Detection of Circulating Tumour Cells in Breast Cancer Patients Using Human Mammaglobin RT-PCR: Association with Clinical Prognostic Factors

PAOLA FERRO[1], MARIA CRISTIANA FRANCESCHINI[1], BARTOLOMEO BACIGALUPO[1], PAOLO DESSANTI[1], EMILIO FALCO[2], VINCENZO FONTANA[3], DANIELA GIANQUINTO[2], MARIA PIA PISTILLO[4], FRANCO FEDELI[1] and SILVIO RONCELLA[1]

[1]Division of Histopathology and Cytopathology, [2]Department of Surgery, Sant'Andrea Hospital, La Spezia, Italy; [3]Unit of Epidemiology and Biostatistics, and [4]Breast Cancer Laboratory of Tumour Genetics Unit, National Institute for Cancer Research, Genova, Italy

Abstract. *Background: So far discordant results regarding the significance of tumour cells circulating in peripheral blood (CTCs) of breast cancer (BC) patients have been reported. Our aim was to evaluate the association of indirect CTC detection by amplification of human mammaglobin (hMAM) gene expression with traditional prognostic markers of clinical outcome in BC at the time of diagnosis. Patients and Methods: Peripheral blood samples from 190 patients with invasive and 12 patients with in situ BC, before therapy and/or surgery, from 184 patients with benign breast disease and from 146 healthy volunteers were tested for hMAM expression by a nested reverse transcriptase-polymerase chain reaction (RT-PCR) assay. Correlations between CTCs and age at diagnosis, tumour type and size, grading, lymph node involvement, oestrogen and progesterone receptor status, HER-2/neu expression and Ki-67/MIB-1 labelling index were assessed through the odds ratio (OR) point estimates, considering OR >2.0 or <0.5 as being clinically relevant. ORs and their corresponding 95% confidence limits (95% CL) were obtained by logistic regression analysis. Results: Expression of hMAM was found only in peripheral blood of patients with invasive BC (9.5%) and multivariate logistic regression analysis indicated its association with lymph node involvement (pN1-pN3 vs. pN0, OR=5.6, 95% CL=1.4-22.6; p=0.009), tumour size (pT2-pT4 vs. pT1, OR=2.3, 95% CL=0.6-9.0; p=0.207) and negative ER status (OR=2.5, 95% CL=0.6-10.0; p=0.227). Conclusion: Our data show that CTC detection in invasive BC at the time of diagnosis is associated with poor prognosis and may also be used as an additional prognostic indicator.*

Correspondence to: Dr. Silvio Roncella, Ph.D., Servizio di Anatomia ed Istologia Patologica, Ospedale Sant'Andrea, Via Mario Asso n. 2, 19124 La Spezia, Italy. Tel: +39 0187604560, Fax: +39 0187533245, e-mail: silvio.roncella@asl5.liguria.it

Key Words: Circulating tumour cells, human mammaglobin, breast cancer, reverse-transcriptase polymerase chain reaction.

Breast cancer (BC) has been shown to shed malignant cells into peripheral blood (PB) at the earliest stages of primary development (1). Circulating tumour cells (CTCs) are potentially able to form metastases (2) and may be regarded as valuable predictors of poor clinical outcome, being associated with shorter disease-free and overall survival (3-6). In addition, CTC levels may be useful for the follow-up evaluation and monitoring of the therapeutic efficacy (3-6). However, although numerous studies suggest the promising role of CTCs in routine management of BC patients, their clinical implication is still under investigation.

Among the numerous molecular markers being currently investigated for the indirect detection of occult micro-metastasis in BC patients, mRNA expression of the human mammaglobin (*hMAM*) gene is considered one of the most promising due to its high sensitivity and specificity (7). The *hMAM* gene, a member of the uteroglobin gene family mapping to human chromosome 11q13, was cloned by Watson and Fleming in 1996 (8). hMAM has been recognized as a breast epithelial cell-associated glycoprotein, expressed in 93% of BC cases (9, 10) and overexpressed in up to 23% (11). hMAM has been proposed as a marker for CTC detection by several authors (11-25). Many studies have reported the association between indirect detection of CTCs using hMAM expression in PB and traditional clinicopathological prognostic factors in BC before therapy and/or surgery (16-19, 25). However, we still do not have a clear view of this issue because the results are often contradictory. Therefore, other studies are still required to enable final conclusion to be drawn.

