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Diagnostic accuracy of small breast epithelial mucin mRNA as a marker for bone marrow micrometastasis in breast cancer: a pilot study

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Abstract
Background. Detection of isolated tumour cells (ITC) in the blood or minimal deposits in distant organs such as bone marrow (BM) could be important to identify breast cancer patients at high risk of relapse or disease progression. PCR amplification of tissue or tumour selective mRNA is the most powerful analytical tool for detection of this micrometastasis. We have evaluated for the first time, the diagnostic accuracy of small breast epithelial mucin (SBEM) as a potential marker for BM micrometastasis in breast cancer.
Methods. A nested RT-PCR assay for detection of *SBEM* mRNA was compared with immunocytochemistry (ICC) with anticytokeratin *AE1/AE3* antibody in paired samples obtained from the BM of breast cancer patients. Associations of *SBEM* mRNA detection in BM and clinical and pathological parameters were evaluated. *SBEM* mRNA status and time to breast cancer progression were analysed using Kaplan–Meyer curves.
Results. Fifty stages I–IV breast cancer female patients were prospectively included in our study. *SBEM* specific transcript was found in BM in 26% of the patients. Detection rate was similar to the percentage of patients with ITCs detected using ICC (24%). SBEM mRNA in BM aspirates were significantly associated with presence of clinically active disease, including locally advanced and metastatic patients (47%, $P = 0.021$) and tumours with positive hormonal receptors (36.7%, $P = 0.035$). In addition association with Her2/neu over-expression (44.4%, $P = 0.051$) and low proliferating tumours (36%, $P = 0.067$) were close to significant levels. When we analysed time to breast cancer progression adjusting for grade or hormone receptor status, presence of *SBEM* mRNA in BM defines distinct prognostic groups.
Conclusions. SBEM might represent a suitable marker for molecular detection of ITCs in BM in breast cancer patients. Analysis of prognostic value for *SBEM* mRNA-based assay should take into account the heterogeneity and different molecular subtypes of breast cancer.

Keywords
Isolated tumour cells; Small breast epithelial mucin Breast tumour cells Minimal disease RNA-based methods

Abbreviations
ITC, Isolated tumour cells; ICC, Immunocytochemistry; CK, Cytokeratin; RT-PCR, Reverse-transcriptase polymerase-chain reaction; SBEM, Small breast epithelial mucin; BM, Bone marrow; TTP, Time to breast cancer progression; HR, Hormonal receptors

Introduction

Breast cancer is the most common type of cancer in European women, accounting for 429,900 cases in 2006 (Ferlay et al. 2007). Relative survival from breast cancer in women has improved steadily in all European countries in recent years. However age-standardized 5-year relative survival remains within a range of 78.1–80% (Verdecchia et al. 2007). Metastatic haematogenous spreading is one of the most important factors affecting the prognosis of carcinoma patients, including breast cancer. Circulating tumour cells and occult metastasis (micrometastasis) are considered early events in the progression of breast cancer. Detection of carcinoma cells in the blood or minimal deposits in distant organs such as bone marrow (BM) could be important to identify patients at high risk of relapse or disease progression (Pantel and Alix-Panabieres 2007). A pooled analysis of data from different clinical studies found strong evidence that the presence of isolated tumour cells (ITC) in BM by means of immunocytochemical-based detection is associated with a poor prognosis in stage I–III breast cancer patients (Braun et al. 2005).

As stated previously, immunocytochemistry (ICC) with anticytokeratin (CK) antibodies would be considered as a benchmark technique for ITC detection. Thus, studies evaluating molecular methods of ITC detection and novel markers would be performed in comparison with standardized ICC (Braun and Naume 2005). PCR amplification of tissue or tumour selective mRNA is the most powerful analytical tool for detection of this circulating or micrometastatic cells. Cytokeratins and mammaglobin are among the most frequent mRNA markers used in different reverse transcriptase-polymerase chain reaction (RT-PCR) assays in breast cancer patients. However down-regulation of mRNA marker in tumour cells (Woelfle et al. 2003) or low-level transcription of selected targets in the haematopoietic compartment (Kruger et al. 2001) could compromise both sensitivity and specificity of molecular methods.

The selection of novel breast-specific transcripts and the development of multi-marker RT-PCR assays are clearly outstanding research questions. In this context we have evaluated the diagnostic accuracy of small breast epithelial mucin (SBEM) as a potential marker for BM micrometastasis in breast cancer. The *SBEM* gene (GenBank No. AF414087] was identified by Miksicek et al. (2002) using the cDNA xProfiler tool. *SBEM* product is similar to proteins *B511 s* (Houghton et al. 2001) and *BS106* (Colpitts et al. 2002). The *SBEM* gene is predicted to code for a low molecular weight glycoprotein with highly similarity to sialomucins, including *MUC1*. Thus the *SBEM* gene is also known as *Mucin-like 1* gene (GeneID: 118430). However *SBEM* gene has shown more specific patterns of expression, limited to breast and salivary glands.

