RESEARCH ARTICLE

Diagnostic Yield of Primary Circulating Tumor Cells in Women Suspected of Breast Cancer: the BEST (Breast Early Screening Test) Study

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Abstract

Purpose: To determine the diagnostic yield of primary circulating tumor cells in women with suspicion of breast cancer, detected as a result of an abnormal mammography. Materials and Methods: Consecutive women presenting for breast biopsy as a result of a mammogram BiRADs of 3 or more, had an 8ml blood sample taken for primary circulating tumor cell (CTC) detection. Mononuclear cells were obtained using differential gel centrifugation and CTCs identified using standard immunocytochemistry using anti-mammoglobin. A test was determined to be positive if 1 CTC was detected. Results: A total of 144 women with a mean age of 54.7 ± 15.6 years participated, 78/144 (53.0%) had breast cancer on biopsy, 65/140 (46.3%) benign pathologies and 1(0.7%) non-Hogkins lymphoma. Increasing BiRADs scores were associated with increased cancer detection (p=0.004, RR 1.00, 4.24, 8.50). CTC mammoglobin positive had a sensitivity of 81.1% and specificity of 90.9%, with positive and negative predictive values of 90.9% and 81.1% respectively. Mammoglobin positive CTCs detected 87% of invasive cancers, while poorly differentiated cancers were negative for mammoglobin. Only 50% of in situ cancers and none of the intraductal cancers had CTCs detected. Menopausal status did not affect the diagnostic yield of the CTC test, which was higher in women with BiRADS 4 mammograms. There was a significant trend (p<0.0001 Chi squared for trends) in CTC detection frequency from intraductal, in situ and invasive (OR 1.00, 8.00, 472.00). Conclusions: The use of primary CTC detection in women suspected of breast cancer has potential uses, especially with invasive cancer, but it failed to detect intra-ductal cancer and 50% of in situ cancer. There was no difference in the diagnostic yield between pre and post menopausal women. To confirm its use in reducing biopsies in women with BIRADs 4a mammagrams and in the detection of interval invasive breast cancer, larger studies are needed.

Keywords: Breast cancer detection - circulating tumor cells - mammogram - mammaglobin - BiRADS

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Introduction

Breast screening is performed in women without any signs or symptoms of breast cancer so that the disease can be detected as early as possible. The 2014 NCCN guidelines recommend annual mammographic screening after the age of 40 years (NCCN, 2014). Randomized clinical trials have shown that annual screening lowers the mortality rate from breast cancer (Barton et al., 1999, Nemec et al., 2007), with a reported sensitivity of 75% (Carey et al., 2004). The latest Cochrane database system review of 2013 (Gotzsche et al., 2013), reported that screening trials did not show a significant reduction in breast cancer mortality at 13 years and that total numbers of lumpectomies and mastectomies were significantly higher in the screened groups. They estimated that for every 2000 women screened for breast cancer, one would avoid dying of the disease but that 10 healthy women would have been treated unnecessarily. This may be due, in part, to interval cancers, those cancers diagnosed after a negative screening mammogram and before the subsequent mammogram. Interval cancers have been shown to occur in 27.7% of women aged 40-49 years and 13.9% of women older than 50 years within 12 months, or in 52.1% of women aged 40-49 years and 24.7% of women older than 50 years when the screening interval was 24 months (Buist et al., 2004). The lower mammographic sensitivity in women with dense breasts and rapid tumor growth being explanations for this failure to detect breast cancer. Thus, the use of complementary tests may improve these figures; one possible candidate is the use of circulating tumor cells.

Ashworth first reported circulating tumor cells in a patient with breast cancer in 1869 (Ashworth, 1869), however it is only in the last few decades that the technology has been available to detect these cells. Published reports have shown that circulating tumor cells (CTCs) are associated with a decreased overall survival

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in metastatic breast cancer (Cristofanilli et al., 2004, 2006), and decreased disease free survival and overall survival in non-metastatic breast cancer (Xenedis et al., 2009). They are a prognostic factor (Tarhan et al., 2013; Turker et al., 2013). However, there are no data published of their detection in women with suspicion of breast cancer. We present a prospective study of Chilean women presenting for a breast biopsy based on the result of an abnormal mammographic screening. We used differential gel centrifugation to obtain mononuclear cells and standard immunocytochemistry with anti-mammaglobin monoclonal antibodies. Results of the detection of CTCs were compared with the results of the core biopsy, to obtain the diagnostic yield.

