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

Michail Ignatiadis,[1,3] Galatea Kallergi,[3] Maria Ntoulia,[5] Maria Perraki,[3] Stella Apostolaki,[3] Maria Kafousi,[2] Grigorios Chlouverakis,[4] Efstathios Stathopoulos,[2] Evi Lianidou,[5] Vassilis Georgoulias,[1,3] and Dimitris Mavroudis[1,3]

Abstract

Purpose: To investigate the prognostic value of the molecular detection of circulating tumor cells (*CTCs*) using three markers [cytokeratin 19 (*CK19*), mammaglobin A (*MGB1*), and *HER2*] in early breast cancer.

Experimental Design: CK19mRNA+, MGB1mRNA+, and HER2mRNA+ cells were detected using real-time (*CK19*) and nested (*MGB1* and *HER2*) reverse transcription-PCR in the peripheral blood of 175 women with stage I to III breast cancer before the initiation of adjuvant chemotherapy. The detection of CTCs was correlated with clinical outcome. In 10 patients, immunofluorescence staining experiments were done to investigate the coexpression of cytokeratin, *MGB1,* and *HER2* in CTCs.

Results: CK19mRNA+, *MGB1*mRNA+, and *HER2*mRNA+ cells were detected in 41.1%, 8%, and 28.6% of the 175 patients, respectively. Patients had one of the following molecular profiles: CK19mRNA+/*MGB1*mRNA+/*HER2*mRNA+ (n = 8), CK19mRNA+/*MGB1*mRNA+/*HER2*mRNA- (n = 1), CK19mRNA+/*MGB1*mRNA-/*HER2*mRNA+ (n = 42), CK19mRNA+/*MGB1*mRNA-/*HER2*mRNA- (n = 21), CK19mRNA-/*MGB1*mRNA+/*HER2*mRNA- (n = 5), and CK19mRNA-/*MGB1*mRNA-/*HER2*mRNA- (n = 98). Double-immunofluorescence experiments confirmed the following CTC phenotypes: CK+/*MGB1*+, CK+/*MGB1*-, CK-/*MGB1*+, CK+/*HER2*+, CK+/*HER2*-, *MGB1*+/*HER2*-, and *MGB1*+/*HER2*+. In univariate analysis, the detection of CK19mRNA+, *MGB1*mRNA+, and HER2mRNA+ cells was associated with shorter disease-free survival (DFS; $P < 0.001$, $P = 0.001$, and $P < 0.001$, respectively), whereas the detection of CK19mRNA+ and *MGB1*mRNA+ cells was associated with worse overall survival ($P = 0.044$ and 0.034, respectively). In multivariate analysis, estrogen receptor–negative tumors and the detection of *CK19*mRNA+ and *MGB1*mRNA+ cells were independently associated with worse DFS.

Conclusion: The detection of peripheral blood *CK19*mRNA+ and *MGB1*mRNA+ cells before adjuvant chemotherapy predicts poor DFS in women with early breast cancer.

Authors' Affiliations: Departments of [1]Medical Oncology and [2]Pathology, University General Hospital of Heraklion; [3]Laboratory of Tumor Cell Biology and [4]Department of Biostatistics, School of Medicine, University of Crete, Crete, Greece and [5]Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, Athens, Greece

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Requests for reprints: Dimitris Mavroudis, Department of Medical Oncology, University General Hospital of Heraklion, P. O. Box 1352, Heraklion 711 10, Crete, Greece. Phone: 30-2810-392823; Fax: 30-2810-392802; E-mail: mavrudis@med.uoc.gr.

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Breast cancer is considered a systemic disease because early tumor dissemination may occur even in patients with small tumors. Micrometastases, which are undetectable by the classic imaging and laboratory studies, can contribute to disease relapse; therefore, their identification in patients with early breast cancer may have substantial effect on determining prognosis and individualizing treatment strategies for these patients. For this purpose, epithelial cells have been identified in the bone marrow (disseminated tumor cells) or the peripheral blood [circulating tumor cells (*CTCs*)] of patients with stage I to III breast cancer using immunocytochemistry or molecular techniques (1–4).

Cytokeratin 19 (*CK19*), a cytoskeletal protein expressed on epithelial but not on mesenchymal cells, has been the marker most extensively studied for the detection of CTCs (3, 5). Using a highly sensitive and specific real-time reverse transcription-PCR (RT-PCR) assay (6), we showed that the detection of

CK19mRNA+ cells before the initiation of adjuvant chemotherapy was an independent prognostic factor for disease recurrence and decreased survival in patients with early breast cancer (7, 8). However, microarray and comparative genomic hybridization studies have shown that breast cancer is a genetically heterogeneous disease (9, 10) and that even the micrometastatic cells of any given patient with early breast cancer may exhibit diverse genomic profiles (11). Moreover, it is known that ~50% of patients with early breast cancer and detectable bone marrow disseminated tumor cells do not relapse even 10 years after diagnosis, whereas there is a significant proportion of patients without detectable disseminated tumor cells who still relapse (12). To increase the sensitivity of detection of CTCs as well as to define clinically relevant subpopulations of CTCs with aggressive biological behavior that could be used more precisely as surrogate markers for relapse, several approaches have been used: (*a*) the use of other markers apart from cytokeratins for detection of minimal residual disease [e.g., mucins, mammaglobin A (*MGB1*), maspin, carcinoembryonic antigen, HER2, and epidermal growth factor receptor; refs. 13–17], (*b*) the use of different multimarker RT-PCR assays (18, 19), and (*c*) the use of assays to distinguish between apoptotic and nonapoptotic or viable micrometastatic cells (20, 21).