Using an in silico approach (Ayerbes et al. 2008) we have analysed the expression of *SBEM* tags in a series of human breast carcinomas SAGE libraries. In addition we developed a model system based on RT-PCR for *SBEM* mRNA to detect isolated breast tumour cells. Using *SBEM* nested approach we could detect up to one cancer cell among 1 μg of normal BM RNA, similar to the results obtained with mammaglobin (*hMAM*) mRNA amplification. No significant expression for *SBEM* was found in haematopoietic cell-lines neither in 23 controls BM analysed.

The aim of our study was to compare directly the detection rate of ITC in BM by *SBEM* RT-PCR and ICC. Molecular assay for detection of *SBEM* mRNA in BM of breast cancer patients was compared with a benchmark technique of disseminated tumour cell detection as ICC with anticytokeratin *AE1/AE3* antibody. Exploratory analysis included associations of *SBEM* mRNA detection in BM and clinical and pathological parameters. In addition *SBEM* mRNA status in BM in relation with time to breast cancer progression were analysed.

Methods

Patients

Consecutive female breast cancer outpatients were included from the medical oncology unit at University hospital in La Coruña, Spain. Inclusion criteria were: Confirmed pathologic diagnosis of invasive breast cancer; stage I–III with no prior systemic therapy for breast cancer; stage IV patients with no previous systemic therapy or in confirmed progression after such treatment; written informed consent. Exclusion criteria were defined as: previous invasive epithelial cancer; coagulopathies or thrombopenia (<20,000); any previous systemic therapy for breast cancer except stage IV patients with confirmed progressive disease; prior pelvic radiation; previous diphosphonate therapy.

The diagnostic work-up included clinical examination, blood sampling with CA 15.3 and CEA serum determination, mammography, chest x-ray, abdominal ultrasound and bone scan. Computed tomography scanning of the chest, abdomen and pelvis was performed on stage IV patients.

After informed, written consent, BM aspiration was performed under local anaesthesia, just before systemic treatment for pathological confirmed breast cancer. In patients who first underwent surgery as loco-regional treatment for primary disease, BM aspirate was obtained at least 2–3 weeks after operation but before 8 weeks. Otherwise BM samples were obtained before neo-adjuvant chemotherapy or in presence of active metastatic disease. BM was aspirated from anterior or posterior iliac crest unilaterally. Skin incision was made to avoid contamination with epidermal cells.

The study was approved by the Institutional Review Board of the Ethic Committee of Clinical Investigation of Galicia (Spain) and written informed consent was obtained from all patients.

Analysis of primary tumour and axillary lymph nodes

The primary tumour and axillaries lymph nodes collected during surgery were processed on a routine diagnostic basis. Histological tumour type, tumour size and nodal involvement were analysed, and the disease was staged according to the TNM system (American Joint Committee on Cancer, AJCC. 5ª edition). Tumour grading was performed according to modified Bloom–Richardson score. Tumour tissue immunostainings were performed using mouse monoclonal antibodies (mAb) against oestrogen receptor (ER) and progesterone receptor (PgR); clones ER-6F11/2 and PGR-312, respectively; Novocastra, Ki-67 antigen (MIB-1, DAKO) and rabbit polyclonal antibody against Her2/neu (DAKO). Immunopositivity was recorded if $\geq 10\%$ (ER, PgR) of the nucleus of tumour cells were immunostained. In addition, Her2/neu required distinct membranous staining for being considered positive.

Preparation of the bone marrow

Unilateral BM aspiration was performed from anterior or posterior iliac crests under local anaesthesia and transferred into heparinized tubes. One aliquot of one-third and at least 1 ml was used for mRNA isolation. Two-thirds were subjected to mononuclear cell (MNC) separation by density centrifugation using Lymphoprep (Nycomed, Oslo, Norway). MNCs were collected from the interphase layer and washed twice in PBS (Life Technologies, Inc.) with 10% FCS. Cytospins were prepared (5×10^5 MNCs/slide) on polylysine-coated slides in a Hettich cytocentrifuge. The cytospins were air-dried at RT overnight before freezing at −80°C or immunostaining.

Bone marrow aliquots destined to mRNA extraction were stabilized immediately after aspiration with the guanidinium lysis buffer RNA/DNA Stabilization Reagent for Blood/BM (Roche) at 10% (vol/vol). Immediate stabilization of the sample using a commercial reagent effectively protects mRNA, avoids loss of target mRNA and ensures higher reproducibility in

clinical samples. Furthermore, sedimentation of all tumour cells in the mononuclear fraction (as needed for ICC analysis) is not warranted by density centrifugation (Choesmel et al. 2004).

Bone marrow lysates were stored at −80°C until mRNA isolation. The mRNA isolation procedure was performed using mRNA Isolation Kit for Blood/BM (Roche) following manufacturer's instructions. Briefly, total nucleic acid fraction was adsorbed to magnetic glass particles and poly (A) + RNA was captured by using biotin-labelled oligo(dT) and streptavidin-coated magnetic particles. Elution was performed on each mRNA preparation in 12 μl RNase-free redistilled water. Purified poly (A) + RNA was further processed in RT-PCR or stored at −80°C until use.