Materials and Methods

The study was carried out between October 2011 and June 2012, in the Hospital de Carabineros de Chile (HOSCAR), biopsy specimens were analyzed in the Pathology Service of HOSCAR and the detection of CTCs was performed at the Instituto de Bio-Oncology, Santiago, Chile. Both the pathologist and immunocytochemist were blinded to the clinical details and the results of the biopsy or CTC test. 144 women with an abnormal mammogram, defined as BiRADs 4 or 5, or a BiRADS 3 where the patient and/or treating physician requested a biopsy participated in the study. These patients had been evaluated by the Breast Cancer Committee of HOSCAR and were deemed to fulfill criteria for a breast biopsy, it was not the purpose of the study to validate the criterios used to recommend a biopsy. Core biopsies were obtained under ultrasound control and local anaesthetic, by a single radiologist and fixed in formalin. Samples were embedded in paraffin wax, sectioned with a thickness of 3μm, rehydrated and examined using H & E staining and analyzed by a single pathologist.

Detection of CPCs: Immediately before breast biopsy, an 8ml venous blood sample was taken in a tube containing EDTA (Beckinson-Vacutainer®), samples were maintained at room temperature and processed within 48 hours. Mononuclear cells were obtained by differential centrifugation using Histopaque 1,077® (Sigma-Aldrich), washed and resuspended in 100 μL of autologous plasma. 25 μL aliquots were used to make slides (sialianized, DAKO, USA), dried in air for 24 hours and fixed in a solution of 70% ethanol, 5% formaldehyde and 25% phosphate buffered saline pH 7.4.

Anti-mammaglobin: CTCs were detected detected using a monoclonal antibody directed against mammaglobin, clone 304-1A5 (Dako, USA) in a dilution of 1:100 and identified using an alkaline phosphatase-anti alkaline phosphatase based system (LSAB2-DAKO, USA) with Vector Blue (Vector Laboratories, USA) as the chromogen according to the manufacturer´s instructions.

Positive samples underwent a second stage using anti-CD45 clone 2B11-PD7/26 (DAKO, USA) and identified with a peroxidase based sysem (LSAB2, DAKO, USA) with DAB (3,3´diaminobenzidine tetrahydrochloride) as the chromogen according to the manufacturer`s instructions.

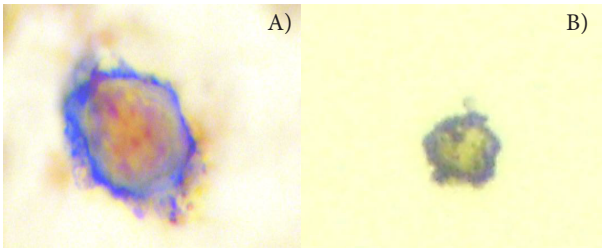

Figure 1. A) CTC Positive for Mammoglobin Stained with Vector Blue®, CD45 negative; B) Leukocyte Positive for CD45 (DAB) Negative for Mammaglobin

A CTC was defined according to the the criteria of the International Society of Hematherapy and Genetic ngineering (ISHAGE) (Borgen et al., 1999) as a cell expressing mammoglobina and negative for CD45. Leuckocytes were CD45 positive, mammoglobin negative (see Figure 1). Breast biopsies were classified as cancer or no-cancer, in the case of being positive for cáncer the biopsy was sub-classified as invasive, in-situ or intra-ductal. The result of the CTC test was classified as positive or negative, a test was considered positive if 1 cell/8ml venous blood was detected.

Analysis of the results The discrimination of the CTC diagnostic test was defined using the normal parameters. true positive (TP); false positive (FP); false negative (FN) and true negative (TN). The predictive values, positive (PPV) as well as negative (NPV) were evaluated, as well as the positive and negative likelihood ratios (+LR and -LR respectively). Comparison with the subtype of cancer, the BiRADS score and menopausal status, defined as natural o surgical cesation of menstruation with an elevated serum level FSH.

