

Detection of Mammaglobin A-mRNA-positive circulating tumor cells in peripheral blood of patients with operable breast cancer with nested RT-PCR

Maria Ntoulia^a, Aliko Stathopoulou^a, Michail Ignatiadis^b, Nikos Malamos^c,
Dimitris Mavroudis^b, Vassilis Georgoulas^b, Evi S. Lianidou^{a,*}

^a *Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, 15771, Greece*

^b *Department of Medical Oncology and Laboratory of Tumour Cell Biology, University Hospital of Heraklion, School of Medicine, University of Crete, 71110, Greece*

^c *Medical Oncology Unit, "Elena Venizelou" Hospital, Athens, 11521, Greece*

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Abstract

Objectives: The development and validation of a nested RT-PCR methodology for the detection of Mammaglobin A-mRNA-positive circulating tumor cells in peripheral blood of patients with operable breast cancer and evaluation of its prognostic significance.

Design and methods: Different combinations of specific primers were in silico designed and selected, so that false positive results due to genomic DNA contamination were avoided. The specificity of the primers used was evaluated in 30 healthy individuals, 20 patients with colorectal cancer and 20 patients with non-small cell lung cancer. The method was applied in 101 patients with operable breast cancer before the administration of adjuvant chemotherapy and 39 patients with metastatic breast cancer.

Results: Mammaglobin A-mRNA-positive cells were detected in 14/101 (13.9%) of early breast cancer patients but not in the control population studied (0%); 9 of them (64.3%) relapsed during the follow-up period. Mammaglobin A was detected in 7/39 (17.9%) of patients with verified metastasis. Multivariate analysis revealed the detection of Mammaglobin A-mRNA-positive cells, as an independent risk factor for reduced DFI.

Conclusions: Mammaglobin A is a highly specific molecular marker for the detection of circulating tumor cells in operable breast cancer, with important prognostic applications.

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Keywords: RT-PCR; Breast cancer; Circulating tumor cells; Peripheral blood; Mammaglobin A

Introduction

The detection of circulating tumor cells (CTCs) in the peripheral blood, bone marrow and lymph nodes is useful for determining prognosis and monitoring of the disease for early intervention in the treatment of breast cancer [1]. RT-PCR methodology has been successfully used to detect circulating breast cancer epithelial cells based on specific molecular markers and it has been shown that breast cancer patients

with metastatic disease have a significantly worse progression-free and overall survival when CTCs are detected in their peripheral blood [2]. Recently, our group has shown that the epithelial molecular marker cytokeratin 19 (CK-19) is an independent prognostic factor for breast cancer micrometastasis [3,4] and we have developed a real-time PCR methodology for the quantitation of CK-19 mRNA in the peripheral blood of early breast cancer patients [5].

In 1996, using a modified differential display technique, Watson and Fleming identified a novel gene, termed Mammaglobin [6]. Mammaglobin A is a highly specific marker for breast tissue since its expression is restricted to the adult

* Corresponding author. Fax: +30 210 7274750.

E-mail address: lianidou@chem.uoa.gr (E.S. Lianidou).

mammary gland and to mammary tumor cell lines and is overexpressed in primary human breast tumors as compared to normal breast tissue [7–9]. High levels of Mammaglobin A in primary breast tumors reflect a less aggressive tumor type and are correlated with the expression of estrogen and progesterone receptors, diploid DNA content, low Ki67 labeling index, low nuclear grade and absence of axillary nodal invasion [10]. Due to its breast-specific expression, Mammaglobin A is of high interest as a candidate diagnostic marker for breast cancer and is considered as the most promising molecular marker for breast cancer today [11]. Evaluation of Mammaglobin A as a molecular marker for lymph node metastasis through the detection of Mammaglobin A-mRNA in lymph nodes demonstrated the utility of Mammaglobin-specific RT-PCR for the detection of disseminated tumor cells (DTCs) [8,12–16]. The increased detection rate of RT-PCR for Mammaglobin relative to histological examination of axillary lymph nodes might identify patients at higher risk of relapse as compared with patients with negative RT-PCR results [17]. Bone marrow micrometastases detected by RT-PCR for Mammaglobin was shown to be a useful predictive marker for early distant recurrence of breast cancer [18–21].