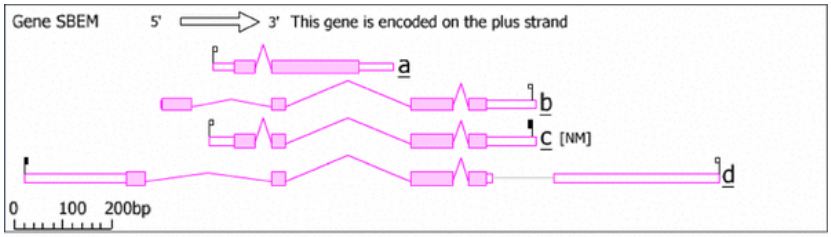
Immunocytochemical staining

Immunocytochemical staining was performed using the Vectastain ABC-AP kit (VECTOR) following manufacturer's procedure. Slides (5×10^5 BM MNCs) were incubated with the anti-cytokeratin mAbs AE1/AE3 (DAKO). At least two slides were incubated with a negative control antibody of the same immunoglobulin isotype (IgG1). The visualization stage included Vector Red alkaline phosphatase substrate kit. Endogenous alkaline phosphatase activity was inhibited by addition of levamisole. The slides were counterstained with Gills's haematoxylin to visualize nuclear morphology. The slides were manually screened by light microscopy by one of the pathologists (PID) with no knowledge about clinical data or RT-PCR status. All of the stained cells were closely evaluated. Categorization of CK immunopositive cells was performed according to the recommended guidelines (Borgen et al. 1999). Presence of micrometastasis was recorded as positive when stained cells shown typical tumour cell morphology or when these immunostained cells lacked haematopoietic characteristics and were not found in negative controls.

RNA isolation and RT-PCR

The mRNA isolation procedure was performed using mRNA Isolation Kit for Blood/BM (Roche) following manufacturer's instructions. Briefly, total nucleic acid fraction was adsorbed to magnetic glass particles and poly(A) + RNA was captured by using biotin-labelled oligo(dT) and streptavidin-coated magnetic particles. Elution was performed on each mRNA preparation in 12 μl RNase-free redistilled water. Purified poly (A) + RNA was further processed in RT-PCR or stored at −80°C until use.

The reverse transcription was performed using the SuperScript II First-Strand Synthesis System for RT-PCR (Invitrogen) using random hexamers according to manufacturer's instructions. PCR amplifications were carried out with AmpliTaq Gold PCR Master Mix (Applied Biosystems). First round PCR amplification of *SBEM* mRNA was performed using specific primers (*SBEM*-U-O and *SBEM*-L-O described by Miksicek et al. (2002). In the nested-PCR new primer pairs (*SBEM*-S-I 5′TGA TCT TCA GGT CAC CAC CA3′ and *SBEM*-A-I 5′TGG ATA CGT GTC AGC TGG AG3′) were used, designed using software available on the Internet (Rozen and Skaletsky 2000). The gene and mRNA structure of *SBEM* and primers are shown in Fig. 1, according to AceView.



SBEM-U-O	**5´ CTT TGA AGC ATT TTT GTC TGT G 3´**
SBEM-L-O	**5´ AAG GTA AGT AGT TGG ATG AAA T 3´**
SBEM-S-I	**5´TGA TCT TCA GGT CAC CAC CA 3´**
SBEM-A-I	**5´TGG ATA CGT GTC AGC TGG AG 3´**

Fig. 1. *Small breast epithelial mucin* gene diagram and primers. The SBEM gene maps on chromosome 12, at 12q13. It contains five introns and four exons. Transcription produces four (**a**–**d**) alternatively spliced mRNAs. Designed primers for RT-PCR are shown

First round PCR was performed in 50 µl of reaction mixture containing 2 µl of template cDNA, deionized water, outer *SBEM* primers U and L, 1 µl 20 µM of each, and PCR Master Mix (2X) 25 µl. In *SBEM* nested reaction 1 µl of first round PCR template and 0.5 µl at 20 µM of each inner *SBEM* primer pair were used. For first round *SBEM* PCR amplification, an initial activation at 95°C was used for 5 min, followed by 35 cycles of 95°C (30 s), 54°C (1 min) and 72°C (1 min). Finally, the last extension was at 72°C for 7 min. For *SBEM* nested reaction, an initial activation at 95°C for 2 min was used, followed by 20 cycles of 95°C 40 s, 62°C 15 s and 72°C 20 s. Last extension was at 72°C for 7 min. PCR products were electrophoresed through agarose gel and stained with 5% ethidium bromide. *Beta-2 microglobulin* serves as a positive control target. Negative controls were included in each experiment.