Statistical Analysis Descriptive statistics were used for demographic variables, expressed as mean and standard deviation in the case of continuous variables with a normal distribution. In case of an asymmetrical distribution the median and interquartile range (IQR) values were used. Noncontiguous variables were presented as frequencies. The Shapiro-Wilk test was used to determine a normal distribution. The Student T-Test was used to compare continuous variables with a normal distribution, the Mann-Whitney test for ordinate and continuous variables with a non-normal distribution and Chi-squared for the differences in frequency. The diagnostic yield for the test detecting CTCs was analyzed using standard parameters. For this purpose patients were classified as having or not having breast cancer. Statistical significance was defined as a p value less than 0.05 to two-sided. Analysis was performed using the Stata 11.0 program (StataCorp LP, College Station, Texas, USA).

Results

144 women with a mean age of 54.7±15.6 years participated; 78/144 (53.0%) had breast cancer diagnosed on biopsy; 65/144 (46.3%) had benign pathologies diagnosed and 1 (0.7%) was diagnosed as having Non-Hodgkin´s Lymphoma. Of the breast cancers detected, 6/78 (7.7%) were classified as intraductal, 12/78 (15.4%) as in-situ and 60/78 (76.9%) as invasive. Women positive

for breast cáncer were significantly older than those with a negative biopsy; 59.2±12.9 years versus 48.7±16.4 years (p=0.004 T-Test). An increasing BiRADS score was associated with an increasing frequency of cancer detection (Table 1), with a relative risk of 1.00, 4.24 and 8.50 for BiRADS 3, 4 and 5 respectively (p=0.004 Chi squared for trends).

Mammaglobin positive primary circulating tumor cells:

64/144 (52.7%) of all patients had mammoglobin positive CTCs detected, the association with the breast biopsy is shown in Table 2.

The diagnostic yield for primary CTCs positive for mammaglobin with an estimated cancer prevalence of 54.17% (95% CI 45.66-62.31 is shown in Table 3.

CTC detection in different subtypes of breast cancer

CTC detection varied depending on the sub-type of breast cancer, there was a significant trend in the presence of CTCs with increasing invasive type (p<0.0001 Chi squared for trends), with a relative risk of 1.00, 8.00 and 472.0 for intra-ductal, in-situ and invasive cancers respectively CTCs being detected in 0/6 intraductal, 6/12 in-situ and 52/60 invasive cancers. The detection of CTCs has the potential to detect invasive cancer but not in-situ or intra-ductal cancer. The use of mammaglobin as a marker for CTCs failed to detect poorly differentiated invasive tumors. Comparing the utility of CTC detection in invasive cancer patients versus other patients, 52/60 (87%) of invasive cancers would have been detected versus 6/84 (7%) in the remaining patients (p<0.0001).

Detection of primary CTCs in patients with Bi-RADS 4 and Bi-RADS 5 mammograms

There was no significant difference in the frequency of false positives or false negatives with respect to the presence or absence of CTCs in patients with/without cancer in patients with BiRADS 4 and BiRADS 5 mammography; 3/36 versus 8/38 (p=0.12) and 1/38 versus 0/12 (p=0.76) respectively (Table 4).

Comparing the diagnostic yield for BiRADS 4 and 5 patients, the cancer prevalence for BiRADS 4 was 48.65% (95% CI 36.85-60.56) was lower than that for BiRADS 5 patients with a cancer prevalence of 76% (95% CI 61.83-86.93). However, the diagnostic yield was better for BiRADS 4 patients using mammaglobin CTCs (Table 5) for specificity and NPV, there was no difference between the sensitivity and PPV of the CTC test in patients with BiRADS 4 and 5 mammographs.

Detection of CTCs according to BiRADS and menopausal state

58 women were defined pre-menopausal and 82

Table 1. Association of BiRADS Score with Breast Cancer

BiRADS score	Nº Patients	Nº Cancer detected (%)	Relative Risk
3	20	4/20 (20%)	1.00
4	74	36/74 (46%)	4.24
5	50	38/50 (76%)	8.50
Total	144	78/144 (54%)	p=0.004

Table 2. Association between the Presence of CTCs and Biopsy Findings

	Breast Bippsy (+)	Breast biopsy (-)	Total
CTC (+)	58	6	64
CTC (-)	20	60	80
Total	78	66	144

Table 3. Diagnostic Yield of CTCs Positive for Mammaglobin

	Value	95% Confidence Interval
Sensitivity	74.4%	63.2-83.6%
Specificity	90.0%	81.3-96.6%
PPV	90.6%	80.7-96.5%
NPV	75.0%	64.1-84.0%
LR (+)	8.18	3.77-17.74
LR (-)	0.28	0.19-041

