

Apoptotic Circulating Tumor Cells in Early and Metastatic Breast Cancer Patients

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Abstract

The detection of circulating tumor cells (CTC) in breast cancer is strongly associated with disease relapse. Since it is unclear whether all CTCs are capable of generating metastasis, we investigated their apoptotic and proliferative status in 56 CTC-positive (29 early and 27 metastatic) patients with breast cancer. Double-staining immunofluorescence experiments were carried out in peripheral blood mononuclear cells (PBMC) cytopins, using the pancytokeratin A45-B/B3 antibody and either M30 (apoptotic marker) or Ki67 (proliferation marker) antibodies. Apoptosis was also evaluated using a polycaspase detection kit. Patients with metastatic disease had significantly lower numbers of apoptotic CTCs compared with patients with early breast cancer (polycaspase kit: 8.1% vs. 47.4% of the total CTC number; $P = 0.0001$; M30-antibody: 32.1% vs. 76.63%; $P = 0.002$). The median percentage of apoptotic CTCs per patient was also lower in patients with advanced compared with those with early disease (polycaspase kit: 0% vs. 53.6%; M30-antibody: 15% vs. 80%). Ki67-positive CTCs were identified in 51.7% and 44% of patients with early and metastatic disease, respectively. Adjuvant chemotherapy reduced both the number of CTCs per patient and the number of proliferating CTCs (63.9% vs. 30%). In conclusion, apoptotic CTCs could be detected in patients with breast cancer irrespective of their clinical status, though the incidence of detection is higher in early compared with metastatic patients. The detection of CTCs that survive despite adjuvant therapy implies that CTC elimination should be attempted using agents targeting their distinctive molecular characteristics. *Mol Cancer Ther*; 12(9); 1886–95. ©2013 AACR.

Introduction

Circulating tumor cells (CTC) are increasingly considered as a "liquid biopsy" that allows the assessment of tumor changes over time simply by repeated blood draws (1). The presence of CTCs in the blood of patients with breast cancer has emerged as a poor prognostic indicator (2–4). Indeed, metastasis is associated with the presence of disseminated (DTC) or circulating (CTC) tumor cells in the bone marrow and peripheral blood, respectively (5, 6). It has been shown that these cells present significant heterogeneity (7–9) and the delineation of their specific molecular characteristics could enhance the understanding of their biology and clinical relevance.

Although many CTCs migrate early from the primary tumor into the circulation, 97% of them will be cleared within a few days (6). According to the seed and soil

hypothesis, the survival of these cells depends on their distinctive biologic characteristics as well as on the micro-environment at the secondary site (10). Only rare subsets of cells finally succeed in establishing a cross-talk with stromal cells in secondary organs that promote tumor cell survival, angiogenesis, and metastatic outgrowth.

Characterizing the viability of CTCs in each patient might be important to improve prognostication and individualize treatment. Thus, the presence of exclusively apoptotic CTCs in a patient may represent a favorable prognostic factor, whereas the preponderance of proliferating cells could be related to poor patient's outcome. Several studies have focused on the apoptotic status of CTCs. Larson and colleagues (11) evaluating the presence of apoptotic and nonapoptotic CTCs in metastatic prostate cancer reported that the number of apoptotic or intact CTCs differs among patients. Similarly, both apoptotic and nonapoptotic CTCs could be detected in a patient with metastatic breast cancer (12). Viable CTCs and DTCs were also evaluated in patients with ovarian cancer where it was shown that patients with viable DTCs after treatment had significantly reduced progression-free survival (13). The detection of DTCs in patients with breast cancer after the completion of neoadjuvant treatment was associated with progressive disease (14). Moreover, apoptotic DTCs were mainly identified in patients with disease remission (14). In addition, serologic cell death biomarkers such as M30 and M65 have been detected in the serum

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of patients with prostate, colorectal, and lung cancer and this has been associated with response to treatment and the status of CTCs or DTCs (15–17).

Evaluation of the prognostic significance of CTCs in early breast cancer revealed that a substantial number of patients with detectable CTCs either before the initiation of any systemic treatment or during adjuvant hormone treatment do not relapse even many years after their detection (3, 18). This observation clearly supports the hypothesis that not all CTCs are capable of metastatic seeding. This could be related to the induction of senescence in CTCs or to a low proliferative potential or, even, to the presence of apoptotic cells. On the other hand, we have reported that activated kinases such as phospho-AKT and phospho-PI3K that are involved in survival pathways are expressed in CTCs from patients with early and metastatic breast cancer (19, 20). There are also conflicting studies concerning the proliferation status of these cells. In 2005, Muller and colleagues (21) reported that CTCs from patients with breast cancer do not express Ki67. However, other studies in patients with prostate and small cell lung revealed Ki67 expression in many patients' CTCs but in different frequencies per patient (22, 23).