We previously developed a nested RT-PCR assay for amplifying the *hMAM* mRNA transcript as an indirect index

of the presence of CTCs in BC patients (12). This methodology proved to be highly sensitive and capable of detecting as few as one BC cell per million normal haematopoietic cells from healthy donors (12). With this level of sensitivity, transcripts of *hMAM* were not detected in PB collected from 58 patients affected by benign breast diseases, nor in PB from 65 healthy volunteers (12).

In the present study, on the basis of the discordant results reported by other authors, we investigated the association of the presence of CTCs with well established prognostic factors of BC at the time of diagnosis. In particular, we focused the statistical analysis on the odds ratio (OR) point estimates as, representing the more relevant index of association between each prognostic factor and the study outcome, they can better help to identify the prognostic implications of CTC detection in PB.

Patients and Methods

Patients. PB from BC patients was collected at the time of diagnosis, before neoadjuvant therapy and/or surgery, at the Division of Surgery of ASL5 La Spezia (Italy) between February 2002 and October 2004. Blood samples (5 ml) were drawn in ethylenediaminetetraacetic acid (EDTA) vacutainer after informed consent and approval of the study design by the Institutional Ethics Committee (protocol n. 53 15/11/2001). The study enrolled 190 patients with invasive BC (median age of 63.2, range 33-93 years), of whom 155 had ductal BC and 35 had other types of BC.

The study also included 12 patients with *in situ* BC (10 ductal and 2 lobular) and 330 controls consisting of 184 patients with benign breast disease and 146 with pathologies different from breast disease, or who were healthy blood donor volunteers.

Enrichment of PB tumour cells and molecular analysis. PB mononuclear cells were obtained by Ficoll-Hipaque (Biochrome AG, Berlin, Germany) density gradient centrifugation, followed by collection of the interface cells. The collected cells, containing the CTCs, were washed twice in phosphate-buffered saline, dissolved in RLT solution (RNeasy Mini Kit, QIAGEN GmbH, Hilden, Germany) and snap-frozen at −30˚C, ready for RNA extraction (12, 26).

Total RNA was extracted using RNeasy Mini Kit (QIAGEN GmbH) following the manufacturer's instructions and 1 μg of total RNA was reverse transcribed into cDNA as previously described (12, 26).

A 10 μl sample of cDNA was amplified by nested PCR described elsewhere (12, 26) using primers that amplify *hMAM* gene mRNA (GenBank accession number U33147). Briefly, the first PCR reaction (total volume 50 μl) contained 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1.5 U Platinum Taq DNA Polymerase (Invitrogen, Milano, Italy) with its buffer and 0.4 μM primers. The cycling conditions were 2 min at 94˚C followed by 40 cycles of 94˚C for 30 s, 64˚C for 20 s, 72˚C for 5 s and a final incubation at 72˚C for 2 min. The nested PCR reaction (total volume 50 μl) was carried out using 5 μl of the first PCR product with conditions identical to those of the first PCR but using the appropriate primers and 30 cycles of amplification. As internal control, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene amplification was carried out for each cDNA sample under conditions identical to those for *hMAM* first amplification. PCR products were separated by standard electrophoresis on 1.5% agarose gel containing ethidium bromide. The MDA-MB415 BC cell line (ICLC, National Institute for Cancer Research, Genova, Italy) was used as an *hMAM* positive control as previously described (26). The *hMAM*-positive samples were confirmed in a second RT-PCR experiment.

Histopathology and immunohistochemistry. The antigens were localized by means of a Ventana Medical Systems/view DAB detection Kit and the reactions were performed by an automated immunostainer NEX ES (Ventana Medical Systems, S.A. Strasbourg, France) programmed for antigen retrieval. Finally, the sections were counterstained with haematoxylin.