DNA sequencing

Polymerase chain reaction products were used as template DNA. Products were purified by enzymatic method (ExoSAP-It, Amersham USB). DNA sequencing was performed in a reference facility on ABI 3700 (Applied Biosystems) using Big Dye Terminators. Sense *SBEM*-specific primers were used.

Study design and statistical analysis

The study was designed as prospective and observational. Its primary objective was to estimate and compare the proportion of breast cancer patients with a positive mRNA transcript in BM with respect to the proportion of patients with CK-positive tumour cells in BM as a reference standard. Both early stage and metastatic patients were included in the study in order to estimate these proportions in a cohort of patients commonly found in every-day clinical practice. Detection rate using anti-CK antibodies is related to different variables including clinical and methodological parameters (Pantel et al. 1994). To obtain a power of 80% and $P = 0.05$ to find out differences using McNemar's test with the continuity correction, sample size was calculated in 47 pairs, considering that CK positive cells are detected in BM in 25% of the patients, mRNA marker expression could be detected in 45% and an estimated 25% of discordant pairs.

Exploratory analysis included associations of *SBEM* mRNA detection in BM and clinical and pathological parameters. The relationship between categorical variables was analysed by Chi-squared test with continuity correction when applicable, or Fisher's exact test. *SBEM* mRNA status in BM and time to breast cancer progression (TTP), defined as the period between BM aspirate and first recurrence, were analysed using Kaplan–Meyer curves and compared with the

log-rank test. The joint effect of covariables was examined using the Cox proportional hazard regression model. *P* values < 0.05 were considered statistically significant. Statistical analysis was performed by SPSS (Version 14.0)

Results

Patients and clinical data

From February 2002 to May 2003, 50 breast cancer female patients were included. Clinical characteristics are shown in Table 1. BM aspiration was performed after R0 surgery in 33 patients. In 17 patients BM samples were obtained before neo-adjuvant chemotherapy for locally advance disease or in presence of active metastatic disease. Thus, this subgroup of patients was prospectively defined to have clinically active disease at the time of BM sampling. The median follow-up time was 258.5 weeks (range 18–298 weeks). Breast cancer progression was detected in 15 patients (30%). There were six relapses from stage I to III patients and nine progressions of metastatic disease. Progression occurred at median time of 61 weeks (range 13–248 weeks). Seven patients died of metastatic disease.

Table 1. Clinical characteristics of the patients

	n	%
Age (years)	53.6 (± 12.18)	32–87
< 50 years	23	46
≥ 50 years	27	54
Stage		
I	13	26
II	19	38
III	7	14
IV	11	22
pT		
pT1	18	36
pT2	19	38
pT3	9	18
pT4	4	8
pN		
pN0	21	42
pN1	25	50
pN2	3	6
pN3	1	2
Hormonal receptors		
Negative	20	40
Positive	30	60
Histology		
Ductal	35	70
Lobular	4	8
Other	11	22
Her2		
Unknown	4	8
0–1	25	50
2	4	8
3	17	34
HR and Her2 negative	12	24
Vascular invasion		
Unknown	8	16
No	23	46
Yes	19	38
Histological grade		
Unknown	5	10
1	10	20
2	17	34
3	18	36
Ki-67		
Unknown	2	4
< 20%	25	50
≥ 20%	23	46

SBEM RT-PCR

First round PCR amplification for *SBEM* mRNA in BM shows positive results in four patients (8%). Nested amplifications were positive in another nine patients (18%). Thus, globally, RT-PCR for *SBEM* was positive in BM in 13 out 50 breast cancer patients (26%; standard error 0.06).

Cytokeratin immunocytochemistry

Isolated tumour cells in BM were detected using anti-cytokeratin mAbs AE1/AE3 and standardized morphological criteria. At least 2×10^6 BM MNC cells were screened per patient. BM aspirate did not obtain enough samples for immunocytochemical analysis in one patient with bone metastasis. Presence of ITC in BM was found in 12 patients (24%; standard error 0.06). The number of tumour cells detected were: one (four patients), two (two patients), three (three patients), six (one patient) and ten or more in two patient.

Diagnostic accuracy of SBEM RT-PCR

The primary objective of our study was to estimate and compare the proportion of breast cancer patients with a positive *SBEM* mRNA transcript in BM in relation to the proportion of patients with CK-positive tumour cells in BM as a standard reference. Results are shown in Table 2. RT-PCR for *SBEM* was positive in 26% of the patients. Presence of ITC using CK staining was found in 24% of the patients. According to McNemar's test, there was no difference in ITC detection in BM between *SBEM* RT-PCR and ICC [two-tailed *P* value = 1 (odds ratio 1.125; 95% confidence interval 0.385–3.351)]. The kappa value was low (0.094, $P = 0.56$).

