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Molecular detection of cancer cells in bone marrow and peripheral blood of patients with operable breast cancer. Comparison of CK19, MUC1 and CEA using RT-PCR

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Abstract

We have compared three different RT-PCR procedures to measure cytokeratin 19 (*CK19*), carcinoembryonic antigen (*CEA*) and mucin *MUC1* gene expression in order to determine their diagnostic value in detecting tumour cells in bone marrow aspirates of patients with operable breast cancer. In an experimental model, the best sensitivity was observed for *CK19* and *MUC1* RT-PCR assays, although only the *CEA* and *CK19* assays showed good specificity. The study of 42 patients showed that a '*CK19* positive/*CEA* positive' RT-PCR assay in bone marrow correlated positively with a positive axillary lymph node status (N_0 versus N_{1-3}, $P < 0.05$). Both assays were also positive in 17% of node negative patients. RT-PCR assays were more sensitive in bone marrow than in peripheral blood. Our results suggest that *CK19* and *CEA* RT-PCR assays are powerful methods for detecting disseminated breast cancer cells. A larger study with long-term follow-up is required in order to clarify their clinical usefullness.

Keywords: Breast cancer; Micrometastatic cells; Bone marrow; RT-PCR; *CEA*; *Cytokeratin 19*; *MUC1*

1. Introduction

Accurate staging of patients diagnosed with breast cancer is important to determine the extent of disease and to plan appropriate therapies. In the last decade there have been remarkable changes in breast cancer treatment. Breast cancer surgery has become more conservative, and an increased number of patients are receiving systemic therapy. The decision to treat breast cancer patients with either chemotherapy or hormonal therapy relies on the tumour size, the nodal status and other prognostic factors. The major problems for prediction of patient's relapse include the heterogeneity of the tumour and the tendency of the disease to spread early. Although 90% of the patients are apparently free of metastases at the time of primary surgery, almost 50% of them will relapse 5 years later [1]. The skeleton represents the most frequent site of metastases caused by breast cancer. These occur in more than 80% of the patients diagnosed with metastatic breast cancer and the skeleton is frequently the first metastatic site to be detected [2]. Therefore, early examination of bone marrow might be helpful in the diagnosis of occult spread of disease.

Using conventional histological methods, disseminated tumour cells are found in only a few cases. The addition of immunohistological methods using monoclonal antibodies (MAbs) directed against surface antigens or cytokeratins has led to improvements in the ability to detect breast cancer cells in bone marrow aspirates and biopsy samples [3]. More recently, the use of polymerase chain amplification to identify cell-specific

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messenger RNA (RT-PCR) has become a highly sensitive tool in the detection of minimal involvement of the blood and bone marrow in a variety of malignancies, including melanoma [4], neuroblastoma [5], prostate cancer [6] and breast cancer [7]. The choice of the messenger RNA (mRNA) target for amplification is determined by the specific characteristics of the malignant cells, but it should not be expressed in bone-marrow cells, lymph node cells or peripheral blood cells. Noguchi and colleagues [8] reported that the expression of the gene coding for the mucin *MUC1* is suitable for the detection of disseminated breast cancer cells in lymph nodes. Gerhard and colleagues [9] demonstrated that the *carcinoembryonic antigen* (*CEA*) is a good target gene for detecting abdominal cancer or breast cancer cells in the bone marrow. Several workers agree that the detection of *cytokeratin 19* (*CK19*) mRNA by RT-PCR is a useful test in order to find a few disseminated tumour cells in the bone marrow of patients with breast cancer [10,11]. We have shown that RT-PCR for *CK19* is a reliable procedure that detects up to one human breast cancer T47D cell per 10^6 peripheral blood mononuclear (PBMN) cells, with higher sensitivity (and the same high specificity) as two different CK19 immunostaining methods [12].

To evaluate if measurement of these three different mRNAs by different mRNA by RT-PCR assays (*MUC1*, *CEA* and *CK19*) have a similar diagnostic value in detecting the presence of disseminated breast cancer cells we compared these procedures in reconstitution experiments that mimic clinical samples, as well as in bone marrow and peripheral blood samples obtained from breast cancer patients before the operation.