The detection of Mammaglobin A in the peripheral blood of metastatic breast cancer patients or at the time of diagnosis using RT-PCR was first reported in 1999 [22] and revealed a 49% positive rate of Mammaglobin mRNA expression in patients with metastatic disease. Since then, many studies have shown the clinical utility of RT-PCR detection of Mammaglobin in the peripheral blood of breast cancer patients. In a report comparing the specificity and sensitivity of Mammaglobin A, epidermal growth factor receptor (EGFR) and CK-19 expression as markers for CTCs in the peripheral blood of metastatic breast cancer patients, Mammaglobin A-mRNA was found to be the most specific molecular marker for the hematogenous spread of breast cancer cells [23]. Mammaglobin A has been evaluated as a molecular marker in combination with other markers in a variety of multigene real-time RT-PCR assays in peripheral blood samples from advanced breast cancer patients and showed high sensitivity and specificity [2,13,14,16,23–27]. Recently, an immunomagnetic cell capture technique coupled with quantitative RT-PCR has been developed for the detection of circulating breast cancer cells in blood, combining a multigene panel [28]. In this paper, Reinholz et al. suggest that the molecular characterization of circulating epithelial cells by using Mammaglobin A and B305D-C offers potential for the early detection of invasive breast cancer [28]. The prognostic and therapeutic implication of Mammaglobin positivity, especially in clinically localized disease (stages I and II) should be evaluated after long-term clinical follow-up of these patients [29]. However, whether Mammaglobin A-mRNA can detect occult tumor cells before the presence of overt metastatic disease still remains elusive [30–32].

In this study, we report the development and validation of a nested RT-PCR methodology for the detection of Mammaglobin A-mRNA-positive occult tumor cells in peripheral blood of patients with operable breast cancer and we further evaluate its prognostic significance. Different combinations of specific

primers were in silico designed and evaluated, so that false positive results due to genomic DNA contamination were avoided. The specificity of the method was evaluated by analyzing peripheral blood samples of healthy female blood donors and patients with metastatic colorectal and non-small cell lung cancer. The method was applied for the detection of Mammaglobin A-mRNA-positive cells in the peripheral blood of 101 patients with operable breast cancer before the administration of adjuvant chemotherapy and 39 patients with metastatic breast cancer. In addition, the relationship between the expression of Mammaglobin A and clinical tumor patient characteristics was investigated.

Methods

Cell samples

The human mammary carcinoma cell lines MDA-MB-415, MDA-MB-435, T-47D, SK-BR-3 and MCF-7 (obtained from the American Type Culture Collection; ATCC) were used as positive controls. All cell lines were cultured and counted by trypan blue exclusion using a hemocytometer, as previously described [3].

Clinical samples

Twenty milliliters of peripheral blood in EDTA was obtained from 101 patients with operable breast cancer (stage I and II) after surgical removal of the primary tumor and before adjuvant chemotherapy, 39 patients with metastatic breast cancer, 20 patients with metastatic colorectal cancer, 20 patients with non-small cell lung cancer (NSCLC) and 30 female healthy volunteers (aged 18–65). To reduce blood contamination by epithelial cells from the skin, the first 5 mL of blood were discarded and the collection tube was at the end disconnected before withdrawing the needle. Peripheral blood samples from healthy blood donors and patients were collected and processed in the same manner. All patients and donors gave their informed consent and the study has been approved by the Ethical and Scientific Committees of the participating Institutions. The peripheral blood mononuclear cells (PBMC) were isolated within 1 h of venipuncture by gradient centrifugation with Ficoll Hypaque-1077 (Sigma Chemical Company, LTD, England) as previously described [3] and cell pellets were kept at -70°C until RNA extraction.

Total RNA isolation and cDNA synthesis

Total RNA isolation was performed by using Trizol LS reagent (Invitrogen, USA) according to the manufacturer's instructions. All preparation and handling steps of RNA took place in a laminar flow hood, under RNase-free conditions. The isolated RNA was dissolved in RNA storage buffer (Ambion, USA) and stored at -70°C until use. RNA concentration was determined, by using the RiboGreen RNA Quantitation Kit (Molecular Probes, Eugene, OR, USA), in the LightCycler (Roche Applied Science, Switzerland). The RNA quantification

Table 1
Sequences and positions of primers used in this study

Name	Sequence 5'–3'	Ref.	Position ^a	Junction	Homology with Mammaglobin B
F1	CAATCAATCCACAAGTgTCTAA^b	New	155–176	No	No
F2	ACggATgAAACTCTgAgCAATg^b	New	268–289	No	No
F3	AgCACTgCTACgCAggCTC	(22)	101–119	Yes	Yes
F4	TgCCATAgATgAATTgAAggAATg	(14)	231–254	No	No
F5	CAGCggCTTCCTTgATCCTTg	(23)	3–23	No	Yes
R1	AACATgTATAgCAggTTTCAAC^b	New	461–482	No	No
R2	CAGTTCTgTgAgCCAAAggT^b	New	354–373	No	No
R3	TgTCATATATTAATTgCATAAACACCTCA	(14)	291–319	Yes	No
R4	ATAAgAAAgAgAAggTgTgg	(23)	412–431	No	No
R5	TgTCATATATTAATTgCATAAAC	New	297–319	Yes	No
R6	TAgCAggTTTCAACAATTgTC	(7)	454–474	No	No

^a Mammaglobin A mRNA, Gene Bank Accession Number U33147.