The following monoclonal antibodies were used: clone 6F11 for oestrogen receptor (ER) (Ventana Medical Systems), clone 16 for progesterone receptor (PGR) (Ventana Medical Systems), and clone MIB-1 for Ki-67 (Dakocytomation Denmark A/S, Glostrup, Denmark). The cells were considered positive for ER, PGR and Ki-67 only when distinct nuclear staining was identified and the percentage of obtained cells was higher than 10. For HER-2 detection, the semi-quantitative detection kit PATHWAY® anti-HER-2/*neu* (4B5) Rabbit Monoclonal Primary Antibody (Ventana Medical Systems) was used. The histopathological grading for invasive BC was evaluated according to Elston and Ellis (27).

The percentage of immunoreactive tumour cells and tumour grading were evaluated independently by two observers (PD and BB or FF) who re-evaluated the conflicting results together.

Statistical analyses. Logistic regression analysis (28) was applied to assess the univariate and multivariate association between CTC status (positive *vs*. negative), indirectly detected by *hMAM* gene expression, and the following selected prognostic factors: age at diagnosis, tumour type and size, grading, lymph node involvement, ER and PGR receptor status, *HER-2/neu* expression and Ki-67/MIB-1 labelling index. The odds ratio (OR) was used as an index of strength of association between each prognostic characteristic and study outcome, considering OR >2.0 or <0.5 as being clinically relevant. For each OR, 95% confidence limits (95% CL) were also computed (28).

In order to evaluate the degree of dependence of each factor on the others in predicting the CTC status, ORs were estimated through both univariate (*i.e.* including only one factor) and multivariate (*i.e.* including all factors) logistic modelling. For each selected factor, the discrepancy between the two ORs was used as a tool for disclosing the degree of correlation with the other prognostic characteristics. Finally, statistical significance of each OR was assessed through the likelihood-ratio chi-square test (28) with *p*-values less than <0.05 being regarded as significant.

All statistical analyses were performed using Stata V10.1 (StataCorp, Stata Statistical Software, College Station, TX, USA).

Results

Detection of CTCs in peripheral blood of BC patients and controls. We investigated the presence of CTCs in PB of BC patients and controls through the hMAM transcript amplification by a previously described RT-PCR approach (12, 26).

hMAM expression was analysed in the mononuclear cell fraction of 532 PB samples derived from 190 patients with

Table I. *Summary of CTC detection in PB from BC patients and controls.*

Group	CTC detection
	Positive/Total (%)
Invasive BC	18/190 (9.5)
In situ BC	0/12 (0)
Benign breast disease	0/184 (0)
Healthy volunteers	0/146 (0)

CTC, Circulating tumour cells; PB, peripheral blood; BC, breast cancer.

Table II. *Selected prognostic factors and CTC detection in PB of patients with invasive BC before neoadjuvant therapy and/or surgery.*

Prognostic factor	Total n=190	CTC detection	
		Negative (%) n=172 (90.5)	Positive (%) n=18 (9.5)
Age (years)			
≤50	42	39 (92.9)	3 (7.1)
>50	148	133 (89.9)	15 (10.1)
Tumour type			
Ductal	155	142 (91.6)	13 (8.4)
Other*	35	30 (85.7)	5 (14.3)
Tumour size (cm)			
≤2	112	108 (96.4)	4 (3.6)
>2	78	64 (82.0)	14 (18.0)
Nodal status			
pN0	110	107 (97.3)	3 (2.7)
pN1-pN3	80	65 (81.2)	15 (18.8)
Grading			
1+2	114	107 (93.9)	7 (6.1)
3	76	65 (85.5)	11 (14.5)
ER			
Positive	159	147 (92.4)	12 (7.6)
Negative	31	25 (80.6)	6 (19.4)
PGR			
Positive	124	113 (91.1)	11 (8.9)
Negative	66	59 (89.4)	7 (10.6)
HER-2/Neu			
1	106	95 (89.6)	11 (10.4)
2	41	39 (95.1)	2 (4.9)
3	43	38 (88.4)	5 (11.6)
Ki-67/MIB			
≤10%	109	102 (93.6)	7 (6.4)
>10%	81	70 (86.4)	11 (13.6)