2. Patients and methods

2.1. Cell samples

The human mammary carcinoma cell line T47D was cultured in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% bovine fetal serum (FBS), 2 mM L-glutamine and 1 mM pyruvate. We harvested the T47D cells grown in monolayer by washing the dishes once with phosphate-buffered saline (PBS) and then incubating the cells with PBS containing 0.53 mM EDTA and 0.05% trypsin (Gibco, Grand Island, USA) for 5 min at 37°C. After washing in PBS, the cells were passed into syringes (25 G 5/8 needle) to dissociate them. PBMN cells were obtained from blood samples collected from healthy donors. Samples were first centrifuged at 1000 *g* for 5 min and the buffy coats removed into fresh tubes. The contaminating red blood cells (RBC) were lysed by resuspending the cells in a RBC lysis buffer (150 mM ammonium chloride, 10 mM potassium bicarbonate, and 0.1 mM EDTA) followed by gentle agitation for 10 min. Cells were counted and viability assessed by trypan blue exclusion.

2.2. Patients and clinical samples

Clinical evaluation was performed in 42 patients with histological diagnosis of operable breast cancer at different stages. The study was examined and approved by the ethical review boards of the Hospital de Clínicas of Montevideo and Sindicato Médico del Uruguay. After they were informed of the purpose, risks and experimental nature of the study, the patients signed a form consenting to the procedure. All patients were screened for metastases by conventional staging (chest X-ray, liver ultrasound, blood tests and bone scan in patients with stage II or III disease). 5 ml samples of bone marrow aspirates were collected by sternal and iliac crest punctures, under anaesthesia before surgery, in EDTA anticoagulant and quickly sent to the laboratory. Additionally, we took a sample of peripheral blood cells under the same conditions. Each sample was processed independently. The blood red cells were lysed as described above and the cells were recovered by centrifugation at 1000*g* for 5 min, washed in PBS, counted and recentrifuged. The cells were resuspended directly into Tri-Reagent (Sigma Chemical Co., St Louis, IL, USA) and total RNA was extracted according to the manufacturer's instructions. As a positive control, RNA was also prepared from the human breast carcinoma T47D cell line.

2.3. Reverse transcriptase polymerase chain reactions

First-strand cDNA was synthesised by using MMLV reverse transcriptase (Amersham) as previously reported [12]. Four different PCR reactions, with the respective negative controls, were performed with each sample in order to amplify fragments of *CK19*, *MUC1*, *CEA* and *β-actin* (a positive control used to indicate that the extraction of an intact mRNA and an adequate cDNA synthesis had taken place in each sample). The sequences of primers utilised (synthesised by Genset, Paris, France) are shown in the Table 1. These primers extend across at least an intron, thus the DNA-derived product was easily distinguished from that expected from amplification of mRNA and, therefore, an eventual DNA contamination would not pose a significant problem. The *CK19* was evaluated by nested PCR as described by Datta and colleagues [10], the only difference was that the second round of PCR was decreased at 30 cycles. The fragment of *MUC1*, was amplified as described previously by Noguchi and colleagues [8]. The *CEA* mRNA was assayed by nested RT-PCR according to the procedure reported by Gerhard and colleagues [9]. The conditions for *β-actin* PCR were the same as for the *MUC1* PCR. 10 μl of all PCR products were

Table 1
Primer sequences

Gene	5′-3′ sequence	Size of PCR product (bp)
Cytokeratin 19 (outer)	AAGCTAACCATGCAGAACCTCAACGACCGC TTATTGGCAGGTCAGGAGAAGAGCC	1069
Cytokeratin 19 (inner)	TCCCGCGACTACAGCCACTACTACACGACC CGCGACTTGATGTCCATGAGCCGCTGGTAC	745
CEA (outer)	TCTGGAACTTCTCCTGGTCTCTCAGCTGG TGTAGCTGTTGCAAATGCTTTAAGGAAGAAGC	160
CEA (inner)	TGTAGCTGTTGCAAATGCTTTAAGGAAGAAGC GGGCCACTGTCGGCATCATGATTGG	131
MUC1	CGTCGTGGACATTGATGGTACC GGTACCTCCTCTCACCTCCTCCAA	288
β-actin	CTCTTCCAGCCTTCCTTCCT AGCACTGTGTTGGCGTACAG	116

electrophoresed on 2% agarose gels and analysed by direct visualisation after ethidium bromide staining.