Here, we sought to improve the prognostic ability of our CK19mRNA assay by simultaneously studying two additional markers (i.e., *MGB1* and *HER2*). *MGB1*, a member of a family of epithelial secretory proteins, the uteroglobulins, is considered to be a specific breast marker (22). *MGB1* has been studied as a marker for the molecular detection of CTCs using different assays mostly in small patient cohorts, consisting of primary and metastatic breast cancer patients who have not been homogeneously treated (14, 18, 23, 24). Therefore, its role as a prognostic marker in early breast cancer still remains unclear. *HER2*/neu gene amplification in breast cancer cells was shown to be associated with resistance to treatment and impaired survival (25–27). Furthermore, we and others have shown that HER2-positive micrometastatic cells define a subpopulation of patients with a high propensity to develop distant metastasis (28, 29).

In the present study, we evaluated the prognostic value of a multimarker RT-PCR assay by simultaneously studying *CK19*, *MGB1*, and *HER2* for the molecular detection of CTCs in women with early breast cancer.

Materials and Methods

Patients. A total of 175 patients who had all received adjuvant chemotherapy for stage I to III breast cancer at the Medical Oncology Department of the University Hospital of Heraklion (Crete, Greece) were included in this retrospective study. For this study, we used frozen RNA samples from the biobank of the laboratory of Tumor Cell Biology for which enough material was available to simultaneously do the three markers plus one housekeeping gene analysis. The 175 patients included in this study were randomly chosen and not selected based on any predefined criteria so that our results would not be biased by any sort of selection criteria. For every patient enrolled, a complete diagnostic evaluation to exclude the presence of distant metastasis was done consisting of chest X-rays, mammography, ultrasound of the liver, and a whole-body bone scan. Computed tomography scans and/or magnetic resonance imaging studies were done if clinically indicated. Surgical treatment was either mastectomy or lumpectomy with axillary lymph node dissection. Radiation treatment was delivered to patients with lumpectomy and those with four or more involved axillary lymph nodes. The administration of adjuvant chemotherapy and hormonal treatment was decided independently of the molecular detection of CTCs. All patients included in this study received adjuvant chemotherapy and most of them were treated in the context of research protocols of the Hellenic Oncology Research Group. Adjuvant chemotherapy regimens consisted of either FEC [5-fluorouracil (700 mg/m^2, day 1) + epirubicin (75 mg/m^2, day 1) + cyclophosphamide (700 mg/m^2, day 1) every 3 weeks for six cycles] or EC-T [epirubicin (75 mg/m^2, day 1) + cyclophosphamide (700 mg/m^2, day 1) every 3 weeks for four cycles followed by docetaxel (100 mg/m^2, day 1) every 3 weeks for four additional cycles] or classic CMF [cyclophosphamide (100 mg/m^2, orally, days 1-14), methotrexate (40 mg/m^2, days 1 and 8), and fluorouracil (600 mg/m^2, days 1 and 8) every 4 weeks for six cycles]. Patients with HER2-positive tumors did not receive adjuvant trastuzumab because all patients were enrolled before the positive results from the adjuvant trastuzumab trials were reported (30–32). All patients with estrogen receptor (ER)-positive and/or progesterone receptor (PR)-positive tumors received 20 mg tamoxifen daily for 5 years; premenopausal women also received luteinizing hormone-releasing hormone analogues for 2 years. Patient follow-up consisted of clinical examination with laboratory and imaging studies every 3 months for the first 2 years, every 6 months for the next 3 years, and yearly thereafter. All patients signed an informed consent to participate in the study, which was approved by the ethics and scientific committees of our institution.

Clinical samples and RT-PCR assays for the detection of CK19mRNA+, MGB1mRNA+, and HER2mRNA+ cells. Peripheral blood (20 mL in EDTA) was obtained from every patient 3 to 4 weeks after primary surgery and before the initiation of any adjuvant treatment. To avoid contamination with epithelial cells from the skin, all blood samples were obtained at the middle of vein puncture after the first 5 mL of blood were discarded.

The procedures of RNA extraction and cDNA synthesis have already been described elsewhere (6, 7). The quality of cDNA was confirmed by amplification of the *GAPDH* housekeeping gene for each individual sample. The sequence of the primers as well as the size of the amplified products for the three studied genes (*CK19*, *MGB1*, and *HER2*) are depicted in Supplementary Table S1.