CTC, Circulating tumour cells; PB, peripheral blood; BC, breast cancer; ER, oestrogen receptor; PGR, progesterone receptor. *Other (n): lobular (23), papillary (3), mioepithelial-rich (1), apocrine (1), sarcomatoid (1), squamous (1), tubular (4), undifferentiated (1).

invasive BC at primary diagnosis, 12 patients with *in situ* BC and 330 controls without BC including 184 patients with benign breast disease and 146 patients without breast disease or healthy blood donors.

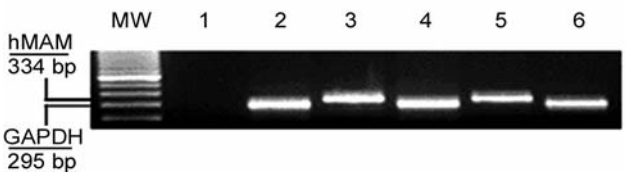

Figure 1. *The Figure shows a representative ethidium bromide-stained 1.5% agarose gel of RT-PCR amplified hMAM and internal control GAPDH. The hMAM band (334 bp) is evident in PB from a patient with breast cancer (lane 3), in the breast cancer cell line MDA-MB415 used as positive control (lane 5), but not in PB from a patient with benign breast disease (lane 1). The internal control GAPDH band (295 bp) is shown for each sample (lanes 2, 4, 6). hMAM, human mammaglobin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PB, peripheral blood; MW, molecular weight marker.*

HMAM transcript was detected in 18/190 (9.5%) PB samples from invasive BC patients but in none of those with *in situ* BC, nor in those without BC, as shown in Table I.

Detailed *hMAM* expression results, according to selected prognostic factors for patients with invasive BC are summarized in Table II.

A representative RT-PCR gel showing the *hMAM* transcript (334 bp) in PB from a patient with BC, but lacking in PB from a patient with benign breast disease, is shown in Figure 1.

Univariate and multivariate analyses. In univariate analysis, clinically remarkable direct correlations between CTC-positive status and selected prognostic factors (*i.e.*, OR >2.0 or OR <0.5) were found for nodal status (positive *vs.* negative, OR=8.2, $p<0.001$), tumour size (>2 cm *vs.* ≤2 cm, OR=5.9, $p<0.001$), histological grading (G3 *vs.* G1+G2, OR=5.6, $p=0.058$) and Ki-67/MIB-1 labelling index (>10% *vs.* ≤10%, OR=2.3, $p=0.098$), as shown in Table III.

A noteworthy association with the ER status (negative *vs.* positive, OR=3.3, $p=0.055$) was also observed, whereas no association with the other prognostic factors was found (Table III).

By comparing univariate to multivariate logistic regression analysis, it is possible to detect changes in OR point estimates basically due to the degree of correlation among the prognostic factors. In particular, a strong attenuation effect (*i.e.* OR towards the value of 1) was seen for some prognostic markers including tumour size (OR=2.3, $p=0.207$), histological grading (OR=1.8, $p=0.379$) and Ki-67/MIB-1 labelling index (OR=1.1, $p=0.849$). Although the OR for lymph node involvement exhibited a discernible reduction on multivariate analysis (OR=5.6), it was the only factor which maintained the highest and statistically significant correlation ($p=0.009$). The multivariate modelling confirmed the association of CTC detection with a negative ER status (OR=2.5, $p=0.227$) whereas it increased the

Table III. *Correlation between CTC detection in PB of patients with invasive BC and selected prognostic factors.*