2.4. Statistical analysis

Correlations between various clinicopathological parameters (stage, tumour size and nodal status) and RT-PCR assays in bone marrow were analysed by Chi square tests.

3. Results

3.1. Sensitivity and specificity of each RNA-based diagnosis

To establish the limits of sensitivity and specificity of the three RT-PCR assays for tumour cell detection, T47D cells were seeded into different tubes containing PBMN cells. A total of 10 million cells were present in each sample at tumour cell — haematopoietic cell ratios ranging from $1:10^3$ to $1:10^7$, thus mimicking the clinical setting for detection of mammary cells in patient's peripheral blood or bone marrow. An additional 20 non-seeded PBMN specimens obtained from different healthy controls were processed. Representative results of a RT-PCR positive assay for each mRNA are shown in the Fig. 1. The comparison of results obtained with the three RT-PCR procedures evaluated for detection of T47D cells, performed in five series of experiments, are shown in Table 2. The highest sensitivity observed corresponded to the identification of one breast cancer cell amongst 1×10^6 PBMN cells using RT-PCR for *MUC1* (in 4/5 samples evaluated) or *CK19* (in 3/5 samples). When the RT-PCR was performed for *CEA* a minor sensitivity was observed (identification of one T47D cell amongst 5×10^5 PBMN cells in 2/5 samples evaluated). Regarding the evaluation of specificity, the RT-PCR assay for *MUC1* showed false-positive results in 12/20 samples obtained from healthy controls. In contrast, none of these 20 control samples were positive for *CK19* or for *CEA* by RT-PCR. Consequently, RT-PCR assays for *CK19* and for *CEA* were chosen for the subsequent evaluation of clinical samples.

Fig. 1. RT-PCR products for each mRNA evaluated. Lane 1, *CK19* (745 bp); lane 2, *MUC1* (288 bp); lane 3, *CEA* (131 bp); lane 4, molecular markers (100 bp DNA Ladder, Gibco BRL); and lane 5, *β-actin* (116 bp).

Table 2
Comparison between the different RT-PCR procedures for carcinoma cell detection

Sample composition			RT-PCR[a]		
T47D	PBMN	Relation T47D/PBMN	CK19	CEA	MUC1
10^6	0		5/5	5/5	5/5
10^4	10^7	1/1000	5/5	5/5	5/5
10^3	10^7	1/10000	5/5	5/5	5/5
200	10^7	1/50000	5/5	5/5	5/5
100	10^7	1/100000	5/5	4/5	5/5
20	10^7	1/500000	5/5	2/5	4/5
10	10^7	1/1000000	3/5	0/5	4/5
5	10^7	1/2000000	0/5	0/5	0/5
0	10^7		0/20	0/20	12/20

[a] Results are number of positive tests/number of tests.

3.2. Analysis of bone marrow samples

We tested 66 bone marrow aspirates of 42 patients with operable breast cancer. The β-actin RT-PCR positive controls gave strong signals in all cases, thus both RNA preparation and cDNA synthesis were successful. Results obtained by RT-PCR assays for each bone marrow aspirate are shown in Table 3. The presence of RT-PCR positive assays was observed in 20/42 patients (48%) for *CK19* and in 12/42 patients (29%) for *CEA*. All assays *CEA* positive were also *CK19* positive, but in 8 patients *CK19* was the only RT-PCR positive test. Correlations between the *CK19* and *CEA* assays in bone marrow and various clinicopathological parameters are shown in Table 4. When the RT-PCR assays were '*CK19* positive' it was not possible to observe a positive correlation with any of the parameters evaluated (stage, tumour size and nodal status). In contrast, when the RT-PCR assays were '*CK19* positive/*CEA* positive' a positive correlation was observed with a positive axillary lymph node status (N_0 versus N_{1-3}, $P=0.05$). Interestingly, the RT-PCR assays were '*CK19* positive/*CEA* positive' in 4/24 (17%) of node-negative patients.