The real-time RT-PCR assay for CK19mRNA+ cells and the primers used have been previously described in detail and were used in this study without any modification (6, 7). According to the analytic detection limit of our assay, the presence of ≥0.6 MCF-7 equivalents/5 μg of total RNA was considered a positive result. The nested RT-PCR assays for the detection of MGB1mRNA+ and HER2mRNA+ cells, respectively, have been used without any modification as previously described (14, 15, 33). The sensitivity of the multimarker assay was tested in spiking experiments with the MCF-7 and SKBR3 cell lines. In three of four experiments, this assay could detect 1 MCF-7 or 1 SKBR3 cell among 10^6 normal peripheral blood mononuclear cells (PBMC). The specificity of the multimarker RT-PCR assay was investigated in 31 healthy women. Among them, 30 were negative for all three markers, whereas 1 woman had the CK19mRNA+/MGB1mRNA-/HER2mRNA- molecular profile.

Immunofluorescent detection of CTCs—confocal laser scanning microscopy. PBMCs were isolated with Ficoll-Hypaque density gradient (d = 1.077 g/mol) centrifugation at 1,800 rpm for 30 min. The isolated PBMCs were washed with PBS and centrifuged at 1,500 rpm for 10 min. Aliquots of 250,000 cells were centrifuged on glass slides.

Double-staining experiments were done to investigate the expression of cytokeratin, *MGB1*, and *HER2* in PBMC cytospins of 10 breast cancer patients. Two different pancytokeratin antibodies were used: A45-B/B3, an anti-mouse monoclonal specific for CK8, CK18, and *CK19* (Micromet), or a pancytokeratin anti-rabbit polyclonal (Santa Cruz Biotechnology) depending on the second examined antigen as previously described (34). All the slides were also stained for 4′,6-diamidino-2-phenylindole

(Invitrogen). Specifically, PBMC cytospins were fixed with cold acetone/methanol 9:1 for 20 min and stained for cytokeratin with the appropriate antibody. Consequently, the same slide was incubated with *MGB1* anti-goat polyclonal (Santa Cruz Biotechnology) or *HER2* anti-mouse monoclonal antibodies (Oncogene), respectively, for 45 min. Cells were then exposed for 1 h to the corresponding secondary antibodies: rhodamine anti-rabbit (Chemicon International, Inc.) for pancytokeratin, FITC anti-mouse (Chemicon International) for A45-B/B3 or *HER2*, and rhodamine anti-goat (Chemicon International) for *MGB1*. Double-staining experiments were done also for *MGB1* and *HER2* following the same protocol. All slides were evaluated using a confocal laser scanning microscope module (Leica Lasertechnik), and images were analyzed with the respective software. Each marker was also tested in combination with anti-CD45 (Santa Cruz Biotechnology) to exclude the presence of marker coexpression on hematopoietic cells.

Primary tumor immunohistochemistry for HER2. *HER2* expression of the primary tumors was detected by immunohistochemistry (IHC) with the monoclonal antibody CB11 (Novocastra) using the Optimax automated system (Biogenex Laboratories). Scoring was based on the criteria recommended by DAKO A/S for the HercepTest.

Statistical analysis. Summary descriptive statistics were expressed as mean (SD) or percent, as appropriate. Continuous variables were compared between the two groups with unpaired *t* test and categorical data with χ^2 or Fisher's exact test, as appropriate. The time from study entry until the day of the first evidence of disease recurrence [disease-free survival (DFS)] and the time from study entry to death [overall survival (OS)] were the main dependent variables of the study. DFS and OS curves for subgroups of patients were constructed using the Kaplan-Meier product limit estimate method (35) and compared by the log-rank test to provide a univariate assessment of the prognostic value of selected clinical risk factors, measured at study entry. These log-rank comparisons were done between subgroups containing at least 20 patients each. Clinicopathologic factors known to be associated with prognosis, such as menopausal status (premenopausal versus postmenopausal), tumor size (T2-3 versus T1), axillary nodal status (positive versus negative), histology grade (3 versus 1-2), ER status (negative versus positive), PR status (negative versus positive), HER2 status (positive versus negative), chemotherapy regimen (FEC/EC-T versus CMF), and additionally the detection of CK19mRNA+ cells (yes versus no), MGB1mRNA+ cells (yes versus no), and HER2mRNA+ cells (yes versus no), were tested in univariate analysis. Variables that were found to be significant at the univariate screen were then entered in a stepwise backward multivariate Cox proportional hazards regression model to identify those with independent prognostic information for DFS and OS (36). Entry into and removal from the model were set at 5% and 10%, respectively. All statistical tests were done at the 5% level of significance. The Statistical Package for the Social Sciences 15 statistical software was used for the analysis.