Table 2. Comparison between *SBEM* RT-PCR and cytokeratin immunocytochemistry for the detection of disseminated epithelial cells in bone marrow from breast cancer patients

		Cytokeratin immunocytochemistry		*P*[a]
		+	−	NS
SBEM mRNA in bone marrow	+	4	9	
	−	8	29	

[a]McNemar's test

A concordance of 66% (33 of 50; 95% CI 51.14–78.41) was found between *SBEM* RT-PCR and ICC. Sensitivity and specificity of *SBEM* RT-PCR, considering ICC as a reference, were 33.3% (95% CI 11.3–64.6) and 76.32% (95% CI 59.38–87.97) respectively.

Breast cancer progression was found in six patients (40 %) with *SBEM* mRNA in BM but only in two patients (13.3 %) with CK positive cells in BM. Diagnostic accuracy for *SBEM* RT-PCR for breast cancer progression was estimated in 68% (95% CI 53.2–80%). By contrast, accuracy for CK-ICC was only 54% (95% CI 39.5–67.9%).

Correlations with clinicopathology

Associations of BM status and clinical and pathologic characteristics of the patients are shown in Table 3. *SBEM* mRNA in BM aspirates were significantly associated with presence of clinically active disease (47%, $P = 0.021$) and tumours with positive hormonal (oestrogen and/or progesterone) receptors (36.7%, $P = 0.035$). Interestingly, association with Her2/neu overexpression (44.4%, $P = 0.051$) and low proliferating tumours, defined as nuclear Ki67 staining < 20% (36%, $P = 0.067$) were nearly significant.

Table 3. Associations of bone marrow status and clinicopathologic characteristics of the patients

Characteristic	*SBEM* RT-PCR positive			CK-ICC positive		
	Number	%	Significance	Number	%	Significance
Age			0.526			0.730
< 50 years	5	10		5	10	
≥ 50 years	8	16		7	14	
Stage			0.180			0.825
I–II	6	18.7		8	25	
III–IV	7	38.9		4	22.2	
Active disease			0.021[a]			0.728
Yes	8	47		5	29.4	
No	5	15.2		7	21.2	
Tumour status			0.149			0.791
T1-2	7	19.4		9	25	
T3-4	6	42.8		3	21.4	
Lymph node status			0.340			0.979
N0	4	19.0		5	23.8	
N+	9	31.0		7	24.1	
Metastasis			0.126			0.609
M0	8	20.5		10	25.6	
M1	5	45.5		2	18.2	
Histological grade			0.891			0.008[a]
1-2	7	25.9		2	7.4	
3	5	27.8		8	44.4	
Unknown	1	20		2	40	
Hormonal receptors			0.035[b]			0.506
Positive	11	36.7		6	30	
Negative	2	10		6	20	
Her2/neu			0.051[b]			0.829
Positive	8	44.4		4	22	
Negative	5	17.9		7	25	
Unknown	0			1	25	
Vascular invasion			0.156			0.155
Positive	7	36.9		3	15.8	
Negative	3	13.0		5	21.7	
Unknown	3	37.5		4	50	
Ki-67 staining			0.067[b]			0.616
<20%	9	36.0		5	20	
≥20%	3	13		6	26.1	
Unknown	1	50		1	50	
CA 15.3			0.719			0.928
≤35	9	24.3		9	24.3	
>35	4	30.8		3	23.1	

[a] *P* value computed by Fisher's exact test
[b] *P* value computed by Pearson's Chi-square test

Although positive results for *SBEM* mRNA were more frequent in patients with stage III–IV (38.9%), T3–T4 tumours (42.8%), positive axillaries lymph nodes (31%), presence of metastasis (45.5%) and vascular invasion (36.9%) these associations did not reach statistically significance.

Presence of CK-positive tumour cells in BM was associated only with histological grade 3 ($P = 0.008$).

Kaplan–Meier survival analysis (Fig. 2) suggest a reduced time to breast cancer progression (TTP) among the *SBEM* mRNA BM positive patients, although this was not significant (log-rank test, $P = 0.150$). Subgroup analysis was performed (Table 4). Interestingly, when TTP was analysed in relation to the grade of the tumours concomitantly with the presence or absence of *SBEM* mRNA in BM, a statistically significant association was observed (long-rank test, $P = 0.045$). In addition and in spite of limited number of patients, presence of *SBEM* in BM defines a very poor prognostic group in hormonal receptors negative patients (log-rank test, $P = 0.027$). Thus, in this HR negative cohort ($n = 20$) the two early breast cancer patients with SBEM mRNA in BM suffered metastatic progression and died of disease (Fig. 2c).