Table 3
Characteristics of patients and RT-PCR assays in bone marrow aspirates. (+) positive assay, (−) negative assay, (ND) not-determined

Patient	Stage	CK19 assay		CEA assay	
		Sternal	Iliac crest	Sternal	Iliac crest
1	I	−	−	−	−
2	I	+	ND	−	ND
3	I	−	−	−	−
4	I	+	−	+	−
5	I	−	ND	−	ND
6	I	+	−	−	−
7	I	+	ND	−	ND
8	I	+	−	+	−
9	I	−	ND	−	ND
10	I	−	−	−	−
11	I	−	−	−	−
12	I	−	ND	−	ND
13	I	+	+	−	−
14	I	+	+	−	−
15	IIa	−	−	−	−
16	IIa	−	−	−	−
17	IIa	+	+	−	+
18	IIa	−	−	−	−
19	IIa	−	ND	−	ND
20	IIa	−	ND	−	ND
21	IIa	−	ND	−	ND
22	IIa	+	+	+	+
23	IIa	+	−	+	−
24	IIa	+	ND	+	ND
25	IIa	−	ND	−	ND
26	IIa	−	−	−	−
27	IIa	+	+	+	+
28	IIa	−	ND	−	ND
29	IIa	−	+	+	+
30	IIa	−	ND	−	ND
31	IIa	−	ND	−	ND
32	IIa	+	−	−	−
33	IIa	−	+	−	−
34	IIb	+	ND	+	ND
35	IIb	+	ND	+	ND
36	IIb	−	+	−	−
37	IIb	−	ND	−	ND
38	IIb	−	−	−	−
39	IIb	−	ND	−	ND
40	IIIa	+	ND	+	ND
41	IIIa	−	−	−	−
42	IIIa	+	+	+	−

We have obtained both sternal and iliac bone marrow aspirates from 24 patients (14 patients positive for the *CK19* assay and 8 patients positive for the *CEA* assay). In order to determine whether aspirates obtained from one versus two different sites in the same patient influence the RT-PCR assay results for the detection of epithelial cells, we have evaluated, in these patients, the distribution of *CK19* and *CEA* positive assays (Table 5). For both RT-PCR procedures we observed more frequently positive results in sternal bone marrow aspirates compared with iliac aspirates. However, RT-PCR assays were positive only in iliac aspirates in 3/14 patients (21%) for *CK19* and in 1/8 patients (13%) for *CEA*, indicating that the best sensitivity could be obtained when bone marrow aspirates are taken from both sites simultaneously.

3.3. Patient blood analysis

RT-PCR assays to evaluate the expression of mRNA for *CK19* and *CEA* were performed in peripheral blood samples obtained at the same time as bone marrow aspirates from 37 of 42 patients included in this work. RT-PCR was positive in 13/37 (35%) blood samples for *CK19* and in 1/37 (3%) blood sample for *CEA* (also positive for *CK19*). As shown in Fig. 2, most blood RT-PCR positive assays (10/13; 77%) were also positive in bone marrow aspirates from the same patient. In 3 cases the RT-PCR assay was positive only in the blood

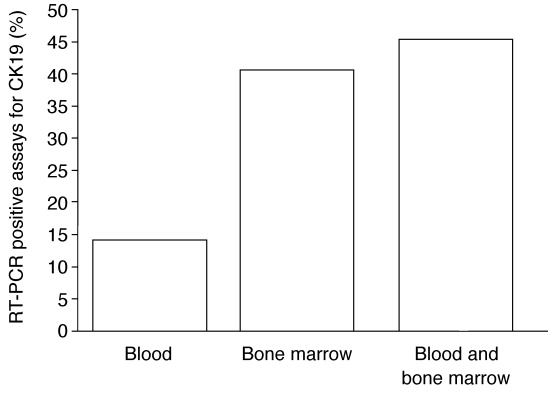

Fig. 2. Comparison of detection rate of *CK19* in peripheral blood or bone marrow aspirates of patients with operable breast cancer.