PPV=positive predictive value; NPV=negative predicitve value; LR(+)=positive likelihood ratio: LR(-)=negative likelihood ratio

Table 4. Detection of CTCs in Patients with BiRADS 4 and BiRADS 5 Mammograms

	BiRADS 4			BiRADS 5		
	Biopsy (+)	Biopsy (-)	Total	Biopsy (+)	Biopsy (-)	Total
CTC (+)	33	1	34	30	0	30
CTC (-)	3	37	40	8	12	20
Total	36	38	74	38	12	50

Table 5. Diagnostic yield of CTC detection in patients with BiRADS 4 and BiRADS 5

	BiRADS 4		BiRADS 5	
	p-value	95%CI	p-value	95% CI
Sensitivity	91.7%	77.5-98.2%	79.0%	62.7-90.4%
Specificity	97.4%	86.1-99.6%	100.0%	73.4-100%
PPV	97.1%	84.6-99.5%	100.0%	88.3-100%
NPV	97.5%	79.6-98.3%	60.0%	36.1-80.8%
LR(+)	34.8	5.0-242	N/C	
LR(-)	0.09	0.03-0.25	0.21	0.11-0.39

*PPV=positive predictive value; NPV=negative predictive value;LR(+)=positive likelihood ratio; LR(-)=negative likelihood ratio

Table 6. Patient Details According to Menopausal and BiRADS Classifiaction

Pre-menopause	Nº Patients	CTC (+)	Nº Cancer (%)	Post-menopause	Nº Patients	CTC (+)	Nº Cancer (%)
BiRADS 3	6	0	0 (%)	BiRADS 3	14	0	4/14 (29%)
BiRADS 4	34	13	14/34 (41%)	BiRADS 4	40	21	22/40 (55%)
BiRADS 5	20	14	16/20 (80%)	BiRADS 5	30	16	22/30 (73%)
Total	58	27	30/58 (52%)	Total	82	37	48/82 (59%)

women post-menopausal.

The details of these women, the BiRADS score, number of women CTC positive and with cancer are shown in Table 5.

30/58 (52%) of premenopausal women had breast cancer compared with 48/82 (59%) of post-menopausal women (p=0.42), there was no significant differences in the frequency of cancer detected between women pre and post-menopausal in the BiRADS catorgories of 3, 4 and 5. (p=0.20, 0,33 and 0,58 respectively). Nor was there a difference in the frequency of CTC detection of the two groups as a whole 27/58 (47%) versus 37/82 (45%) (p=0.87).

Discussion

Currently there are a number of analytical methods for isolating and detecting CTCs, using a combination of two steps; firstly isolation and enrichment of mononuclear cells which includes the CTCs and secondly their detection. There is a substantial variability in the rates of samples positive for CTCs using existing isolation and detection methods and as such the clinical results may differ. Although most of these methods are highly specific and sensitive, there are no large studies comparing different methods when using the same clinical samples (Lianidou et al., 2011, 2011a). The pitfalls of the differing detection methods has been extensively reviewed by Panteleakou et al (2009). However, the presence and detection of CTC would appear to be rarer in early breast cancer when using EpCAM based systems, however prospective randomised study data is limited in these patients.

CellSearch (Veridex, LLC, Raritan, NJ) is a widely used semi-automatic commercial system that relies on immunomagnetic capture of CTCs using epithelial cell adhesion molecule (EpCAM) which is expressed on the surface of epithelial malignancies, followed by positive selection with cytokeratin and negative selection of leukocytes. The widely accepted concept that all cytokeratin and/or EpCAM positive, CD45 negative cells with a nucleus in cancer pacients are circulating tumor cells (CTCs) has imposed a clear bias on the study of CTCs. Mainly the failure to include tumor cells that have reduced or absent cytokeratin and/ or EpCAM expression and the failure to identify such cell types limits investigations into additional tumor types. EpCam is expressed in most but not all tumors (Went et al., 2004), there is downregulation with cancer progression and metastasis, cytokeratins are heterogeneously expressed in tumor cells and also may be down regulated during disease progression or in poorly differentiated tumors. During the progression of epithelial to mesenchymal transition both markers are downregulated (Paterlini-Brechot et al., 2007); EpCAM may be down regulated to allow tumor cell dissociation from the epithelium and cytokeratin downregulated to facilitate tumor cell plasticity and migration (Raimondi et al., 2011). The incidence of CTC detection in breast cancer was reported to be 12-50% in the early setting and 25-80% in advanced and metastatic settings depending on the methods used in different clinical studies (Riethdorf S et al.,2008; Franken et al.,2012). Using the CellSearch system ≥1 CTC was detected in 15% of benign tumors, 19% of in-situ tumors and 19% of invasive cancers (Franken et al., 2012).