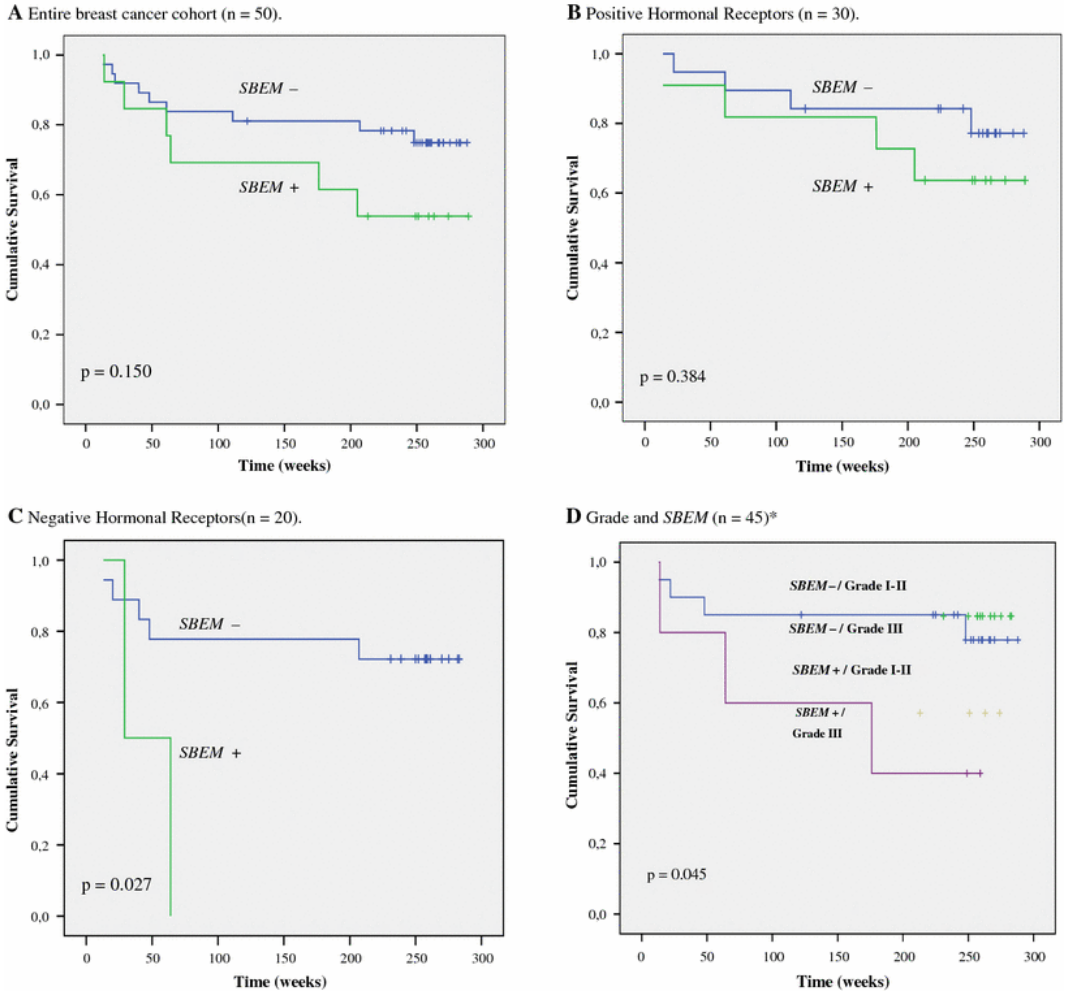


Fig. 2. Kaplan–Meier graphs for time to breast cancer progression according to *SBEM* mRNA status in bone marrow. Kaplan–Meier estimates are shown for the entire breast cancer cohort (**a**), positive (**b**) and negative (**c**) hormonal receptors and adjusted for grade (**d**). *Symbols on the graph lines* represent censored data; *P*-values are given for log rank tests. *Asterisk* indicates grade was unknown in five patients

Table 4. Time to breast cancer progression and *SBEM* bone marrow status determined by RT-PCR: subgroup analysis

	Bone marrow status	Patients (*n*)	Time to progression (weeks)	
			Mean	Standard error
Grade 1–2	SBEM (−)	20	246.1	20.7
	SBEM (+)	7	198.7	37.9
Grade 3	SBEM (−)	13	244.1	25.3
	SBEM (+)	5	154.4	44.8
Hormonal receptors (+)	SBEM (−)	19	249.9	18.9
	SBEM (+)	11	225.4	29.2
Hormonal receptors (−)	SBEM (−)	18	222.3	24.6
	SBEM (+)	2	46.5	17.5

The effect of covariables in TTP was examined using the Cox proportional hazard regression model. Results are showed in Table 5. In multivariate analysis only stage at the time of BM aspirate was statistical significant.