Table 4
Correlation between stage of disease, tumour size, nodal status and RT-PCR assays

Factor evaluated	*n*	CK19 negative CEA negative	CK19 positive CEA negative	CK19 negative CEA positive *n* (%)	CK19 positive CEA positive	CK19 positive and/or CEA positive
Stage						
I	14	7 (50)	5 (36)	0	2 (14)	7 (50)
IIa	19	11 (58)	2 (11)	0	6 (32)	8 (42)
IIb	6	3 (50)	1 (17)	0	2 (33)	3 (50)
IIIa	3	1 (33)	0	0	2 (67)	2 (67)
Tumour size						
T1	23	11 (48)	6 (26)	0	6 (26)	12 (52)
T2	17	10 (59)	2 (12)	0	5 (29)	7 (41)
T3	2	1 (50)	0	0	1 (50)	1 (50)
Nodal status						
N−	24	14 (58)	6 (25)	0	4 (17)	10 (42)
N+	18	8 (44)	2 (11)	0	8 (44)	10 (56)

sample, but in 9 patients the test was positive only in bone marrow aspirates (making a total of 19/37; 51%).

4. Discussion

Detection of small numbers of breast cancer cells in patient blood and bone marrow has become increasingly important. The ability to rule out the presence of micrometastatic disease at any stage of the clinical management protocol, whether before, during, or after therapy, would provide a useful monitoring and diagnostic tool. In the past few years it has been reported that the RT-PCR is a method with high sensitivity and could be applicable to the diagnosis of minimal residual disease in cancer patients [7,13]. Here we have compared three different RT-PCR procedures (detection of *CEA*, *MUC1* and *CK19* mRNAs) in order to determine the limits of sensitivity and specificity of these three assays for human breast tumour cell detection, as well as whether the presence of 'epithelial' mRNAs in bone marrow aspirates of patients with operable breast cancer correlates with clinicopathological parameters.

When the sensitivity was evaluated, *CK19* and *MUC1* assays were the most sensitive (up to one T47D cell per 10^6 PBMN cells) followed by the *CEA* assay (up to one T47D cell per 5×10^5 PBMN cells). Regarding the specificity, we found the same high specificity (100% in PBMN cells obtained from 20 healthy controls) for both *CK19* and *CEA* assays. Our results dealing with the specificity of these RT-PCR assays agree with previous studies where a lack of *CK19* nor *CEA* expression has been reported in bone marrow or peripheral blood cells [10,14]. These results differ from the findings reported by Burchill and colleagues [15] who identified *CK19* expression in 6/15 blood samples analysed from healthy controls. These apparent contradictions could be explained by the different experimental conditions used to detect *CK19* expression. In this study, a band of 745 bp was amplified by nested PCR whereas Burchill and colleagues identified a band of 214 bp after one round of PCR. Recently, Jung and colleagues have reported that neither *CEA* nor *CK19* messenger RNAs could be amplified from bone marrow samples of healthy subjects. In contrast, the authors have observed that the transcription of both genes could be induced in haemopoietic tissues under certain conditions, e.g. inflammatory diseases [16]. Inappropriate transcription of epithelial-specific genes in haematopoietic cells or the presence of pseudogenes could be factors limiting the specificity of RT-PCR assays [17]. We have utilised here the primers published by Datta and colleagues which were designed to avoid amplification of *CK19* pseudogenes [10]. However, the use of a reduced number of cycles (5 less) in the second round of the nested procedure improved the specificity of the original protocol [12]. A quantitative methodology could improve the specificity of the RT-PCR assay for detection of disseminated cells in patients with breast cancer [18]. Using the same RT-PCR procedure used by Noguchi and colleagues to assess *MUC1* gene expression [8] we have observed 12/20 RT-PCR positive assays in normal peripheral blood samples. However, Noguchi and colleagues have shown that this procedure was highly specific for the detection of metastatic breast cancer cells in lymph nodes. In contrast, Hoon and colleagues reported *MUC1* gene expression in seven of eight normal donor peripheral blood cells

Table 5
Distribution of RT-PCR positive assays when bone marrow aspirates were obtained simultaneously from sternum and iliac crest

Positive sample	CK19 positive assay ($n=14$) *n* (%)	CEA positive assay ($n=8$) *n* (%)
Sternal aspirate	5 (36)	4 (50)
Iliac crest aspirate	3 (21)	1 (13)
Sternal and iliac crest aspirates	6 (43)	3 (38)

and in four of eight lymph nodes obtained from patients without cancer [19]. Our results suggest that this procedure for *MUC1* gene evaluation could be non-specific for epithelial cells. However, to conclude about the definitive clinical value of RT-PCR for *MUC1*, a standardisation of methods is required in order to compare the results obtained from different laboratories.