Prognostic factor	Univariate			Multivariate		
	OR	95% CL	P-value	OR	95% CL	P-value
Age (years)			0.547			0.538
≤50	1.0	-		1.0	-	
>50	1.5	0.4-5.3		1.6	0.4-7.1	
Tumour type			0.305			0.135
Invasive ductal	1.0	-		1.0	-	
Other*	1.8	0.6-5.5		2.8	0.8-10.5	
Tumour size (cm)			<0.001			0.207
≤2 (pT1)	1.0	-		1.0	-	
>2 (pT2-pT4)	5.9	1.9-18.7		2.3	0.6-9	
Nodal status			<0.001			0.009
pN0	1.0	-		1.0	-	
pN1-pN3	8.2	2.3-29.5		5.6	1.4-22.6	
Grading			0.058			0.379
1+2	1.0			1.0	-	
3	5.6	0.9-7.0		1.8	0.5-6.9	
ER			0.055			0.227
Positive	1.0	-		1.0	-	
Negative	3.3	1.0-10.0		2.5	0.6-10.0	
PGR			0.700			0.397
Positive	1.0	-		1.0	-	
Negative	1.25	0.5-3.3		0.6	0.16-2.0	
HER-2/Neu			0.467			0.578
0-1+	1.0	-		1.0	-	
2+	0.4	0.1-2.1		0.5	0.1-2.6	
3+	1.1	0.4-3.5		1.2	0.3-4.3	
Ki-67/MIB			0.098			0.849
≤10%	1.0	-		1.0	-	
>10%	2.3	0.9-6.2		1.1	0.3-4.2	

CTC, Circulating tumour cells; PB, peripheral blood; BC, breast cancer; ER, oestrogen receptor; PGR, progesterone receptor; OR, odds ratio; CL, confidence limits. *Other (n): lobular (23), papillary (3), mioepithelial-rich (1), apocrine (1), sarcomatoid (1), squamous (1), tubular (4), undifferentiated (1).

prognostic role of tumour type (OR=2.8, p=0.135). Finally, no substantial changes in the remaining prognostic characteristics (age at diagnosis, PGR status and HER-2 overexpression) were detected.

Discussion

hMAM gene expression has been regarded as a breast tissue-associated marker and a promising molecular indicator of micrometastatic disease in BC (8). hMAM for the indirect detection of CTCs in BC has been investigated by many authors who provided clear evidence for its high specificity (11-24). In this study, we evaluated the presence of CTCs through the analysis of *hMAM* gene expression by RT-PCR in PB samples from BC patients at the time of diagnosis and from controls.

Our results, obtained in a larger number of patients and controls, confirm those reported supporting the crucial importance of *hMAM* as a marker for CTC detection, specificity being an absolute requirement in this field. In fact, misleading identification of tumour cells may lead to wrong choices in clinical decisions and may represent a source of dilemma and anxiety for the patients.

The frequency of *hMAM* expression in PB from invasive BC varies among the studies. Our finding of 9.5% positive samples at primary diagnosis is in line with the frequencies reported by some authors: 8% by Grünewald *et al.* (16) and Gargano *et al.* (15), 13% by Mikhitarian *et al.* (17), but in contrast to the higher frequencies reported by others: 18% by Marques *et al.* (21), 20% by Benoy *et al.* (25), 38% by Lin *et al.* (18), 41% by Cerveira *et al.* (13) and 61% by Zehentner *et al.* (19).

In addition, in the multivariate analysis, we found that the following four factors, nodal status, tumour size, tumour type, and negative ER status, played a role in predicting *hMAM*-positivity in our series of patients. A significant association of *hMAM* expression in PB with nodal status was reported by Grünewald *et al.* in patients with invasive BC at the time of diagnosis or during the follow-up (16).

A trend towards a positive correlation of *hMAM* expression with lymph node involvement and with increased tumour size was reported by Suchy *et al.* in PB of patients with invasive BC (20), whereas a significant association with increased tumour size was reported by Ignatiadis *et al.* in early invasive BC (14).

Concerning the association of *hMAM* expression with high histological grading, we confirmed the data reported by Mikhitarian *et al.* (17), as we found significant association of *hMAM* expression in PB samples obtained before surgery with G3 grading in univariate analysis, although this finding was not confirmed by multivariate analysis. Moreover, at variance with Mikhitarian *et al.* (17), who reported only a trend towards an association of *hMAM* expression with ER-negative tumours, we found this association to be statistically significant.