Table 5. Time to breast cancer progression in relation to clinicopathological characteristics and *SBEM* bone marrow status determined by RT-PCR: Cox proportional hazard regression model

Variable	Subset	Hazard ratio (95% confidence interval)	*P*
Univariate analysis			
Active disease	No/yes	5.47 (1.86–16.08)	0.002
Ca 15.3	Normal/high	4.97 (1.79–13.79)	0.002
Stage	I–II/II–IV	12.20 (3.39–43.81)	0.0001
Lymph Nodes	Negative/positive	5.22 (1.17–23.16)	0.030
SBEM	Negative/positive	2.10 (0.75–5.91)	0.160
Grade	I–II/III	1.076 (0.341–3.393)	0.9
Hormonal receptors	Negative/positive	0.686 (0.249–1.893)	0.467
Multivariate analysis			
Active disease	No/yes	0.26 (0.051–1.287)	0.098
Ca 15.3	Normal/high	3.07 (0.89–10.51)	0.074
Stage	I–II/III–IV	16.73 (2.99–93.62)	0.001
Lymph nodes	Negative/positive	2.88 (0.58–14.22)	0.195
SBEM	Negative/positive	2.17 (0.59–7.822)	0.239

Discussion

Isolated tumour cell detection in BM by means of immunocytochemical staining has been shown to be an independent prognostic factor in early breast cancer (Pantel and Alix-Panabieres 2007; Braun et al. 2005; Wiedswang et al. 2003). ICC has been considered the gold standard for ITC detection and objective criteria for the evaluation of stained cells has been developed (Borgen et al. 1999). Reverse transcription (RT)-PCR has been used to indirectly detect tumour cells through the expression of epithelial or breast cancer-associated mRNA transcripts in BM, blood and other compartments. Although RT-PCR has been considered more sensitive than antibody-based techniques very few studies have compared the detection rates and diagnostic accuracy of both methodologies (Schoenfeld et al. 1997; Lambrechts et al. 1999; Slade et al. 1999; Ring et al. 2005; Benoy et al. 2004) in BM aspirate. Moreover, different markers have been evaluated in ITC by RT-PCR in breast cancer, and conflicting results about sensitivity and specificity have been reported (reviewed in Lacroix 2006).

In our study we have evaluated for the first time the diagnostic accuracy of SBEM as a potential marker for BM micrometastasis in breast cancer. RT-PCR assay for detection of *SBEM* mRNA was compared with ICC with *AE1/AE3* antibody in paired samples obtained from the BM of breast cancer patients. *SBEM*-specific transcript was found in 26% of patients, and the detection rate was similar to the percentage of patients with ITC detected, using ICC (24%). However, concordant results for both tests were found in 66%. Our results are comparable to those previously reported. Slade et al. (1999) reported a concordance of only 50% between ICC (based in CK staining) and quantitative RT-PCR for CK-19 mRNA when analysing BM samples of primary breast cancer patients. Benoy et al. (2004) compared the detection rates of ICC based in CK staining with RT-PCR in BM in a subgroup of 20 metastatic breast cancer patients. Concordances of 75 and 70% for CK-19 and *hMAM* were found respectively.

It has been suggested (Schoenfeld et al. 1997) that some of CK positive cells were not viable or that they were dormant with low metabolic activity as defined by their inability to synthesise mRNA. In fact a single marker to detect and quantify ITC in BM or other samples might lead both to false-negative and false-positive analyses. Moreover and due to the low number of ITC usually present in BM and sensitivity of both methods of detection a stochastic effect and sampling errors cannot be ruled out (Slade et al. 1999).

A previous work (Woelfle et al. 2003) had shown that BM micrometastases are associated to a specific transcriptional signature. Lower expression (at mRNA and protein level) of luminal CKs (CK8, CK18 and CK19) has been found in primary breast tumours with BM micrometastasis. It could be argued that a fraction of disseminated tumour cells in these patients might show a negative CK phenotype and missed in ICC detection. In addition, recent studies have clearly demonstrated that isolated and micrometastatic tumour cells included a remarkably heterogeneous population (Klein et al. 2002; Gangnus et al. 2004; Watson et al. 2007). Loss of luminal CKs and over expression of vimentin suggest that ITC have acquired an epithelial to mesenchymal transition phenotype (Willipinski-Stapelfeldt et al. 2005). We hypothesized that *SBEM* RT-PCR assay could detect a different population of disseminated tumour cells in BM than those detected in a CK-based assay.

Variability in *SBEM* mRNA expression levels was identified among different human breast cancer cell lines tested in our previous study (Ayerbes et al. 2008). In mammary tissues, including breast cancer, *SBEM* expression has been shown to be restricted to luminal epithelial cells (Allinen et al. 2004). Although *SBEM* expression has been found in the ER-positive, well-differentiated, "luminal epithelial-like" breast cancer cell lines (Lacroix and Leclercq 2004), other studies have shown *SBEM* gene expression, as assessed by RT-PCR, in more than 90% of primary or metastatic breast cancers (Miksicek et al. 2002; Colpitts et al. 2002; Brown et al. 2006). In a small subset of primary breast tumours Skliris et al. (2008) have recently shown that *SBEM* mRNA was detected by RT-PCR in all cases. Furthermore, there was a significant correlation between *SBEM* protein expression (determined by immunohistochemistry) and *SBEM* gene expression (determined by RT-PCR and by Northern blot analysis).

