RESEARCH ARTICLE

Secondary Circulating Tumor Cells (CTCs) but not Primary CTCs are Associated with the Clinico-Pathological Parameters in Chilean Patients With Colo-Rectal Cancer

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Abstract

Background: The aim of this study was to assess detection of circulating tumor cells (CTC) using anti-CEA pre and post surgery in Chilean patients with colo-rectal cancer. Materials and Methods: The presence of CTCs was evaluated in 80 colorectal cancer patients pre and post surgery using standard immunocytochemistry and the results were compared with findings for standard clinico-pathological parameters. Results: In patients pre-surgery CEA (+) CTCs were frequently found, with no relation to tumor size or nodal status. After surgery, the presence of CTCs was associated with such clinico-pathological parameters. The frequency of CTC detection in node positive patients did not change after surgery. In patients with metastasis there was also no change in the frequency of CTC detection, and clusters of 3 or more CTCs were evident. Conclusions: Secondary CTCs are associated with clinico-pathological parameters only after surgical removal of the primary tumor, and might be important in identifying patients at high risk of relapse. Primary CTCs detected before surgical removal are frequently found, are not associated with the clinico-pathological parameters and might have a role in cancer screening. These findings suggest the need for studies with a larger population of patients.

Keywords: Colorectal cancer - circulating tumor cells - CEA

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Introduction

Although the first report of circulating tumor cells (CTCs) was published in 1869 (Ashworth, 1869), it was not until fifty years ago that CTCs were first reported in colo-rectal cancer (CRC) (Engell, 1955). CTCs can be detected early in the natural history of solid tumors and before the development of detectable metastasis (Glaves, 1983; Allard et al., 2004). Although they are not thought to circulate in healthy individual or in non-maligant disease this has been recently questioned. They had been reported in patients with benign colonic disease (Pantel et al., 2012) and benign prostate disease (Murray et al., 2013). Using the EpCAM based CellSearch (Veridex)® detection system, circulating epitelial cells were found in between 0% and 18.7% of patients with benign colonic disease, while the Epispot® cytokeratin-19 assay detected them in between 8.3-28.6% of patients (Pantel et al., 2012). What remains to be confirmed is whether or not trafficking of normal epitelial cells could occur in benign conditions and thus contribute to the "*false positive*" findings in control patients detected using current assay methods. The detection of these benign epitelial cells is important when considering the detection of "*CTCs*" before the removal of the primary tumor and after surgical removal if there is benign tissue persisting, such as after colectomy for colonic cancer. This is further confounded by the fact that not all CTCs express EpCAM and/or cytokeratins (Mikolajczyk et al., 2011) and that both markers may have decreased expression during the epitelial-mesenchymal transition which facilitates the dissemination of cancer cells from the primary tumor, by increasing the plasticity of the tumor cells and thus facilitates their migration through the basement membrane (Raimondi et al., 2011).

Thus the selection of biomarkers to identify CTCs needs to address these factors and to establish unambiguous criteria por the malignant nature of these circulating cells. With the increasing number of patients with colorectal cancer (Abdifard etal, 2013; Zheng et al., 2014) this is becoming increasingly important.

The aim of the present study was to use immunocytochemistry to detect CTCs which expressed carcino-embrionic antigen (CEA) in Chilean patients with histologically confirmed colo-rectal cancer, before

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surgery and three months after primary tumor removal. CEA was selected as the detecting biomarker as it is highly overexpressed in colorectal cancer (Shiveley et el, 1985); routinely used as a tumor biomarker and as an auxillary indicator for the preoperative diagnosis of CRC (Kojima et al., 2009).

Materials and Methods

A prospective single institution study of consecutive patients who complied with the following criteria: *i)* newly diagnosed non-metastatic or metastatic CRC, *ii)* absence of previous colon cancer o inflammatory colon disease, *iii)* written informed consent A group of 20 patients with non-malignant colo-rectal disease with a colonoscopy negative for cancer were used as controls.

CTC detection

Blood samples were collected the week prior to surgery and from 4 to 8 weeks after surgery, before adjuvant or palliative chemotherapy for those patients with advanced or metastatic disease.

Venous blood was collected using a 21G bufferfly needle; the first 5ml was discarded to prevent posible contamination by epitelial cells and the second 8ml was collected into tubes containing EDTA (Beckinson-Vacutainer®). The samples were transported at 4ºC and processed within 24 hours.

Mononuclear cells were obtainerd using differential gel centrifugation with Histopaque 1,077® (Sigma-Aldrich, USA) according to the manufacturer's instructions. The obtained cells were re-suspended in 100µl of autologous plasma and 25µl aliquots of cell suspensión used to prepare slides (sialinized, DAKO, USA), the nair dried for 24 hours and finally fixed using a solution of 70% etanol, 5% formaldehyde and 25% phosphate buffered saline (PBS) pH 7.4 (DAKO, USA) for five minutes and washed twice with PBS.