On the other hand, our findings are in contrast with those of Zehentner *et al.* (19), Lin *et al.* (18), and Benoy *et al.* (25), who did not find any association between poor prognostic factors and *hMAM* expression in PB samples from BC patients collected before surgery.

The discrepancies in CTC detection through the use of hMAM marker may be partially linked to the number of patients studied, age or disease stage, amount of cellular *hMAM* mRNA, or to other variables, including the amount of PB analysed, blood collected and its storage, PCR methodology and RT-PCR conditions (primers, markers, reagents, *etc.*).

In general, further conflicting findings regarding CTC detection in BC may arise from the non-uniformity of the

methodologies applied (*i.e.* direct versus indirect methods and markers used). Finally, the results are difficult to interpret and compare owing to the scarce knowledge of the functional biology of CTCs (4, 5). In particular, the time and mumber of CTC shed in the systemic circulation, besides their half-life in PB, not yet completely known, may contribute to the variability of results.

Therefore, in spite of the large number of studies, we still do not have a clear view of the clinical impact of CTCs. The concordance of results represents the basis conferring a clinical significance on CTCs and supports the application of CTC detection in routine clinical decisions.

In this regard, important progress has arisen from the use of an automated enrichment and immunocytochemical detection system (CellSearch system, Veridex, Warren, NJ, USA) reported by Riethdorf *et al*. providing evidence for a robust tool allowing standardized detection and quantification of CTCs in PB of patients with metastatic BC (29). In addition, the American Society of Clinical Oncology (ASCO), for the first time in 2007, included CTC detection among the specific markers recommended in the guidelines for the use of tumour markers in BC. However, ASCO advises that CTCs "should not be used to make the diagnosis or to influence any treatment decisions until further validation confirms the clinical value of this test" (30). On the basis of our experience, we are in line with ASCO guidelines and we believe that further research is necessary to standardize methodologies and to fill the gap in the incomplete understanding of the functional biology of CTCs in BC.

Acknowledgements

This work was supported by grants from Ricerca Sanitaria Regione Liguria, from Comitato Assistenza Malati e Lotta Contro i Tumori (Sarzana, Italy) and Associazione Italiana Contro le Leucemie/Linfoma e Mieloma (AIL, Sezione Francesca Lanzone, La Spezia, Italy). The Authors thank the staff of the Laboratory of Histopathology and Cytopathology of Sant'Andrea Hospital (La Spezia, Italy), Dr. Gaetano Leto for critically reading the manuscript, and Mrs. Anna Morabito for her valuable technical assistance.

References

1 Kohn EC and Liotta LA: Molecular insights into cancer invasion: strategies for prevention and intervention. Cancer Res *55*: 1856-1862, 1995.

2 Pretlow TG, Schwartz S, Giaconia JM, Wright AL, Grimm HA, Edgehouse NL, Murphy JR, Markowitz SD, Jamison JM, Summers JL, Hamlin CR, MacLennan GT, Resnick MI, Pretlow TP and Connell CF: Prostate cancer and other xenografts from cells in peripheral blood of patients. Cancer Res *60*: 4033-4036, 2000.

3 Pantel K, Alix-Panabières C and Riethdorf S: Cancer micrometastases. Nat Rev Clin Oncol *6*: 339-351, 2009.

4 Fehm T, Müller V, Alix-Panabières C and Pantel K: Micrometastatic spread in breast cancer: detection, molecular characterization and clinical relevance. Breast Cancer Res *10(Suppl 1)*: S1, 2008.

5 Paterlini-Brechot P and Benali NL: Circulating tumour cells (CTC) detection: clinical impact and future directions. Cancer Lett *253*: 180-204, 2007.

6 Dawood S, Broglio K, Valero V, Reuben J, Handy B, Islam R, Jackson S, Hortobagyi GN, Fritsche H and Cristofanilli M: Circulating tumour cells in metastatic breast cancer: from prognostic stratification to modification of the staging system? Cancer *113*: 2422-2430, 2008.