To try to avoid the posible decreased EpCAM expression in primary CTCs we used anti-mammaglobin to identify these cells. Previously published studies have shown that mammaglobin CTCs are only detected in invasive breast cancer (Ferro et al., 2010), is the most specific marker for breast cancer (Li et al., 2011), however there is a variability in the frequency of detection between 14%-54% (Ntoulia M et al., 2006, Chen et al., 2010). The majority of these studies have used RT-PCR to detect CTCs and not immunocytochemistry.

We chose differential gel centrifugation and standard immunocytochemistry as an analytical method for its relatively low cost, that it could be implemented in a routine immuncytochemical laboratory of a general hospital, without the need for capital investment in high technology equipment and/or training of laboratory staff. Pre-analytical variables such as sample taking, conditions of transport to the laboratory are factors which are controlled and reproducible. Isolation and enrichment of the mononuclear cell fraction are variables which are reproducible using differential gel centrifugation with a >80% cell retrieval rate. The main variable is the manual method of detection and observer variability. This variability occurs in the interpretation of the mammogram by the radiologist and that of the core biopsy by the pathologist. With adequate training we consider that this variability can be reduced to a minimum.

The test was designed as a sequential test to mammographic screening, being used as a complementary test in women with suspicion of breast cancer. It was designed to give a positive/negative answer, with a positive test being the detection of one CTC in 8ml of venous blood, giving the treating physician an answer to the question "does the patient need a biopsy?"

Accepting the limitations of our study, of the small numbers of intra-ductal and in-situ tumors as well as being a single centre study, we are able to draw some conclusions; firstly that primary CTCs are detected in the majority of invasive cancers but not non-invasive cancers. Thus the test does not answer the fundamental question of biospy or no biopsy, and could not be used to exclude patients from this diagnostic procedure, especially as in-situ cancers represent approximately 18% of all diagnosed breast cancers (Lynge et al., 2014).

In-situ tumors have been classified as low, intermediate and high grade depending on the morphological characteristics. Although high grade in-situ cancer is considered to have a greater propensity to recur or progress, several studies indicate that in situ cancers of all grades has a similar potential to progress to invasive disease, but that high grade lesions are likely to progress more rapidly and lead to metastatic disease and death (Badve et al., 1998). Among the women who developed invasive breast carcinoma, after a mis-diagnosis of benign disease and not cancer in-situ, the mean time to the development of detected invasive carcinoma was 9.0 years after the initial "benign" breast biopsy was performed (range, 4-18 years) (Collins et al., 2005). The

numbers of in situ cancers in our study is too small to draw conclusions, but it is possible that CTCs only detect high grade in situ tumors, this warrants further investigation.

In BiRADS 4 patients 70% of the biopsied lesions are benign (Orel et al., 1999), thus decreasing the number of these biopsies is important, however not at the cost of missing clinically significant cancers. In the group of patients studied, 51% of BiRADS 4 had benign pathologies, nearly all CTC positive patients had cancer found on biopsy, but 8% of CTC negative patients also had cáncer, 1 of which was invasive. The number of patients was too small to permit an analysis of the subtypes of BiRADS 4 patients, an expanded study of the differing diagnostic yields in the subtypes of BiRADS 4 patients may be warranted, especially the results in patients classified as 4a and 4b.

The use of primary CTC detection may be useful in the detection of interval cancers, especially in high risk patients, as determined by the modified McGail model (NCCN, 2014). This may be especially so when mammographic screening is every two years. Detection of primary CTCs between screenings would be used to prompt re-evaluation of the patient. This could detect the fast growing invasive tumors. Slow growing intra-ductal or in situ cancers would not be detected, but hopefully detected at the next screening mammogram.

In conclusion, as a sequential test to detect breast cáncer, the test failed to detect intra-ductal and in-situ cancer; in patients with BiRADS 4 mammography there may be a place in subtypes 4a and 4b to avoid the need for breast biopsy, and in the detection of inter-interval screening invasive breast cancer. This would need additional studies with a greater number of patients..

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