^b Selected primers are shown in bold.

was performed in the following way: 5 µL of an RNA solution of known concentration supplied with the kit or the unknown sample was added along with 5 µL of the fluorophore RiboGreen reagent in LightCycler glass capillaries. A standard curve was created by using the relative fluorescence units of the RNA standard solutions as measured using the LightCycler Instrument in the Real Fluorimeter Mode (concentration range 5–500 ng/mL). Every sample was measured in triplicate and the RNA concentration was calculated with the use of the standard curve.

Reverse transcription of RNA was carried out with the THERMOSCRIPT RT-PCR System (Invitrogen, USA) according to the manufacturer's instructions, using 5 µg of total RNA as template. RNA integrity was tested in the cDNA preparation by real-time PCR amplification of the human hypoxanthine guanine phosphoribosyl transferase (HPRT) gene using the LightCycler h-HPRT gene set (Roche Diagnostics) according to the manufacturer's instructions.

Primer design

In the beginning, eleven different primers (Table 1) were checked in ten different combinations (Table 2). Six of these primers were previously described [7,14,22,23] while the

oligonucleotide sequences for five primers (F₁, F₂, R₁, R₂, R₅) were de novo designed In Silico by using the Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA) in order to avoid primer-dimer formation, false priming sites, formation of hairpin structures and homology with Mammaglobin B gene. The primer pairs that we finally selected (F1-R1 and F2-R2) among the ten different combinations were evaluated as shown in Table 2 and produced specific products only with Mammaglobin A and not with Mammaglobin B according to a search in the BLAST Sequence Similarity Search tool (NCBI, NIH) and did not amplify genomic DNA. All oligonucleotides were synthesized at the Microchemistry Laboratory of the Foundation for Research and Technology at Heraklion (FORTH), Crete, Greece.

Nested RT-PCR

The first PCR reaction was performed in a total volume of 25 µL. One microliter of cDNA was placed into a 24 µL reaction volume containing 1.25 µL of the sense primer F1 (3 µM), 1.25 µL of the antisense primer R1 (3 µM), 0.5 µL of dNTPs (10 mM), 1.25 µL of MgCl₂ (50 mM), 2.5 µL of 1× PCR buffer (10× concentration), 0.2 µL of Platinum Taq polymerase (Invitrogen, USA) (5U/µL) and 17.05 µL of DEPC-H₂O. PCR reaction was initiated with a 5-min denaturation at 94°C followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 30 s and extension at 72°C for 45 s, and a final extension step at 72°C for 5 min.

The second PCR reaction was also performed in a total volume of 25 µL. One microliter of the first PCR product was placed into a 24 µL reaction volume containing 1.25 µL of the sense primer F2 (3 µM), 1.25 µL of the antisense primer R2 (3 µM), 0.5 µL of dNTPs (10 mM), 1.25 µL of MgCl₂ (50 mM), 2.5 µL of 1× PCR buffer (10× concentration), 0.2 µL of Platinum Taq polymerase (5U/µL) and 17.05 µL of DEPC-H₂O. PCR reaction was initiated with a 5-min denaturation at 94°C followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 62°C for 30 s and extension at 72°C for 45 s, and a final extension step at 72°C for 5 min.

Table 2
Evaluation of specificity of primer combinations for Mammaglobin A-mRNA detection with nested RT-PCR

1st PCR	Product (bp)	2nd PCR	Product (bp)	Amplification of genomic DNA
F1-R1	328	F2-R2	106	No
F3-R3	219	F4-R3	89	Yes
F3-R4	331	F4-R3	89	Yes
F3-R5	219	F4-R3	89	Yes
F5-R4	429	F4-R3	89	Yes
F5-R6	472	F4-R3	89	Yes
F5-R5	317	F4-R3	89	Yes
F5-R3	317	F4-R3	89	Yes
F5-R4	429	F3-R5	219	Yes
F5-R6	472	F3-R5	219	Yes

Statistics

Data were analyzed by means of nonparametric tests. Differences were analyzed by two-tailed Chi-squared or Fischer's Exact Test, when appropriate. A *p*-value of less than 0.05 was considered as significant. Data processing was performed by means of SPSS for Windows (SPSS, Chicago, Illinois) and Statmost (DataMost Corp., USA). All clinical and biological tumor variables potentially associated with Mammaglobin A expression were individually tested in a univariate model. Those associations attaining or showing a trend towards statistical significance were subsequently included in a multivariate model, in which Mammaglobin A expression was taken as dependent variable. Multivariate and univariate analyses for prognostic factors were performed using the Cox proportional hazards models and Kaplan–Meier analysis with log rank tests.