In our study, the detection of *SBEM* transcript in the BM of patients with breast cancer was significantly correlated with known clinicopathological prognostic factors. *SBEM* mRNA in BM aspirates were significantly associated with the presence of clinically active disease (47%, $P = 0.021$) and tumours with positive hormonal receptors (36.7%, $P = 0.035$). In addition, association with Her2/neu over-expression (44.4%, $P = 0.051$) was close to significance level. A previous report has shown significant association between Her2/neu determined by IHC and increasing amounts of *SBEM* mRNA (Lacroix and Leclercq 2004) in breast tumours. Using an in silico approach (Ayerbes et al. 2008) we have analysed the expression of *SBEM* tags in a series of human breast carcinomas SAGE libraries ($n = 27$). *SBEM* expression were quantified and correlated with the tag numbers of different molecular markers associated with breast cancer progression. Expression of *SBEM* was significantly correlated (Spearman's rho) to Her2/neu expression (0.662; $P = 0.000$).

Although it could appear paradoxical, in our study we found an association of *SBEM* mRNA in BM and low proliferating tumours, that was of borderline significance (36%, $P = 0.067$). Other authors have found no correlation between the presence of CK-positive cells in BM and Ki67 staining in primary breast tumours (Schindlbeck et al. 2005). However, in a recent study that analysed *SBEM* protein expression in a large cohort ($n = 300$) of invasive breast cancers, negative association with Ki67 staining was found (Brown et al. 2006). Interestingly, most circulating and micrometastatic tumour cells do not express the proliferation antigen Ki67 and may therefore remain in the state of dormant cell-cycle arrest (Pantel et al. 1993; Muller et al. 2005).

In our study, high tumour grade was significantly associated with ICC detection of ITC in BM as previously described (Braun et al. 2000, 2005). No correlations with other clinical or pathologic characteristic were found. Lack of association of BM status and other prognostic indicators could be dependent of different factors including stage of disease, study design and other methodological aspects. In stages I–III patients, the presence of CK positive cells in BM has been correlated with established prognostic factors, including pT and pN status (Braun et al. 2005; Wiedswang et al. 2003) and hormonal receptor (Braun et al. 2005). However, Braun et al. (2000) found that the incidence of BM micrometastases was similar in patients with lymph-node metastasis and those without it and failed to detect an association between hormonal receptor status and positive CK staining in BM. In addition, in a recently published paper Bidard et al. (2008a) found that ITC detection in BM, using CK antibodies and specific morphologic features, did not correlate with any of the patient's clinical or pathologic characteristics. Furthermore, in patients with locoregional and distant recurrent breast cancer, the presence of CK positive cells in BM at the time of relapse was not correlated with any of the clinical characteristics at the time of primary diagnosis (Janni et al. 2000).

In order to explore the clinical significance of the detection of *SBEM* mRNA in BM in our series of breast cancer patients, we correlated the RT-PCR results and breast cancer progression. Breast cancer progression was found in six patients (40%) with *SBEM* mRNA in BM but only in two patients (13.3 %) with CK positive cells in BM. Diagnostic accuracy for *SBEM* RT-PCR for breast cancer progression was estimated in 68% (95%CI 53.2–80%). In addition, Kaplan–Meier survival analysis (Fig. 2) suggests a reduced time for breast cancer progression (TTP) among the *SBEM* mRNA BM positive patients, although this was not significant (log-rank test, $P = 0.150$).

Prognostic impact for ITC in BM in breast cancer has shown to be dependent of several factors, including stage of disease, detection method (Bidard et al. 2008b) and molecular subtypes of breast cancer (Naume et al. 2007). Clearly, to ascertain a clinical utility, if any, for *SBEM* mRNA as a marker for ITC detection, a large study with homogeneous patient cohort and adequate sampled size is needed.

However when we analysed TTP stratifying according to different prognostic factors, presence of *SBEM* in BM defines poor-prognosis groups adjusting for grade or hormone receptor status (Fig. 2). Tumour grade has been a highly valuable prognostic factor for breast cancer, as high-grade lesions are associated with significantly poorer clinical outcome (Soerjomataram et al. 2008). In addition, distinct transcriptional signatures had been found to be associated with different tumour grades (Ma et al. 2003). Patients with HR-negative breast cancer experienced relapse and progression more often than those with HR-positive tumours during the first 5 years.

This is not surprising, taking into account the differences in genetic profiles between HR-negative and HR-positive tumours. In line with this, Ignatiadis et al. (2007) have recently analysed the prognostic significance of cytokeratin19 mRNA detection in blood in early breast cancer patients according to the molecular subtype. They found that *CK-19* mRNA in blood was an independent prognostic factor for disease-free survival and overall survival only in patients with ER-negative tumours.

Conclusions

Small breast epithelial mucin might represent a suitable marker for molecular detection of ITCs in BM in breast cancer patients. However, and when we considered the different transcriptional molecular profiles of breast cancer, a multi-marker and quantitative approach could obtain a higher efficacy. Analysis of prognostic value for *SBEM* mRNA-based assay should take the heterogeneity and different molecular subtypes of breast cancer into account.

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Conflict of interest statement

None declared.

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