The slides were processed within one hour of fixation and incubated with monoclonal anti-CEA clone 11-7 (DAKO, USA) for one hour at room temperatura. CTCs were identified using an alkaline phosphatase-antialkaline phosphatase based system (LSAB2, DAKO, USA) with neofuschin as the chromogen and levisamole as an endogenous alkaline phosphatase inhibitor. Positive samples underwent a second process using anti-CD45 clone 2B11 + PD7/26 (DAKO, USA); incubated for one hour at room temperatura and identified using a peroxidase based system (LSAB2, DAKO, USA) with DAB (3,3´diaminobenzadine tetrachloride) as the chromogen.

A CTC was defined according to the morphophological criteria of ISHAGE (Borgen et al., 1999), as a nucleated cell expressing CEA but not CD45. Primary CTCs were defined as those CTCs detected before definitive surgery and secondary CTCs as those detected after surgery. A positive test was defined as the detection of at least 1 cell/8ml venous blood (Figure 1A-E).

The clinical parameters used were that of the TNM classification of 2010 (Edge et al, (2010). Surgical specimens were analyzed by a single experienced pathologist.

Statistical analysis

descriptive statistics were used to describe the demographic and clínico-pathological features and the two tailed Chi squared and Fisher Exact Test used to compare frequencies.

Ethical considerations

The study was approved by the Hospital Ethics Committee.

Results

80 consecutive patients, 56 (70%) women, with a mean age of all patients of 63.8±13.0 years and treated for CRC at the Hospital de Carabineros de Chile between July 2010 and December 2012 participated in the study. Of these 80 patients, 5 died before the second blood simple was taken at 3 months, these 5 patients were all primary CTC positive.

20 controls participated, of whom 1 was CTC positive, the histological diagnosis was a 1cm tubulo-villous adenoma, moderately differentiated which presented as an incidental finding at colonoscopy.

Of the 80 patients with CRC, 72/80 (90%) had primary CTCs detected which was significantly higher than the control group 1/20 (5%) ($p<0.001$).

a) Comparison of the presence of primary CTCs with secondary CTCs: 75/80 patients had blood samples taken for the detection of primary and secondary CTCs, of these 75 patients, 67 (89%) were primary CTC positive. After surgery 33/67 (49%) were negative for secondary CTCs. 8/75 (11%) of patients were primary CTC negative, of whom 1/8 (13%) became positive after surgery (Table 1).

b) Comparison of primary and secondary CTCs with clinico-pathological findings: In patients without evidence of metastasis 60/80 patients pre-surgery did not have metastasis detected, and 59/75 post surgery were metastasis free.

Comparing all the patients without evidence of metastasis, 48/60 (80%) were primary CTC positive and after surgery 21/59 (47%) were secondary CTC positive ($p<0.001$).

Considering only tumor size, there was no relation pre-surgery between the frequency of primary CTC detection and tumor size ($p=0.10$ Chi squared for trends); however post-surgery, tumor size was associated with the frequency of secondary CTC detection ($p=0.003$, Chi squared for trends) with a relative risk of 1.00, 3.73 and 48.00 fot tumors T1+2, T3 and T4 respectively.

Considering only nodal status, there was a significant reduction in the frequency of patients positive for

Table 1. Detection of CTCs before and after Surgery

	Negative after Surgery	Positive after Surgery
Negative before surgery	7	1
Posiitive before surgery	33	34

Table 2. Frequency of CTC Detection According to TN Status Pre and Post Surgery

	Pre surgery	Post surgery	
T1N0M0 + T2N0M0`	5/9 (56%)	1/9 (11%)	
T1N1M0 + T2N1M0	0	0	p=0.29 (Fisher Test)
T3N0M0	25/30 (83%)	4/30 (13%)	p=0.0001 (Chi squared)
T3N1M0	11/14 (79%)	10/14 (71%)	p=1.00 (Chi squared)
T4N0M0	1/1 (100%)	1/1 (100%)	p=1.00 (Fisher test)
T4N1M0	6/6 (100%)	5/5 (100%)	p=1.00 (Fisher test)
Total	48/60 (80%)	21/59 (47%)	p<0.001 (Chi squared)