7 Lacroix M: Significance, detection and markers of disseminated breast cancer cells. Endocr Relat Cancer *13*: 1033-1067, 2006.

8 Watson MA and Fleming TP: Mammaglobin, a mammary-specific member of the uteroglobin gene family, is overexpressed in human breast cancer. Cancer Res *56*: 860-865, 1996.

9 Roncella S, Ferro P, Bacigalupo B, Dessanti P, Giannico A, Gorji N, Moroni M, Tozzini S, Pensa F, Gianquinto D, Fais F, Pronzato P and Fedeli F: Relationship between human mammaglobin mRNA expression in breast cancer tissue and clinico-pathologic features of the tumors. J Exp Clin Cancer Res *25*: 65-72, 2006.

10 Span PN, Waanders E, Manders P, Heuvel JJ, Foekens JA, Watson MA, Beex LV and Sweep FC: Mammaglobin is associated with low-grade, steroid receptor-positive breast tumors from postmenopausal patients, and has independent prognostic value for relapse-free survival time. J Clin Oncol *22*: 691-698, 2004.

11 Zach O, Kasparu H, Krieger O, Hehenwarter W, Girschikofsky M and Lutz D: Detection of circulating mammary carcinoma cells in the peripheral blood of breast cancer patients *via* a nested reverse transcriptase polymerase chain reaction assay for mammaglobin mRNA. J Clin Oncol *17*: 2015-2019, 1999.

12 Roncella S, Ferro P, Bacigalupo B, Pronzato P, Tognoni A, Falco E, Gianquinto D, Ansaldo V, Dessanti P, Fais F, Rosai J and Fedeli F: Human mammaglobin mRNA is a reliable molecular marker for detecting occult breast cancer cells in peripheral blood. J Exp Clin Cancer Res *24*: 265-271, 2005.

13 Cerveira N, Torres L, Rocha P, Bizarro S, Pereira D, Abreu J, Henrique R, Teixeira MR and Castedo S: Highly sensitive detection of the *MGB1* transcript (mammaglobin) in the peripheral blood of breast cancer patients. Int J Cancer *108*: 592-595, 2004.

14 Ignatiadis M, Kallergi G, Ntoulia M, Perraki M, Apostolaki S, Kafousi M, Chlouverakis G, Stathopoulos E, Lianidou E, Georgoulias V and Mavroudis D: Prognostic value of the molecular detection of circulating tumor cells using a multimarker reverse transcription-PCR assay for cytokeratin 19, mammaglobin A, and HER2 in early breast cancer. Clin Cancer Res *14*: 2593-2600, 2008.

15 Gargano G, Agnese V, Calò V, Corsale S, Augello C, Bruno L, La Paglia L, Gullo A, Ottini L, Russo A, Fulfaro F, Rinaldi G, Crosta A, Cicero G, Majorana O, Palmeri L, Cipolla C, Agrusa A, Gulotta G, Morello V, Di Fede G, Adamo V, Colucci G, Tomasino RM, Valerio MR, Bazan V and Russo A: Detection and quantification of mammaglobin in the blood of breast cancer patients: can it be useful as a potential clinical marker? Preliminary results of a GOIM (Gruppo Oncologico dell'Italia Meridionale) prospective study. Ann Oncol *17*: 41-45, 2006.

16 Grünewald K, Haun M, Urbanek M, Fiegl M, Müller-Holzner E, Gunsilius E, Dünser M, Marth C and Gastl G: Mammaglobin gene expression: a superior marker of breast cancer cells in peripheral blood in comparison to epidermal growth factor receptor and cytokeratin-19. Lab Invest *80*: 1071-1077, 2000.

17 Mikhitarian K, Martin RH, Ruppel MB, Gillanders WE, Hoda R, Schutte del H, Callahan K, Mitas M and Cole DJ: Detection of mammaglobin mRNA in peripheral blood is associated with high grade breast cancer: interim results of a prospective cohort study. BMC Cancer *8*: 55, 2008.