Results

Selection of primer pairs

Ten different combinations of primer pairs were evaluated for their ability to amplify specifically only Mammaglobin A-mRNA and no traces of genomic DNA, coextracted with total RNA (Table 1). The primer pairs were selected so that in 9 out of 10 combinations, primers that hybridize to exon–exon boundaries (junctions) were used. Pure genomic DNA, extracted from peripheral blood of three healthy blood donors, was used as a positive control for genomic DNA to evaluate the specificity of these primer combinations for Mammaglobin A-mRNA. As can be seen in Table 2, only the first combination, involving the primer pairs F1-R1 and F2-R2, did not amplify genomic DNA, while the other nine combinations did. Due to the presence of the junction of exons 2 and 3 in this area, amplification of genomic DNA or unspliced mRNA generates a 1994-bp product in second PCR, whereas the corresponding sizes for Mammaglobin A-mRNA PCR products for the first

and second PCR were 328 bp and 106 bp, respectively. We further optimized selected primer pair concentrations, as well as reaction temperatures, Mg²⁺ concentration and incubation times.

Sensitivity

In order to define the sensitivity of the proposed assay, total RNA was prepared from 1×10^6 tumor cells (as counted in the hemocytometer) from the five cell lines studied (MDA-MB-415, MDA-MB-435, T-47D, SK-BR-3 and MCF-7). Serial dilutions of these RNA preparations in DEPC-treated water, corresponding to 1, 10, 10^2 , 10^3 and 10^4 tumor cells for each cell line were used for cDNA synthesis and these cDNAs were kept in aliquots at -20°C . As can be seen in Fig. 1, the expression of Mammaglobin A differs in the five cell lines studied. The nested RT-PCR assay could detect Mammaglobin A-mRNA corresponding to 1 MDA-MB-415 tumor cell, 10 SK-BR-3 and 10^3 MDA-MB-435 and T-47D tumor cells. Mammaglobin A-mRNA-positive cells were very faintly detected in MCF-7 cell line.

Specificity

To determine the specificity of the proposed assay, peripheral blood mononuclear cells (PBMC) from 30 healthy female blood donors, 20 patients with metastatic colorectal cancer, as well as 20 patients with NSCLC, were analyzed. None of those samples was found positive for Mammaglobin A-mRNA, indicating the high specificity of this primer combination for Mammaglobin A-mRNA.

Detection of Mammaglobin A-mRNA-positive cells in peripheral blood of patients with operable and metastatic breast cancer

Mammaglobin A-mRNA-positive cells were detected in the peripheral blood of 7 out of 39 (17.9%) patients with metastatic