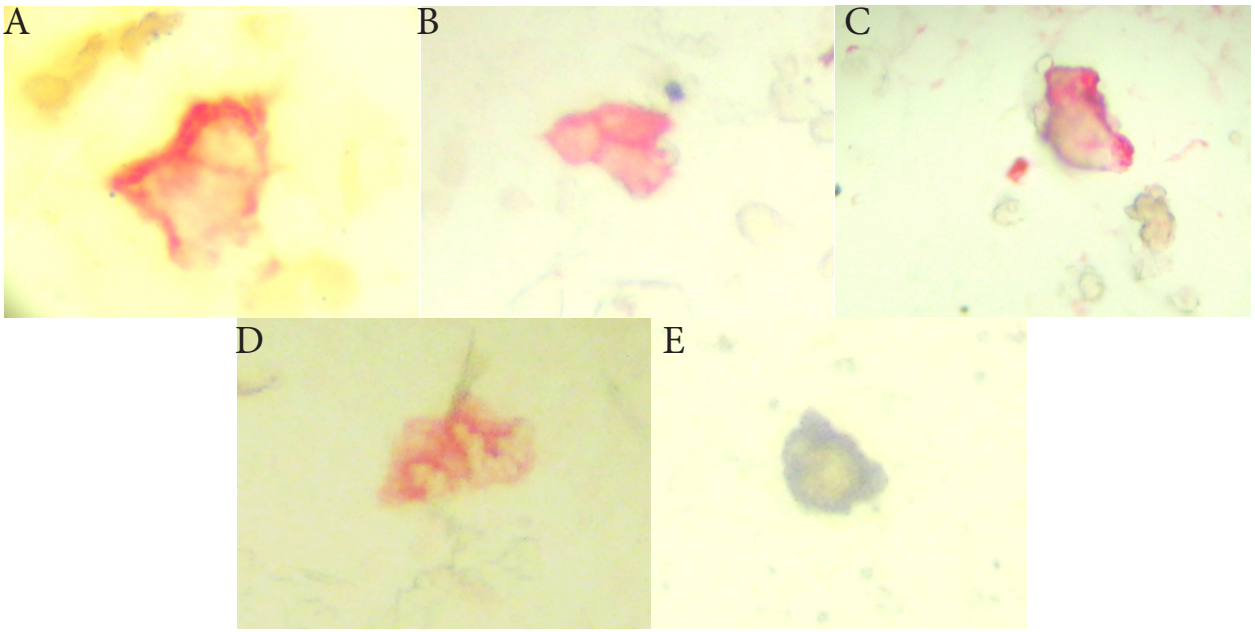

Figure 1. A: duplet of CEA (+) CTCs in CRC metastasica, B: triplet of CEA (+) CTCs in CRC metastasica, C: Single CEA (+) in CRC non-metastasica, D: Duplet of mucin secreting CTCs in CRC mucin secreting primary tumor, E: Leucocyte negative for CEA, positive for CD45

secondary CTCs, both in N0 patients 36/42 (86%) versus 8/40 (20%) (p<0.001, Chi squared) and N1 patients 36/38 (95%) versus 27/35 (74%) (p=0.041, Chi squared). The frequency of secondary CTC detection after surgery was significantly lower in N0 patients in comparison with N1 patients (p<0.001, Chi squared).

There was no difference in pre and post surgery frequency of CTC detection in T4 patients, irrespective of nodal status.

Comparing T3N0M0 versus T3N1M0 patients, there was no difference in the frequency of CTC detection pre-surgery between these groups, After surgery T3N0M0 patients had a significantly lower frequency of secondary CTC detection 4/30 (13%) versus 10/14 (71%) (p<0.001, Chi squared) than T3N1M0 patients. There was a significant reduction in the frequency of CTCs detected in T3N0M0 patients after surgery, 25/30 (83%) versus 4/30 (13%) (p=0.001 Chi squared). Comparing T3N1M0 patients there was no such significant reduction (Table 2).

In patients with metastasis: there was no difference between pre and post-surgery irrespective of T or N status, 17/20 (85%) versus 14/16 (88%) (p=1.00) respectively. However, patients with metastasis frequently had clusters of 3 or more cells detected which was not seen in M0 patients (Figure 1B).

Discussion

Studies using reverse transcriptase polymerase chain reaction (mRNA-rT-PCR) have reported that over 70% of patients were positive for CTCs pre-surgery with little variation in the frequency of positive patients by tumor size or nodal status (Wharton et al., 1999). Using anti-EpCAM, 71% of patients with CRC had CTCs detected pre-surgery, which decreased to 21% post-surgery (Galizia et al., 2013), similar to the frequency of detection using anti-CEA in our study.

2/3 patients with carcinoma in situ and 3/6 patients with small T2N0M0 tumors had primary CTCs detected. It is unlikely that these patients with CTCs detected pre-surgery are destined to develop recurrent disease, experimental and clinical studies indicate that CTC positivity before surgery does not imply that metastasis has occurred (Fidler, 1973; Glaves, 1983). This is because hematogenous metastasis is thought to be an inefficient process, where less than 0.01% of CTCs will implant at distant sites and eventually form metastasis (Fidler, 1973), the majority being cleared from the blood within 24 hours (Fidler, 1970).