The aims of the current study were to assess the apoptotic status of CTCs in patients with early and metastatic breast cancer using two different methods as well as their proliferative potential. In addition, the apoptotic and proliferative status of CTCs before and after adjuvant treatment was also evaluated.

Materials and Methods

Patient samples and cytospin preparation

Fifty-six patients with early ($n = 29$) and metastatic ($n = 27$) breast cancer were enrolled in the study. These patients harvested cytokeratin-positive CTCs when routinely screened in the context of the initial evaluation of their disease. CK-positive CTCs were detected by double immunofluorescence using anti-cytokeratin and anti-CD45 antibodies. Patients were enrolled in this study if they had at least two CTCs per 10^6 peripheral blood mononuclear cells (PBMC).

In addition, to evaluate CTCs before and after chemotherapy in a cohort of 10 patients we have obtained blood before and after the end of adjuvant chemotherapy whenever this occurred; samples were obtained after the eighth chemotherapy dose-dense cycle in 6 of 10 patients (corresponding to a 4-month treatment), after the sixth cycle in 3 patients, and after the fourth chemotherapy cycle in one patient, respectively.

In addition, 10 female normal blood donors were included as negative controls. Peripheral blood (10 mL in EDTA) was obtained before the initiation of adjuvant treatment (usually 4–6 weeks after primary surgery) or before first-line chemotherapy for metastatic disease.

All blood samples were obtained at the middle of vein puncture after the first 5 mL of blood was discarded. These precautions were undertaken to avoid contamination of the blood sample with epithelial cells from the skin during

sample collection. All patients gave their informed consent to participate in the study, which has been approved by the Ethics and Scientific Committees of our Institution.

PBMCs were isolated by Ficoll–Hypaque density gradient ($d = 1,077$ g/mL) centrifugation at 1,800 rpm for 30 minutes. Aliquots of 5×10^5 PBMCs were cytocentrifuged at 2,000 rpm for 2 minutes on glass slides. Cytospins were dried and stored at -80°C until use. At least two slides with a total of 5×10^5 PBMCs were analyzed for each examined molecule in individual patients.

Cell cultures

MCF7 and SKBR3 breast cancer cells (obtained from American Type Culture Collection). Authentication was done by staining experiments with antibodies for estrogen, progesterone, and HER2 and EGFR receptors, just before the beginning of the study and spontaneously during the research. No other authentication test was done. All cell lines revealed the expected phenotype.

MCF7 and SKBR3 cell lines were centrifuged on cytopins according to the procedure followed for patients' samples and they were used as controls for apoptotic and Ki67 staining experiments, respectively.

MCF7 cells (mammary adenocarcinoma cell line) were incubated with $2 \mu\text{mol/L}$ staurosporine for 2 hours induce apoptosis (24). MCF7 cells were cultured in 1:1 (v/v) Dulbecco's modified Eagle medium (DMEM)/Ham's F12 medium (GIBCO-BRL Co) supplemented with 10% FBS (GIBCO-BRL), 2 mmol/L L-glutamine (GIBCO-BRL), 30 mmol/L NaHCO₃, 16 ng/mL insulin, and 50 mg/mL penicillin/streptomycin (GIBCO-BRL). SKBR3 cells were cultured in RPMI supplemented with 10% FBS. Cells were maintained in a humidified atmosphere of 5% CO₂ in air. Subcultivation was conducted with 0.25% trypsin and 5 mmol/L EDTA (GIBCO-BRL). All experiments were carried out during the logarithmic growth phase. Twenty to 24 hours before the experiments, cells were transferred in serum-free medium containing only L-glutamine, NaHCO₃ and penicillin/streptomycin. After incubation, cells were centrifuged on cytopins according to the same procedure followed for patients' samples.

Detection of apoptotic CTCs

Two different methods that evaluate sequential steps of the apoptotic procedure were used for the detection of apoptosis in CTCs. The first one was based on the identification of activated caspases using the Polycaspase Detection Kit (Invitrogen). The kit uses fluorescent-labeled inhibitors of caspases (FLICA) that react with the enzymatic reactive center of activated caspases and allows the detection of caspase activity (25, 26). The second was based on the detection of M30-positive (Roche) CTCs. The M30 antibody binds to the respective neo-epitope exposed after caspase cleavage of human cytokeratin (18). For either method, cytopins were evaluated using a confocal laser scanning microscope module (Leica Lasertechnik) and the Ariol microscopy system (Genetix), an automated system for CTC identification (27, 28).