18 Lin YC, Chen SC, Hsueh S, Lo YF, Chow-Wu YH, Liaw IC and Cheng AJ: Lack of correlation between expression of human mammaglobin mRNA in peripheral blood and known prognostic factors for breast cancer patients. Cancer Sci *94*: 99-102, 2003.

19 Zehentner BK, Persing DH, Deme A, Toure P, Hawes SE, Brooks L, Feng Q, Hayes DC, Critichlow CW, Houghton RL and Kiviat NB: Mammaglobin as a novel breast cancerbiomarker: multigene reverse transcription-PCR assay and sandwich ELISA. Clin Chem *50*: 2069-2076, 2004.

20 Suchy B, Austrup F, Driesel G, Eder C, Kusiak I, Uciechowski P, Grill HJ and Giesing M: Detection of mammaglobin expressing cells in blood of breast cancer patients. Cancer Lett *158*: 171-178, 2000.

21 Marques AR, Teixeira E, Diamond J, Correia H, Santos S, Neto L, Ribeiro M, Miranda A and Passos-Coelho JL: Detection of human mammaglobin mRNA in serial peripheral blood samples from patients with non-metastatic breast cancer is not predictive of disease recurrence. Breast Cancer Res Treat *114*: 223-232, 2009.

22 Corradini P, Voena C, Astolfi M, Delloro S, Pilotti S, Arrigoni G, Bregni M, Pileri A and Gianni AM: Maspin and mammaglobin genes are specific markers for RT-PCR detection of minimal residual disease in patients with breast cancer. Ann Oncol *12*: 1693-1698, 2001.

23 Ntoulia M, Stathopoulou A, Ignatiadis M, Malamos N, Mavroudis D, Georgoulias V and Lianidou ES: Detection of Mammaglobin A mRNA-positive circulating tumour cells in peripheral blood of patients with operable breast cancer with nested RT-PCR. Clin Biochem *39*: 879-887, 2006.

24 Ring AE, Zabaglo L, Ormerod MG, Smith IE and Dowsett M: Detection of circulating epithelial cells in the blood of patients with breast cancer: comparison of three techniques. Br J Cancer *92*: 906-912, 2005.

25 Benoy IH, Elst H, Philips M, Wuyts H, Van Dam P, Scharpé S, Van Marck E and Vermeulen PB and Dirix LY: Real-time RT-PCR detection of disseminated tumour cells in bone marrow has superior prognostic significance in comparison with circulating tumour cells in patients with breast cancer. Br J Cancer *94*: 672-680, 2006.

26 Roncella S, Ferro P, Bacigalupo B, Dessanti P, Pronzato P, Franceschini MC, Pratticò L, Carletti AM, Canessa PA, Fontana V, Fais F, Pistillo MP and Fedeli F: Assessment of RT-PCR detection of human mammaglobin for the diagnosis of breast cancer derived pleural effusions. Diagn Mol Pathol *17*: 28-33, 2008.

27 Elston C.W and Ellis IO: Pathological prognostic factors in breast cancer. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology *19*: 403-410, 1991.

28 Hosmer DW and Lemeshow S: Applied Logistic Regression, 2nd Edition. Wiley & Sons, 2000.

29 Riethdorf S, Fritsche H, Müller V, Rau T, Schindlbeck C, Rack B, Janni W, Coith C, Beck K, Jänicke F, Jackson S, Gornet T, Cristofanilli M and Pantel K: Detection of circulating tumour cells in peripheral blood of patients with metastatic breast cancer: a validation study of the CellSearch system. Clin Cancer Res *13*: 920-928, 2007.

30 Harris L, Fritsche H, Mennel R, Norton L, Ravdin P, Taube S, Somerfield MR, Hayes DF and Bast RC Jr: American Society of Clinical Oncology. American Society of Clinical Oncology 2007 update of recommendations for the use of tumour markers in breast cancer. J Clin Oncol *25*: 5287-5312, 2007.

Received February 17, 2010
Revised April 22, 2010
Accepted April 27, 2010