This suggests that primary CTCs are not a biomarker that could be useful as a prognostic parameter, in the

majority of cases they are eliminated after surgery. This lack of an association between primary CTCs and the AJCC staging system has been reported using rT-PCR assay methods (Patel et al., 2002). However, if primary CTCs are frequently found pre-surgery the question arises if they could be useful in detection of CRC, however the lack of a specific colorectal biomarker would hinder this application and the fact that EpCAM positive CTCs can be detected in benign colonic disease (Pantel et al., 2012).

The detection of secondary CTCs is associated with prognosis (Albuquerque et al., 2012; Galizia et al., 2013), and associated with disease stage (Gazzaniga et al., 2013). Our study supports the results of Gazzaniga; that with increasing tumor size and nodal infiltration the frequency of secondary CTC detection is increased. Together these data suggest that secondary CTC detection might help in the selection of high risk stage II colorectal cancer patients for adjuvant therapy. This is further supported by the fact that patients secondary CTC positive had an increased risk of relapse, while patients secondary CTC negative had a reduction of <90% in the risk of tumor relapse (Galiza et al., 2013). Galiza (2013), also reported that after surgery the number of patients positive for CTCs decreased significantly, that high postoperative levels of CTCs was the only independent variable related to cancer relapse. Galiza ret al used flow cytometry with anti-EpCAM/CD326 to detect CTCs; van Dalum (2015) using the CellSearch system® which using EpCAM to capture CTCs, reported that in patients with non-metastatic CRC CTCs detected before surgery and two to three years after surgery were associated with a poorer prognosis. They also reported that CTCs detected 2-3 weeks after surgery were not significantly associated with prognosis. A meta.-analysis of 1847 patients reported that although the detection of CTCs pre- treatment, during treatment and post-treatment were associated with the prognosis, that the association between prognosis and CTC detection was more pronounced when samples were collected during and after treatment (Huang et al., 2015). Thus samples collected pre-surgery and early post-surgery as a result of tumor manipulation may be cleared by the immune system or are not capable of implanting in distant tissues and thus not affect the prognosis (Weitz et al., 1998; Lim SH et al., 2014). This may explain the conflicting results reported by von Dalum.

Importantly, the detection of secondary CTCs may be used to monitor the effects of treatment, a meta-analysis showed that CTC response predicted tumor response to chemotherapy (Huang et al., 2014), that the timing of sampling for CTCs was important, changes in tumor activity or proliferation during chemotherapy may impact CTC numbers, thus changes in CTC levels better reflect chemotherapeutic sensitivity and tumor proliferation (Molnar et al., 2003). Changes in the dominant tumor phenotype is an important consequence of chemotherapy, sensitive cells are eliminated while those resistant to a particular line of treatment survive and proliferate. Detection of K-ras and BRAF mutations in CTCs represents a non-invasive method of monitoring changes in tumor phenotype, especially with the use of cetuximab or panitumumab (Musella et al., 2015; Suhaimi et al., 2015).

CTCs detected pre-surgery do not reflect micrometastatic disease as the majority are cleared from the circulation after tumor removal. This differs from other biomarkers, Yang et al. (2011) reported that the use of serum Carcinoembryionic antigen, serum CA19-9 and CA-125 preoperatively was associated with the 5 year disease free survival. However, they could be useful in detecting colorectal cancer, whereas secondary CTCs detected after surgery would be more appropriate for prognosis.

In this study, we report the results of an inhouse immunocytochemical assay for the detection of CTCs in colorectal cancer patients. Assay development and validation (addressing technical terms such as specificity, contaminants, efficiency, sensitivity and simple quality) have been extensively explained elsewhere (Albuquerque et al., 2012a; 2012b). By applying simple routine immunocytochemistry, the implementation costs were minimal as equipment and reagents are part of the routine histocytochemical laboratory of a general hospital. No enrichment or detection method has yet proven to be the gold standard and continuing efforts are needed to improve the reliability of CTC detection techniques. The CellSearch® system has been validated via multicentre studies and is the only FDA approved device for enumeration of CTCs, its use presents some limitations that may be crucial for elucidating and optimizing the use of CTCs in cancer management (Hayes et al., 2008). The limitations of our study must be considered, with a relatively small study population which may influence result interpretation.

In this small population of CRC patients the use of in house routine low cost technology can be used to detect CTCs. They are associated with the clínico-pathological parameters only after surgical removal of the primary tumor; secondary CTCs might be important in identifying patients at high risk of relapse. Primary CTCs detected before surgical removal are frequently found, are not associated with the clínico-pathological parameters and might have a role in cancer screening.

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