Results

Patient characteristics and detection of CTCs. Patients' characteristics are shown in Table 1. The patients' median age

Table 1. Patient characteristics

	All patients, *n* (%)	CK19mRNA+, *n* (%)	MGB1mRNA+, *n* (%)	HER2mRNA+, *n* (%)
Patients enrolled	175	72 (41.1)	14 (8)	50 (28.6)
Age (y)				
Median (min/max)	54 (28-75)	54.5 (28-74)	57.5 (37-75)	55 (28-74)
Menopausal status				
Premenopausal	72 (41.1)	31 (43.1)	4 (5.6)	21 (29.2)
Postmenopausal	103 (58.9)	41 (39.8)	10 (9.7)	29 (28.2)
Tumor size (cm)				
≤2.0	58 (33.1)	21 (36.2)	1 (1.7)	14 (24.1)
>2.0	117 (66.9)	51 (43.6)	13 (11.1)	36 (30.8)
Histology grade				
1/2	84 (48)	35 (41.7)	8 (9.5)	24 (28.6)
3	78 (44.6)	31 (39.7)	4 (5.1)	23 (29.5)
Lobular/unknown	13 (7.4)	6	2	3
Lymph nodes				
0	56 (32)	24 (42.9)	4 (7.1)	16 (28.6)
1-3	49 (28)	19 (38.8)	4 (8.2)	14 (28.6)
≥4	70 (40)	29 (41.4)	6 (8.6)	20 (28.6)
ER				
Positive	101 (57.7)	41 (40.6)	7 (6.9)	29 (28.7)
Negative	72 (41.1)	31 (43.1)	7 (9.7)	21 (29.2)
Unknown	2 (1.1)			
PR				
Positive	67 (38.3)	31 (46.3)	5 (7.5)	21 (31.3)
Negative	106 (60.6)	41 (38.7)	9 (8.5)	29 (27.4)
Unknown	2 (1.1)			
HER2/neu status				
Negative (0, 1+, 2+ IHC)	124 (70.9)	55 (44.4)	10 (8.1)	38 (30.6)
Positive (3+ IHC)	48 (27.4)	16 (33.3)	3 (6.3)	11 (22.9)
Unknown	3 (1.7)	1	1	1
Surgery				
Lumpectomy	117 (66.9)	47 (40.2)	9 (7.7)	33 (28.2)
Mastectomy	58 (33.1)	25 (43.1)	5 (8.6)	17 (29.3)
Chemotherapy				
CMF	26 (14.9)	11 (42.3)	3 (11.5)	8 (30.8)
FEC	95 (54.3)	39 (41.1)	7 (7.4)	27 (28.4)
EC-T	54 (30.9)	22 (40.7)	4 (7.4)	15 (27.8)
Relapses	49 (28)	32 (65.3)	9 (18.4)	25 (51)
Deaths	26 (14.9)	16 (61.5)	5 (19.2)	11 (42.3)

was 54 years (range, 28-75 years) and 66.9% had undergone a lumpectomy. Among the 175 patients, 41.1% were premenopausal, 66.9% had tumors >2 cm, 44.6% were histology grade 3, 41.1% were *ER negative,* and 68% had infiltrated axillary lymph nodes. Overall, CK19mRNA+, MGB1mRNA+, and HER2mRNA+ cells were detected in 72 (41.1%), 14 (8%), and 50 (28.6%) of the 175 patients, respectively. The detection of CTCs was not significantly associated with any of the known clinicopathologic characteristics, except for the detection of MGB1mRNA+ cells that was significantly associated with tumor size >2 cm (Table 1). Based on the detection of the three molecular markers, patients had one of the following molecular profiles: CK19mRNA+/MGB1mRNA+/HER2mRNA+ (n = 8), CK19mRNA+/MGB1mRNA+/HER2mRNA- (n = 1), *CK19*mRNA+/MGB1mRNA-/HER2mRNA+ (n = 42), *CK19*-mRNA+/MGB1mRNA-/HER2mRNA- (n = 21), *CK19*mRNA-/MGB1mRNA+/HER2mRNA- (n = 5), and CK19mRNA-/MGB1MGB1mRNA-/HER2mRNA- (n = 98).

HER2 expression on the primary tumor and detection of CTCs. Among the 175 patients, 48 (27.4%) patients were HER2-positive (IHC 3+). Interestingly, both CK19mRNA+ and HER2mRNA+ cells were detected in 11 of 48 (22.9%) patients with HER2-positive primary tumors and in 38 of 124 (30.6%) patients with HER2-negative primary tumors. All the patients who had HER2mRNA+ blood samples were also CK19mRNA+.