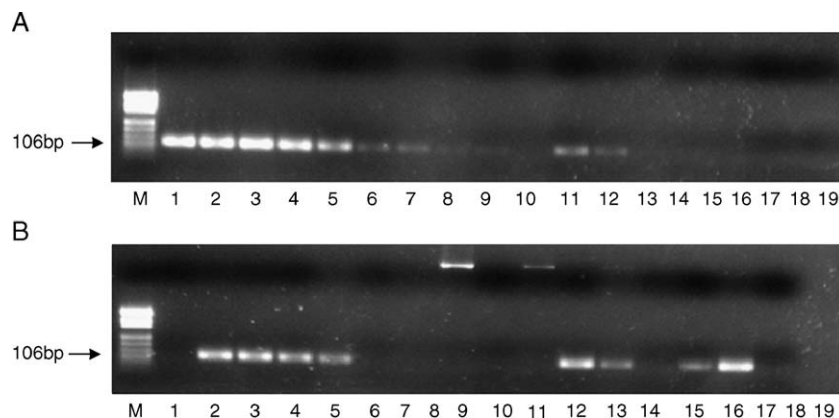


Fig. 1. Nested RT-PCR assay for Mammaglobin A-mRNA. (A) M: DNA ladder, 1: MDA-MB-415, 10^4 cells; 2: MDA-MB-415, 10^3 cells; 3: MDA-MB-415, 10^2 cells; 4: MDA-MB-415, 10 cells; 5: MDA-MB-415, 1 cell; 6: MDA-MB-435, 10^4 cells; 7: MDA-MB-435, 10^3 cells; 8: MDA-MB-435, 10^2 cells; 9: MDA-MB-435, 10 cells; 10: MDA-MB-435, 1 cell; 11: T-47D, 10^4 cells; 12: T-47D, 10^3 cells; 13: T-47D, 10^2 cells; 14: T-47D, 10 cells; 15: T-47D, 1 cell; 16: MCF-7, 10^4 cells; 17: MCF-7, 10^3 cells; 18: MCF-7, 10^2 cells; 19: MCF-7, 10 cells. (B) M: DNA ladder, 1: MCF-7, 1 cell; 2: SK-BR-3, 10^4 cells; 3: SK-BR-3, 10^3 cells; 4: SK-BR-3, 10^2 cells; 5: SK-BR-3, 10 cells; 6: SK-BR-3, 1 cell; 7–18: patients' samples; 19: negative control.

breast cancer. In order to evaluate the clinical significance of the presence of Mammaglobin A-mRNA-positive circulating tumor cells in peripheral blood of operable breast cancer patients, the proposed nested RT-PCR assay was applied in a total of 101 peripheral blood samples obtained from patients with operable breast cancer (stages I and II) before the administration of adjuvant chemotherapy. Mammaglobin A-mRNA-positive cells could be detected in the peripheral blood of 14 (13.9%) out of 101 patients with operable breast cancer (Fig. 1B).

The clinical and pathologic tumor characteristics of these patients, in respect to Mammaglobin A-mRNA expression in PBMC, are presented in Table 3. Their median age was 53.3 years and 57 (56.4%) patients were postmenopausal. Seventy (69.3%) patients had tumors measuring from 1 to 4 cm, 32 (31.7%) had one to three involved lymph nodes and 58 (57.4%) were estrogen receptor (ER) negative. Fischer's Exact Test analyses showed that there was no statistically significant association between the detection of Mammaglobin A-mRNA-

positive cells and the patients' menstrual status, stage of the disease, size and histological grade of the tumor, number of involved lymph nodes, estrogen or progesterone receptor positivity (Table 3).

During the follow-up period (median follow-up period: 24 months; range: 1–80 months), 36 (35.6%) out of 101 patients with operable breast cancer developed distant metastases. Nine (64.3%) out of 14 patients that were found positive for Mammaglobin A-mRNA and 27 (31%) out of 87 patients that were found negative, relapsed. These results indicate that there is statistically significant higher probability ($p=0.01$) that Mammaglobin A positive patients will relapse (Table 4). During the same follow-up period, 22 (21.8%) of these 101 patients, died of breast cancer (Table 4).

Univariate analysis has shown that the presence of Mammaglobin A-positive cells in the blood, as well as, the presence of more than four involved axillary lymph nodes, tumor size, premenopausal status, ER-negative and PR-negative status were associated with a decreased disease-free interval (DFI) ($p<0.05$)

Table 3
Early breast cancer patient characteristics and detection of Mammaglobin A-mRNA-positive cells in peripheral blood

	All patients		Mammaglobin mRNA ⁺		Mammaglobin mRNA ⁻		<i>p</i> -value ^a
	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	
Patients enrolled	101	–	14	13.9	87	86.1	
<i>Age, years</i>							
Median	53		56		52		0.750
Range	(26–75)		(45–75)		(26–74)		
<i>Menopausal status</i>							
Premenopausal	44	43.6	5	11.4	39	88.6	0.190
Postmenopausal	57	56.4	9	15.8	48	84.2	
<i>Tumor size (cm)</i>							
≤1	4	4	0	–	4	100	0.242
1–1.9	14	13.9	1	7.1	13	92.9	
2.0–3.9	56	55.5	7	50	7	50	
≥4.0	25	24.8	6	24	19	76	
Unknown	2	2	0	0	2	100	
<i>Grade</i>							
I	6	5.9	1	16.7	5	83.3	0.234
II	45	44.6	6	13.3	39	86.7	
III	45	44.6	6	13.3	39	86.7	
Unknown	5	5	1	20	4	80	
<i>Lymph nodes</i>							
0	24	23.8	3	12.5	21	87.5	0.201
1–3	32	31.7	4	12.5	28	87.5	
≥4	44	43.6	7	15.9	37	84.1	
Unknown	1	1	0	–	1	100	
<i>Receptor status</i>							
ER+	40	36.6	5	12.5	35	87.5	0.244
ER–	58	57.4	7	12.1	51	87.9	
ER unknown	3	3	2	66.7	1	33.3	
PR+	36	35.6	4	11.1	32	88.9	0.222
PR–	62	61.4	9	14.5	53	85.5	
PR unknown	3	3	1	33.3	2	66.6	

Abbreviations: ER, estrogen receptor; PR, progesterone receptor

^a Fischer's Exact Test.