The current study disclosed that 20 out of 42 patients who underwent curative surgery (48%) showed positive *CK19* mRNA and 12 (29%) showed positive *CEA* mRNA in bone marrow aspirates obtained prior to surgery. The fact that all *CEA* positive patients were also *CK19* positives, but in 8 patients RT-PCR assays were only positive for *CK19*, agrees with the higher sensitivity observed in the experimental model for the *CK19* assay compared with the *CEA* assay for human breast cancer cell detection. However, the best direct correlation with clinicopathological parameters (lymph node involvement) was observed when *CEA* assay was positive, suggesting that the clinical value of a *CK19* positive assay or a *CEA* positive assay is not the same. In addition, 4 out of 24 (17%) patients without involvement of the axillary nodes (N-) showed a positive result for both RT-PCR assays in the samples obtained before surgery. This finding supports the hypothesis that early tumour-cell dissemination occurs in breast cancer, as a systemic disease component. Some studies suggest an independent impact of a positive bone marrow status on unfavourable disease-free survival [20,21].

Regarding the potential clinical application of RT-PCR assays for the detection of epithelial cells, it could be of interest to know whether the site and number of bone marrow aspirates influence the results, and also whether there is a correlation with the results obtained in peripheral blood. For both *CK19* and *CEA*, we observed the highest frequency of RT-PCR positive results in sternal bone marrow aspirates compared with iliac aspirates, although the numbers obtained are small and thus limit any conclusions. The greatest sensitivity was obtained when bone marrow aspirates were obtained simultaneously from both sites. In addition, our study disclosed that the detection of disseminated carcinoma cells by RT-PCR could be more accurately evaluated on bone marrow aspirates than in peripheral blood samples. The *CK19* positive assays in peripheral blood samples (13/37 patients) correlated well with *CK19* positive assays in bone marrow (19/37), although in 9 patients the test was positive only in bone marrow samples. We detected cells expressing *CEA* in only 1/37 peripheral blood samples from patients with breast cancer. Mori and colleagues detected *CEA* mRNA in 12/62 (19%) of peripheral blood samples from patients with gastrointestinal or breast carcinomas who had undergone a curative operation. Moreover, 4/12 RT-PCR positive cases developed metastatic disease after surgery, whereas none of 50 cases that were negative by RT-PCR developed metastases [22]. However, the authors did not specify the number of breast cancer patients where the RT-PCR for *CEA* was positive in peripheral blood. Carcinoma cells may be intermittently shed into the bloodstream [23], which could result in a sampling error if a single-point sampling is evaluated. It is not yet clear whether circulating cancer cells in peripheral blood can establish tumours at other sites. Nevertheless, it is generally believed that very few cancer cells shed into the bloodstream succeed in establishing secondary tumours [24]. A more accurate molecular study on circulating cells could give information about their metastatic potential. Recently, a set of molecules was identified that may determine interactions between epithelial tumour cells and bone marrow [25].

In conclusion, we have shown that determination of *CEA* and *CK19* gene expression by RT-PCR are powerful tools for detecting human breast cancer cells in bone marrow, not only in patients with advanced disease but also in those with early disease. The clinical application of *MUC1* RT-PCR assay could be limited by its low specificity. Our results suggest that a simultaneous positive RT-PCR assay for both *CK19* and *CEA* in bone marrow aspirates could be useful in identifying patients at high risk of developing metastases. Considering that the number of patients in this study was too small for meaningful conclusions, a larger study with long-term follow-up is in progress in order to clarify the clinical usefulness of searching breast cancer cells in bone marrow using *CEA* and *CK19* RT-PCR assays.

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