CTC immunophenotype. To further validate the presence of the different CTC molecular profiles obtained by the multimarker assay, double-immunofluorescence experiments were done in a small cohort of 10 cytokeratin-positive breast cancer patients. Confocal laser scanning microscopy confirmed the presence of the following CTC phenotypes: CK+/*MGB1*+, CK+/*MGB1*-, CK-/*MGB1*+, CK+/*HER2*+, CK+/*HER2*-, *MGB1*+/*HER2*-, and *MGB1*+/*HER2*+. Six of the 10 patients had more than one CTC immunophenotype. All the patients with CK+/*MGB1*+ cells had also CK+/*MGB1*- cells, implying profound heterogeneity of protein expression pattern in CTCs. Two of the patients with HER2-positive CTCs had *HER2*+/*MGB1*+ and *HER2*+/*MGB1*- cells in the same slide. Remarkably, CK-/*MGB1*+ cells (Fig. 1) were detected in PBMC cytospins from two patients with *CK19*-/*MGB1*+/*HER2*- molecular profile. Five characteristic CTC immunophenotypes from five different patients are depicted in Fig. 1. Interestingly, the concordance between the CTC phenotype detected by immunofluorescence and the molecular profile detected by RT-PCR was 100% for the 10 patients studied.

CTC molecular profiles and clinical outcome. During the follow-up of this study, 15 (15.3%) relapses were observed in the 98 patients with no positive marker detected in their peripheral blood (*CK19*mRNA-/*MGB1*mRNA-/*HER2*mRNA-) versus 8 (30.7%) relapses in the 26 patients with one positive marker (*CK19*mRNA+/*MGB1*mRNA-/*HER2*mRNA- or *CK19*mRNA-/*MGB1*mRNA+/*HER2*mRNA-), 20 (46.5%) relapses in the 43 patients with two positive markers (*CK19*mRNA+/*MGB1*mRNA+/*HER2*mRNA- or *CK19*mRNA+/*MGB1*mRNA-/*HER2*mRNA+), and 6 (75%) relapses in the 8 patients with three positive markers (*CK19*mRNA+/*MGB1*mRNA+/

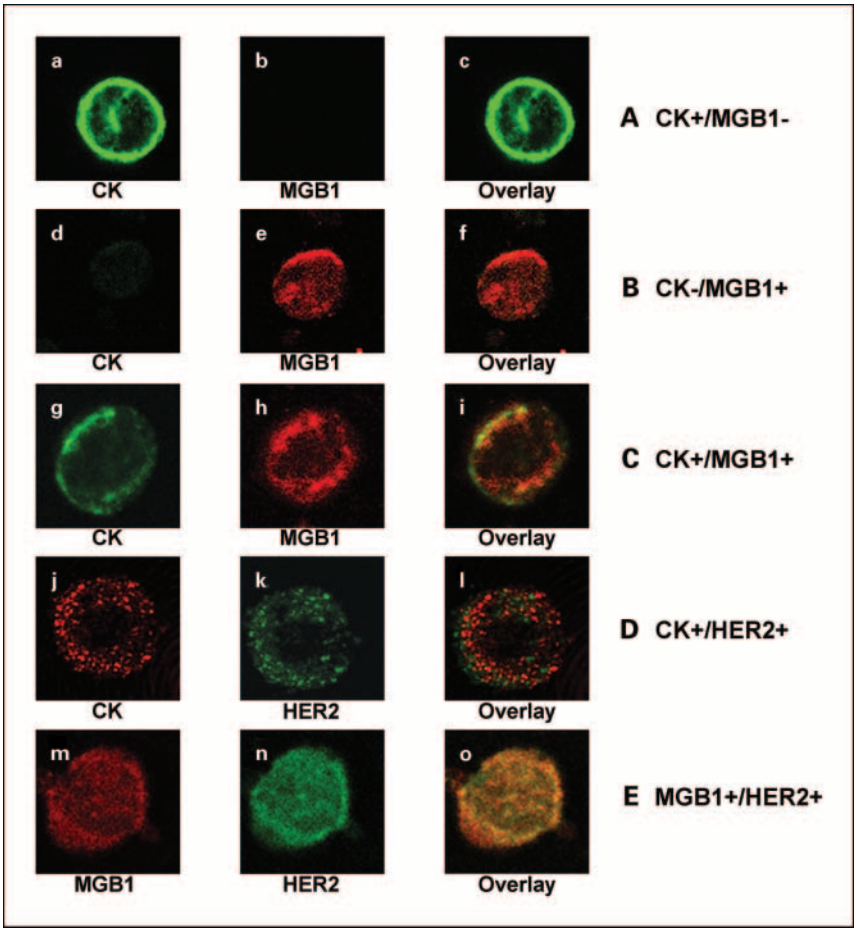

Fig. 1. *A* to *E,* five different CTC immunophenotypes from five different patients: CK+/*MGB1*- (*A*), CK-/*MGB1*+ (*B*), CK+/*MGB1*+ (*C*), CK+/*HER2*+ (*D*), and *MGB1*+/*HER2*+ (*E*). *A* to *C,* representative micrograph of confocal laser scanning microscopic sections of PBMC cytospins double stained with pancytokeratins A45-B/B3 monoclonal antibody (*green*; *a, d*, and *g*) and *MGB1* (*red*; *b, e*, and *h*) goat polyclonal antibody and the overlay sections (*c, f*, and *i*). Magnification, ×600. *D,* representative micrograph of confocal laser scanning microscopic sections of PBMC cytospins double stained with pancytokeratins polyclonal antibody (*red*; *j*) and *HER2* monoclonal antibody (*green*; *k*) and overlay section (*l*). Magnification, ×600. *E,* representative micrograph of confocal laser scanning microscopic sections of PBMC cytospins double stained with MGB1 goat polyclonal antibody (*red*; *m*) and HER2 mouse monoclonal antibody (*green*; *n*) and overlay section (*o*). Magnification, ×600.

