for CTC and patients negative for CTC before surgery. Uen et al. used a multi-marker membrane array method to detect CTC in 438 patients with TNM-

stage I-III colorectal cancer [21]. Presence of all four markers (hTERT, CK19, CK20 and CEA) were considered as a positive result for CTC. The authors demonstrated that patients with persistent presence of CTC after surgery had a significantly poorer relapse-free survival compared with patients without CTC ($p < 0.001$) [21]. These results are supported by Wang et al. who used the same set of markers (hTERT, CK19, CK20 and CEA) on 157 patients with TNM-stage I-III colorectal cancer and found the expression of all four markers to be an independent predictor of early postoperative relapse [22]. Patients with all four markers had a relative risk of 18.7 of developing postoperative relapse compared with patients without any marker ($P = 0.019$).

With a single marker (CEA mRNA) and a RT-PCR approach, Sadahiro et al. detected CTC in peripheral blood from patients with TNM-stage I-III colorectal cancer [23]. Blood samples were taken 7-10 days after resection and CEA mRNA positive patients showed significantly poorer disease-free survival ($p = 0.007$) and overall survival ($p = 0.04$) than the CEA-negative patients. Ito et al. have also reported a shorter disease-free survival for patients positive for CEA mRNA compared with patients negative for CEA mRNA after curative resection of non-metastatic colorectal cancer ($p = 0.03$) [24].

Lloyd et al. used RT-PCR and multiple markers including ephrin-B4, laminin-Y2, matrilysin, CK20 and CEA mRNA to detect CTC [26]. Samples were taken from peripheral blood before and after surgery and samples of peritoneal lavage were taken during the operation, before and after tumor resection. There was no significant correlation between CTC from the blood samples and prognosis, but the authors found that 33% were positive for CTC in post-resection peritoneal fluid and that these patients had a significantly poorer prognosis ($p=0.002$) [26].

Discussion

Detection methods

The clinical significance of CTC in non-metastatic colorectal cancer is being investigated. Preliminary results seem promising, but are to some degree difficult to compare and interpret, due to heterogeneous study designs and detection methods. As demonstrated in the reviewed studies the detection rates of CTC varied markedly. The explanation is most likely the different detection techniques rather than a biological variation of CTC. Utilizing an immunological approach is very interesting because it allows further analysis of CTC, such as gene-expression profiles or evaluation of the expression of biological therapeutic targets as Epidermal Growth Factor Receptor (EGFR). Opposite to the results in metastatic colorectal cancer, there are no prognostic data supporting the use of the only commercial available immunological assay CellSearch in non-metastatic colorectal cancer. The RT-PCR approach may be the method of choice at the moment, but there is an absence of an international standard on choice of markers, enrichment procedures and laboratory techniques. As demonstrated in this review, some investigators use single marker assays others use multiple markers. Because of the heterogeneity within tumors it seems more likely that a multiple marker assay will provide the greatest CTC yield. A standardization of detection methods is of great importance for the comparability of future large-scale studies.

Prognostic significance

Despite the differences in detection techniques, CTC detection shows promising results regarding prognostic information in patients with non-metastatic colorectal cancer. The prognostic significance of CTC was evaluated in eight of nine studies included in this

review. Five of the studies found the presence of CTC postoperatively to predict poor disease-free survival and two studies demonstrated preoperative CTC to predict early recurrence [19-25]. These findings are supported by similar results in a review by Peach et al., where six of nine studies found postoperative detection of CTC to be an independent predictor of cancer recurrence [17]. In a meta-analysis Rahbari et al. also found postoperative tumor cell detection to predict poor recurrence free survival in curatively resected colorectal cancer patients [16]. CTC detected after surgery is the most frequently applied sampling time, but as demonstrated in the present review, also preoperatively detected CTC can predict early relapse. However, from a clinical point of view postoperatively elevated CTC is more relevant as it may represent sign of residual disease activity.

Only one study by Lloyd et al. found no correlation between CTC and prognosis [26]. This study had a very low detection rate of only 4% which may have caused a lack of power to demonstrate a prognostic significance.

Future perspectives

The TNM staging system is the most frequently used clinical tool to predict outcome and direct postoperative adjuvant chemotherapy treatment. Survival data show that the 5-year stage-specific survival rate for TNM-stage IIIa colon cancer (83%) is significantly better than stage IIb (72%) ($p < 0.001$) [8]. None of the included studies had divided the TNM-stages into subgroups and no data exist on whether CTC are useful to predict patients at risk of developing recurrence within these subgroups. Still, the presences of CTC may be supplementary to the TNM staging system, as colorectal cancer patients with either lymph node metastasis, vascular invasion or postoperative presence of CTC had a seven fold

risk of developing relapse [21]. A future scenario could be to combine CTC and the TNM staging system to increase the prognostic accuracy. Allen-Mersh et al. demonstrated that adding CTC to lymph node metastasis increased the hazard ratio for predicting recurrence from 8 (lymph node metastasis alone) to 18.5 in patients with non-metastatic colorectal cancer [20].

In conclusion, the presence of CTC in peripheral blood is a prognostic marker of poor disease-free survival in patients with TNM-stage I-III colorectal cancer. Combining the TNM staging system and CTC, could aid decision-making as to which patients should be offered adjuvant chemotherapy. However, an international consensus on choice of detection method, supported in large-scale studies, is warranted before incorporating CTC into risk stratification in the clinical setting.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

This work was supported by Hilleroed Hospital Research Foundation and Region Sealand Research Grant.

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Table 1. Characteristics of the included studies

Reference	Detection method	Marker	Number of patients	TNM-stage	Sampling time	Detection rate (No/%)	Correlation to stage	Prognostic significance
Maestro, 2009 [18]	CellSearch	EpCAM	164	I-III	Post-operative	25/15%	Correlation to stage (localized versus metastatic)	Not tested
Wong, 2009 [19]	Immuno-Cytochemical	CK20	101	I-III	Pre-operative	58/57%	Correlation to TNM-stage	Early recurrence
Allen-Mersh, 2007 [20]	RT-PCR	CEA CK20	113	I-III	Post-operative	35/31%	Not tested	Poor disease-free survival
Uen, 2008 [21]	RT-PCR	hTERT CK19 CK20 CEA	438	I-III	Post-operative	137/31%	Not tested	Poor relapse-free survival
Wang, 2007 [22]	RT-PCR	hTERT CK19 CK20 CEA	157	I-III	Post-operative	90/57%	Correlation to TNM-stage	Poor relapse-free and overall survival
Sadahiro, 2007 [23]	RT-PCR	CEA	200	I-III	Post-operative	44/22%	No significance	Poor disease-free survival and overall survival
Ito, 2002 [24]	RT-PCR	CEA	99	I-III	Post-operative	50/50%	No significance	Poor disease-free survival
Iinuma, 2006 [25]	RT-PCR	CEA CK20	128	I-III	Pre-operative	8/6%	Correlation to Duke stage	Poor disease-free survival
Lloyd, 2006 [26]	RT-PCR	CK20 CEA EphB4 LAMy2 MAT	125	I-II	Post-operative	5/4%	Not tested	No significant results

Table 2. Advantages and disadvantages of the two main methods for detection of circulating tumor cells

Assay	**Advantages**	**Disadvantages**
Immunological technique (CellSearch)	- High specificity - Further cytological analysis possible - Semi-automated process	- Moderate sensitivity, EpCAM negative cells may be missed - Subjective CTC verification
RT-PCR technique	- High sensitivity - Objective detection of CTC	- Low specificity due to target genes expressed in other cells - Instability of RNA - No further cytological analysis possible