Table 4
Disease relapses and deaths in operable breast cancer patients in respect to Mammaglobin A-mRNA status

Mammaglobin A-mRNA	Relapses			Deaths		
	<i>n</i>	%	<i>p</i> -value ^a	<i>n</i>	%	<i>p</i> -value ^a
Positive (<i>n</i> =14)	9	64.3		3	21.4	
Negative (<i>n</i> =87)	27	31	0.01	19	21.9	0.271

^a Fischer's Exact Test.

(Table 5). The Kaplan–Meier estimates of the cumulative DFI for Mammaglobin A-mRNA-positive and Mammaglobin A-mRNA-negative groups were significantly different (log rank test, $p=0.0291$) in favor of patients who were Mammaglobin A-mRNA-negative in the peripheral blood (Fig. 2A).

As shown in Table 5, only tumor size was associated with decreased overall survival. The Kaplan–Meier estimates of the OS rates for Mammaglobin A-mRNA-positive and Mammaglobin A-mRNA-negative groups were not significantly different (log rank test, $p=0.5630$) (Fig. 2B). In the Cox proportional hazards analysis, tumor size was the only significant parameter influencing overall survival, since the hazard of death was increased by a factor of 1.307 for each extra centimeter of tumor size (Table 6).

Furthermore, the multivariate application of the Cox proportional model yielded only four factors with significant independent influence on the DFI: the presence of Mammaglobin A-mRNA-positive cells in the peripheral blood, tumor size, four or more involved lymph nodes and ER-negative status (Table 6).

Table 5
Predictive and prognostic factors by univariate analyses (unadjusted relative risks) for DFI and OS

Parameter	Log rank test	<i>p</i> -value	Hazard ratio	<i>p</i>	95% CI
<i>Disease-free interval</i>					
Premenopausal status (post)	4.42	0.0356	2.106	0.041	1.031–4.302
Tumor size	–	–	1.178	0.020	1.026–1.354
Lymph nodes (≥4 positive)	7.84	0.0051	2.665	0.007	1.303–5.451
Histology grade (III)	2.46	0.1166	1.746	0.123	0.861–3.542
ER (negative)	4.15	0.0415	2.079	0.047	1.010–4.283
PR (negative)	5.43	0.0198	2.484	0.025	1.122–5.497
Mammaglobin A (positive)	4.76	0.0291	2.341	0.035	1.061–5.165
<i>Overall survival</i>					
Premenopausal status (post)	0.92	0.3386	1.555	0.343	0.625–3.872
Tumor size	–	–	1.278	0.009	1.064–1.534
Lymph nodes (≥4 positive)	1.59	0.2076	1.801	0.214	0.712–4.556
Histology grade (III)	1.87	0.1714	1.909	0.179	0.744–4.898
ER (negative)	0.75	0.3853	1.503	0.389	0.595–3.798
PR (negative)	0.53	0.4663	1.427	0.469	0.545–3.732
Mammaglobin A (negative)	0.33	0.5630	1.538	0.566	0.353–6.693

Discussion

Mammaglobin A is a very promising specific diagnostic tumor marker for breast cancer and can become an important tool for detecting primary and metastatic breast cancer, monitoring lymph nodes during and after surgery and predicting disease outcome and recurrence. However, the clinical relevance and application of Mammaglobin-A-specific assays still has to be evaluated in larger prospective studies [26]. The detection of Mammaglobin A-mRNA-positive cells in peripheral blood of