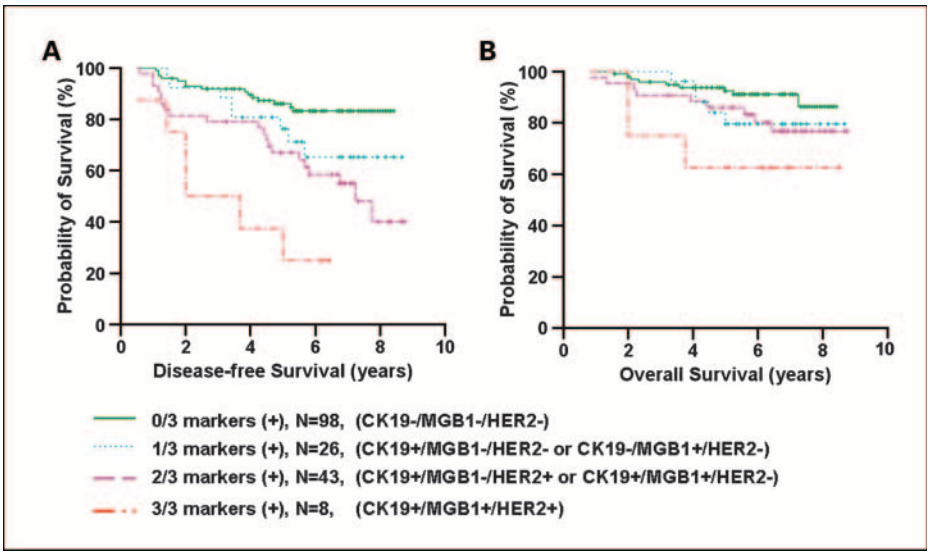

Fig. 2. DFS (*A*) and OS (*B*) in early breast cancer patient groups based on the molecular detection of CTCs using a panel of three markers (*CK19*, MGB1, and HER2).

HER2 mRNA+) Table 2. The estimated median DFS was 2 years (95% confidence interval, 0-4.1 years) for patients with three positive markers, 7.2 years (95% confidence interval, 6-8.5 years) for patients with two positive markers, whereas the median DFS has not been reached for patients with one or none positive markers (Fig. 2A). Moreover, compared with patients with no positive markers, patients with one positive marker had a nonsignificant trend for shorter DFS ($P = 0.092$, log-rank test), whereas patients with two positive markers had significantly shorter DFS ($P < 0.001$, log-rank test). The estimated 5-year DFS for patients with three, two, one, and zero positive markers was 25%, 67%, 76.3%, and 86%, respectively.

Similarly, 9 (9.1%) deaths occurred in the 98 patients with no positive marker, 5 (19.2%) in the 26 patients with one positive marker, 9 (20.9%) in the 43 patients with two positive markers, and 3 (37.5%) in the 8 patients with three positive markers Table 2. The median OS for these patient subgroups cannot as of yet be estimated (Fig. 2B). However, compared with patients who had no positive markers, patients with one and two positive markers had a nonsignificant trend for worse OS ($P = 0.202$ and 0.104, respectively, log-rank test). The estimated 5-year OS for patients with three, two, one, and zero positive markers was 62.5%, 85.9%, 79.5%, and 92.4%, respectively.

The association between the detection of single-marker (*CK19*, *MGB1*, and HER2) or two-marker combinations (*CK19/MGB1*, *CK19/HER2*, and *MGB1/HER2*) and DFS/OS is presented in Supplementary Figs. S1 and S2.

Univariate and multivariate analysis. For DFS, primary tumor size >2 cm, ER-negative tumors, and the detection of CK19mRNA+, *MGB1* mRNA+, and *HER2* mRNA+ cells were all significantly associated with unfavorable outcome in the univariate analysis (Table 3A), whereas ER-negative tumors and the detection of CK19mRNA+ and *MGB1* mRNA+ cells emerged as independent adverse prognostic factors in the multivariate analysis (Table 3B). For OS, in the univariate analysis, the detection of *CK19* mRNA+ and *MGB1* mRNA+ cells was significantly associated with reduced survival (Table 3A), whereas they marginally failed to emerge as independent prognostic factors in the multivariate analysis (Table 3B).

Discussion

We have previously shown that the detection of peripheral blood *CK19* mRNA+ cells before the initiation of adjuvant chemotherapy is an independent prognostic factor for shorter DFS and OS (7, 8). In the present study, we report for the first time that, using a three-marker RT-PCR analysis, the detection of *CK19* mRNA+ cells as well as the detection of *MGB1* mRNA+ cells were both independent prognostic factors for reduced DFS. The detection of *HER2* mRNA+ cells was a prognostic factor for shorter DFS in the univariate screen but did not retain independent prognostic value in the multivariate analysis. Therefore, *MGB1* but not *HER2* adds statistically independent and complementary prognostic information to the knowledge gained by *CK19* alone.

The multimarker RT-PCR assay used in the present study showed slightly increased sensitivity for the detection of CTCs over the single-marker (*CK19*) RT-PCR by detecting five additional *CK19-/MGB1+/HER2-* patients. Interestingly, two of these five patients relapsed and one died of breast cancer. In two of these five patients, PBMC cytospins were available and the presence of CK-/*MGB1+* cells was confirmed by immunofluorescence (Fig. 1B). CK-/*MGB1+* has already been reported as a rare immunophenotypic profile of CTCs (37). Additionally, the following CTC phenotypes were confirmed in the 10 patients studied by immunofluorescence: CK+/*MGB1+*, CK+/*MGB1-*, CK+/*HER2+*, CK+/*HER2-*, *MGB1+/HER2-*, and *MGB1+/HER2+*. Interestingly, we observed a complete concordance (100%) between the CTC phenotype as detected by immunofluorescence and the molecular profile as detected by RT-PCR in these 10 patients. The fact that the CTC molecular profiles obtained by our multimarker RT-PCR assay were confirmed by immunofluorescence in a small cohort of 10 patients provides further evidence for the validity of our findings. This is especially true for the two patients with the rare *CK19* mRNA-/*MGB1* mRNA+/*HER2* mRNA- molecular profile. However, these results should be interpreted with caution due to the small number of patients examined.

Although in our study the detection of *MGB1* emerged as an independent prognostic factor for DFS, its low sensitivity (only 8% positive patients) limits its further development as a single

Table 2. Patient groups based on CTC molecular profiles and clinical outcome

No positive markers	No. patients	CK19mRNA	MGB1mRNA	HER2mRNA	Relapses	Deaths	Median DFS, y (95% CI)	P*	Median OS	P*
0	98	-	-	-	15	9	NR		NR	
1	26	+	-	-	8	5	NR	0.092	NR	0.202
		-	+	-						
2	43	+	-	+	20	9	7.25 (6-8.5)	<0.001	NR	0.104
		+	+	-						
3	8	+	+	+	6	3	2 (0-4.1)		NR	
Total	175				49	26	NR		NR	

Abbreviations: NR, not reached; 95% CI, 95 confidence interval.
*Refers to the comparison with the zero/three marker (CK19mRNA-/MGB1mRNA-/HER2mRNA-) group; log-rank comparisons were done between subgroups containing at least 20 patients each.

marker for clinical use. The *MGB1* detection could be improved by using an epithelial cell enrichment step with immunomagnetic beads as has been reported by other investigators (18). *MGB1* detection rates in early breast cancer have varied considerably in published studies from 2% to 22% (23, 24, 38, 39). This variation can be attributed to the different detection techniques used with different sensitivities and specificities, the different patient populations studied, and possibly the lack of correlation data about *MGB1* expression in the primary tumor. Indeed, one limitation of the present study is the lack of information about *MGB1* expression on primary tumors. *MGB1* detection by IHC has been reported in only a

Table 3. Univariate (A) and multivariate (B) analysis for DFS and OS in women with early breast cancer

Variable	Hazard ratio (95% CI)	P
A.		
DFS		
Menopausal status (pre vs post)	0.739 (0.410-1.331)	0.313
Tumor size (T2-3 vs T1)	2.292 (1.112-4.725)	0.025
Histology grade (3 vs 1/2)	1.661 (0.911-3.030)	0.098
Lymph nodes (positive vs negative)	1.587 (0.827-3.046)	0.165
ER (negative vs positive)	2.446 (1.375-4.350)	0.002
PR (negative vs positive)	1.072 (0.597-1.923)	0.816
HER2, primary tumor (positive vs negative)	1.651 (0.920-2.963)	0.093
Adjuvant chemotherapy (FEC/EC-T vs CMF)	1.708 (0.677-4.310)	0.257
CK19mRNA+ cells (yes vs no)	2.967 (1.647-5.344)	<0.001
MGB1mRNA+ cells (yes vs no)	3.275 (1.586-6.763)	0.001
HER2mRNA+ cells (yes vs no)	2.869 (1.638-5.025)	<0.001
OS		
Menopausal status (pre vs post)	1.032 (0.474-2.246)	0.938
Tumor size (T2-3 vs T1)	2.670 (0.920-7.750)	0.071
Histology grade (3 vs 1/2)	1.806 (0.781-4.173)	0.167
Lymph nodes (positive vs negative)	2.836 (0.977-8.234)	0.055
ER (negative vs positive)	2.122 (0.973-4.626)	0.058
PR (negative vs positive)	1.040 (0.472-2.293)	0.922
HER2, primary tumor (positive vs negative)	1.249 (0.539-2.896)	0.604
Adjuvant chemotherapy (FEC/EC-T vs CMF)	1.459 (0.438-4.863)	0.538
CK19mRNA+ cells (yes vs no)	2.257 (1.024-4.977)	0.044
MGB1mRNA+ cells (yes vs no)	2.867 (1.081-7.606)	0.034
HER2mRNA+ cells (yes vs no)	1.806 (0.828-3.938)	0.137
B.		
DFS		
Tumor size (T2-3 vs T1)	1.954 (0.899-4.248)	0.091
ER (negative vs positive)	2.459 (1.375-4.398)	0.002
CK19mRNA+ cells (yes vs no)	3.085 (1.687-5.642)	<0.001
MGB1mRNA+ cells (yes vs no)	2.633 (1.251-5.541)	0.011
HER2mRNA+ cells (yes vs no)	1.781 (0.750-4.229)	0.191
OS		
CK19mRNA+ cells (yes vs no)	2.102 (0.947-4.665)	0.068
MGB1mRNA+ cells (yes vs no)	2.521 (0.942-6.745)	0.066