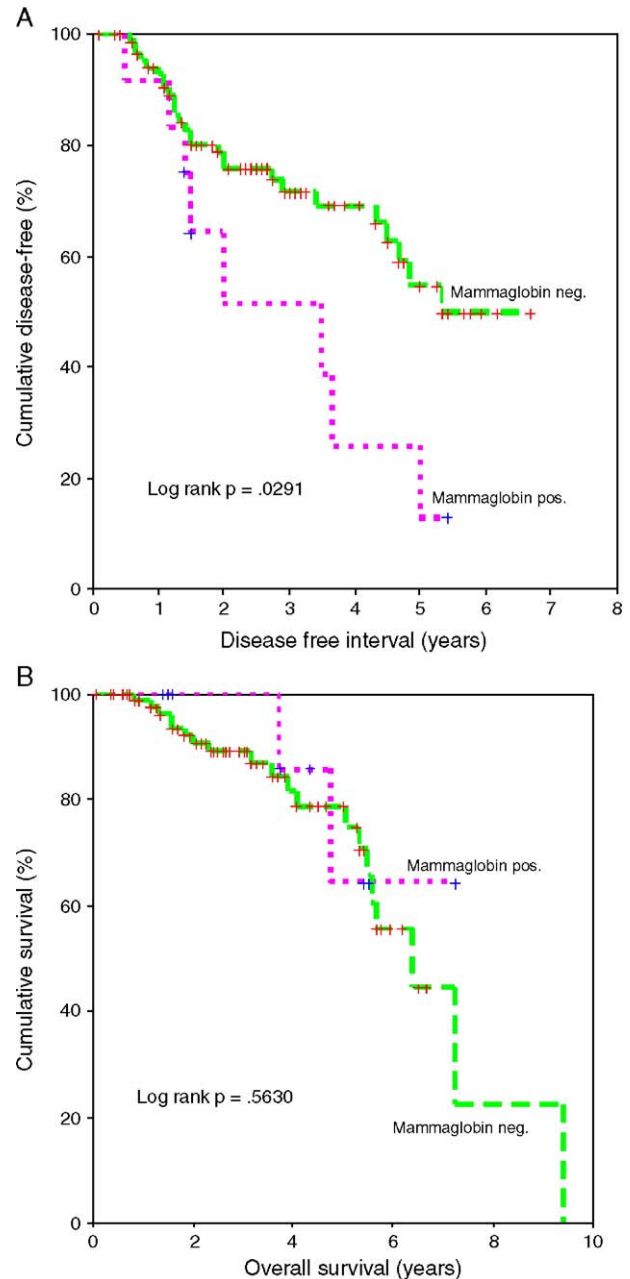


Fig. 2. (A) Kaplan–Meier estimates of the cumulative DFI for the patients with Mammaglobin A-mRNA-positive and Mammaglobin A-mRNA-negative cells in the peripheral blood. (B) Kaplan–Meier analysis of the OS for the patients with Mammaglobin A-mRNA-positive and Mammaglobin A-mRNA-negative cells in the peripheral blood.

Table 6
Independent predictive and prognostic factors by multivariate analysis for DFI and OS

Parameter	Hazard	<i>p</i> -value	95% CI
<i>Disease-free interval</i>			
Mammaglobin A (positive)	2.840	0.020	1.182–6.828
Tumor size (cm)	1.216	0.021	1.030–1.436
Estrogen receptor (negative)	2.497	0.029	1.096–5.693
Lymph nodes (≥ 4 positive)	2.316	0.037	1.050–5.104
<i>Overall survival</i>			
Tumor size (cm)	1.307	0.009	1.068–1.599

advanced breast cancer patients has been extensively evaluated [22,23,25,29]. However, the clinical utility of the detection of Mammaglobin A-mRNA-positive cells in peripheral blood of operable breast cancer patients still remains elusive, since most studies report conflicting results with different percentages of Mammaglobin A-mRNA positivity [22,30,32]. This could be partially explained by the differences in methodology used primarily due to the primer sequences selected for Mammaglobin A-mRNA amplification.

In the present study, ten different primer pair combinations were initially evaluated for their specificity for Mammaglobin A-mRNA by a nested PCR approach. The specificity of the developed assay was based on the selection of a primer pair combination that was *in silico* designed and based on the exclusion of amplification of highly homologous Mammaglobin B as well as of genomic DNA traces, coextracted with total RNA. It is important to note that despite the fact that primer R3 hybridizes on the junction of exons 2/3, it does amplify genomic DNA (Table 2). Although this was quite unexpected, it could partly be explained by the fact that it is a quite long primer (29 bases) and 13 bases of its 3' end can hybridize to the exon 2 of genomic DNA. It is also interesting to note that despite the fact that both primers F3 and R5 are designed on exon–intron boundaries and do not show homology with Mammaglobin B, they do give an amplification product with genomic DNA (Table 2). The selected primer pairs are not located on exon junctions; however, the amplification of genomic DNA was completely avoided because of a significant difference in size of the expected PCR products. Moreover, none of the selected primers had homology with Mammaglobin B, so when the PCR products were run in a gel no other non-specific bands were observed besides the appropriate 106-bp band (Fig. 1). The high degree of specificity of the method is supported by the fact that no expression was detected in the PBMC of healthy donors and patients with other neoplastic diseases.