Abbreviations: NR, not reached; 95% CI, 95 confidence interval.

fraction of primary breast tumors ranging from 48% to 80% (40, 41). Similarly, at the mRNA level, although *MGB1* has been reported to be expressed in 93% of patients with breast cancer, *MGB1* expression levels have varied over 10,000-fold (42). High levels of *MGB1* in the primary tumor have been associated with tumors with a less aggressive phenotype (low-grade, ER-positive tumors; ref. 42). At present, it is not known if the differential expression of *MGB1* in the primary tumor has an effect on the detection of peripheral blood *MGB1* mRNA+ cells.

Although the multimarker RT-PCR only slightly improved the sensitivity of detection of CTCs over the single-marker CK19 assay, the presence of two or three positive markers in the peripheral blood was associated with progressively worse prognosis compared with that of one positive marker. Therefore, the three-marker assay could provide important prognostic information for patients' risk stratification in adjuvant breast cancer trials.

Until now, only molecularly-based detection of CTCs is a prognostic marker for clinical outcome (7, 8). A more standardized, automated, immunofluorescence-based approach (CellSearch, Veridex) has yielded a low CTC detection rate (10%) in a large cohort of 1,767 early breast cancer patients, whereas correlation with clinical outcome is still pending (43). In this context, the proposed multimarker RT-PCR assay could be a more suitable tool for the assessment of CTCs in early breast cancer. However, an important limitation of our study is that all patients were recruited in one center and the CTC analysis was done in one laboratory. An international, prospective, multicenter trial with different participating laboratories in which issues of stability during shipment of the samples, interlaboratory reproducibility of the multimarker assay, and validation of our results in a diverse patient population is urgently needed. Further development of this assay should take into consideration the problems related to tumor marker prognostic studies described by McShane et al. (44).

In the present study, patients with positive CTCs detected by our multimarker assay had worse prognosis compared with those with nondetectable CTCs despite the fact that they had received both adjuvant chemotherapy and, if appropriate, tamoxifen. Therefore, in future trials, the value of secondary adjuvant treatment strategies should be prospectively investigated in patients with detectable CTCs to improve their clinical outcome. For example, the presence of peripheral blood *HER2* mRNA+ cells in early breast cancer could be used to select patients that could benefit from anti-*HER2* targeted agents (45). Recently, preliminary results from subset analysis of the NSABP B-31 trial have suggested that benefit from adjuvant trastuzumab may not be exclusively confined to patients with IHC 3+–positive or fluorescence *in situ* hybridization–positive primary tumors (46). The benefit of trastuzumab in these patients could be related to effective targeting of HER2-positive CTCs (45). In our study, both *CK19* mRNA+ and *HER2* mRNA+ cells were detected in 38 of 124 (30.6%) patients with *HER2*-negative primary tumors and those women would have been ineligible for adjuvant trastuzumab based on current criteria. Although the finding of *HER2* mRNA+ cells in women with "HER2-negative" primary tumors could be simply due to the fact that the RT-PCR for *HER2* is more sensitive than immunocytochemistry for *HER2*, it might also have important therapeutic implications. We and others have shown that trastuzumab can effectively eliminate CTCs and disseminated tumor cells overexpressing HER2 regardless of the *HER2* status of the primary tumor in patients with early or metastatic breast cancer (45, 47). Similarly, the presence of peripheral blood MGB1mRNA+ cells in early breast cancer could be used to select patients that could derive potential benefit from vaccine strategies against MGB1-expressing cells (48, 49).

The role of the above multimarker RT-PCR assay as a prognostic tool in early breast cancer as well as a predictive tool to define patient groups that could benefit from anti-*HER2* and/or anti-*MGB1* targeted therapies should be validated in prospective clinical trials where the prognostic or predictive value of detection of CTCs will be the primary objective of the trial (50).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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