The sensitivity of the developed nested RT-PCR assay was evaluated by using five different breast cancer cell lines. RT-PCR could detect 1 MDA-MB-415, 10 SK-BR-3, 10^3 MDA-MB-435 and 10^3 T-47D cells (Fig. 1A). Mammaglobin A-mRNA-positive cells were very faintly detected in the MCF-7 cell line. These results are in agreement with other reports that have also used MDA-MB-415, MDA-MB-435, T-47D and SK-BR-3 cancer cell lines [6,19,31] and MCF-7 cell line [20,22].

Mammaglobin A-mRNA-positive cells were detected in the peripheral blood of 14 (13.9%) out of 101 patients with operable breast cancer before adjuvant chemotherapy and 7 (17.9%) out of 39 patients with metastatic breast cancer. The peripheral blood samples of our early breast cancer patients were collected 4–6 weeks after surgery. So it is unlikely, that these cells disseminated because of surgery, since tumor cells shed in peripheral blood after surgery are very quickly eliminated and are not detected after 2–3 weeks [1]. These data are in agreement with other reports that have also shown low positivity for Mammaglobin A-mRNA [23,30]. On the contrary, there are investigators who have shown a higher detection rate for Mammaglobin A-mRNA in peripheral blood from early breast cancer patients by using nested RT-PCR [22], immunomagnetic enrichment of epithelial cells followed by real-time RT-PCR assay [26,28] or after synthesizing cDNA with gene-specific primers [29]. The clinical utility of circulating tumor cells in peripheral blood has been recently shown in metastatic breast cancer patients [33]. Moreover, a recent study has clearly verified the prognostic significance of the presence of disseminated tumor cells in the bone marrow of breast cancer patients [34].

According to our findings, the detection of Mammaglobin A-mRNA-positive cells in peripheral blood of operable breast cancer patients was significantly associated with clinical relapse; 9 clinical relapses were observed among the 14 Mammaglobin A-mRNA-positive patients (64.3%, $p=0.01$). Among the 101 patients with operable breast cancer studied, 36 (35.7%) relapsed. Nine (25%) out of these 36 patients that developed distant metastases were found positive for Mammaglobin A-mRNA-positive cells in the peripheral blood. Moreover, the presence of Mammaglobin A in patients with operable breast cancer could be related with the use of adjuvant chemotherapy. As can be seen in Table 4, among the 87 patients who were negative for Mammaglobin A, there were 27 patients that later relapsed. This could be explained by the fact that since not all breast cancer cells express Mammaglobin, tumor cells that are shed from Mammaglobin-negative tumors will not be detected using Mammaglobin-specific RT-PCR [35].

However, the question whether Mammaglobin detection in CTCs can improve management of patients with operable breast cancer can only be answered through a future large prospective study.

More importantly, the detection of Mammaglobin A-mRNA-positive cells in the blood had prognostic implications for the patients with stages I and II breast cancer because it was associated with reduced DFI ($p=0.0291$). The Cox proportional hazards analysis indicated that the detection of Mammaglobin A-mRNA-positive cells in the peripheral blood, the premenopausal status, the number of involved axillary lymph nodes, the tumor size and the status of ER and PR expression by the tumor were the only important factors affecting duration of DFI, while this was not the case for overall survival, where the only factor was tumor size. In the multivariate analysis, the presence of Mammaglobin A-mRNA-positive cells in the peripheral blood, the number of involved axillary lymph nodes, the tumor size and the status of ER expression by the tumor were the only

important factors affecting duration of DFI, while this was not the case for OS, where the only factor was also tumor size. These results clearly demonstrate the significant and independent prognostic value of Mammaglobin A-mRNA detection by RT-PCR in the peripheral blood of patients with stages I and II breast cancer. Our results are in accordance with very recently published information which has shown that Mammaglobin A detection in PBMCs represents a negative prognostic marker in breast cancer [36].

In conclusion, the most interesting finding of the present work is the high incidence of relapses in the group of patients with operable breast cancer who displayed Mammaglobin A-mRNA-positive cells in their peripheral blood. We also found that the detection of Mammaglobin A-mRNA-positive cells in the peripheral blood of patients with stages I and II breast cancer before the initiation adjuvant chemotherapy is a significant adverse prognostic factor associated with shortened DFI. We strongly believe that Mammaglobin's prognostic potential could be further increased by using an additional cell enrichment step, such as antibody coated magnetic particles and moreover by using a combination of tumor biomarkers [28]. We conclude that Mammaglobin A-mRNA is an important molecular marker for the detection of occult mammary carcinoma cells in the peripheral blood of patients with operable breast cancer before the administration of adjuvant chemotherapy.

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