Detection of apoptotic CTCs using the polycaspase detection kit. Cytospins from patients' PBMCs were washed with PBS and incubated with 0.5× FLICA reagent for 1 hour. Slides were then fixed with the appropriate solution provided by the manufacturer. Subsequently, slides were stained with the A45-B/B3 antibody (detecting CK8, CK18, and CK19; Micromet Munich) followed by the secondary anti-mouse antibody Alexa 555 (Invitrogen). The pancytokeratin A45-B/B3 antibody was used as marker for epithelial cells. The cytomorphologic criteria proposed by Meng and colleagues (ref. 29; i.e. high nuclear/cytoplasmic ratio, larger cells than white blood cells, etc.) were used to characterize a CK-positive cell as a CTC. Cells were also stained with 4', 6-diamidino-2-phenylindole (DAPI) conjugated with antifade (Invitrogen).

Detection apoptotic M30-positive CTCs. Cytospins from the same cohort of patients were fixed with cold acetone:methanol 9:1 (v/v) for 20 minutes and stained for cytokeratin with A45-B/B3 antibody (Micromet) and Alexa 555 (Invitrogen) as a secondary antibody. Consequently, slides were incubated with M30-FITC conjugated antibody (Roche) for 1 hour. Cells were then stained with DAPI (Invitrogen) conjugated with antifade.

In both assays (polycaspase and M30 antibody), the following controls were used in each experiment: (i) positive controls: MCF7 cells incubated with 2 μmol/L staurosporine (Merck); (ii) two negative controls: (a) untreated MCF7 cells stained with M30 antibody or FLICA reagent; and (b) cells treated with staurosporine and incubated with the corresponding IgG secondary antibody, without prior exposure to M30 or FLICA reagent.

Double immunofluorescence experiments for cytokeratin and Ki67 or CD45: PBMC cytospins fixed, as described previously, were double-stained with the anti-cytokeratin A45-B/B3 antibody (Micromet) and Alexa 555 (Invitrogen), Ki67 (Abcam) or CD45 (Santa Cruz Biotechnology) antibodies were added and slides were finally incubated with DAPI conjugated with antifade. Positive and negative controls of SKBR3 cytospins were used in every experiment. Only the nuclear staining of Ki67 antigen was considered as positive.

Results

Patients

Patients with early ($n = 29$) and metastatic ($n = 27$) breast cancer and detectable CK-positive CTCs before the initiation of adjuvant or first-line treatment, respectively, were enrolled. Patients' characteristics listed in Table 1. A total of 656 CTCs were analyzed in patients with early breast cancer (median: 2.5 CTCs/per patient; range: 1–27) and 505 CTCs (median: 3 CTCs/patient; range: 1–40) in patients with metastatic breast cancer. CTC counts per patient correspond to CTCs/ 5×10^5 PBMCs which is approximately included into 0.5 mL of blood. After a median follow-up period of 54.5 months (range, 42–94), 4 (14%) of the patients with early breast cancer presented a distant relapse.

Detection of apoptotic CTCs in patients with early and metastatic breast cancer

MCF7 control cells with and without staurosporine were analyzed with Ariols system (Fig. 1A and B) and confocal laser scanning microscopy (Fig. 1A (III) and B (III)). Control experiments have shown that in slides without staurosporine (Fig. 1A (II) and B (II)) the percentage of apoptotic MCF7 cells was less than 1%, whereas after 2-hour treatment with staurosporine, the percentage of apoptotic cells was about 30%. These findings clearly indicate that apoptosis is not induced *ex vivo* due to manipulations during sample preparation (Fig. 1A (I) and B (I)). In addition, the patients' PBMCs nearby were not apoptotic (Fig. 2B and C) indicating that the observed apoptosis in CTCs is not induced *ex vivo* by the experimental procedure.

Polycaspase kit. Using the polycaspase detection kit, a total of 135 and 179 CTCs were analyzed in patients with early and metastatic breast cancer, respectively. The incidence of detection of apoptotic CTCs was significantly higher in patients with early breast cancer compared with patients with metastatic disease (78.6% vs. 33%, respectively; $P = 0.001$; Fig. 2A, I). Exclusively apoptotic CTCs were observed in 42.9% versus 7.4% of early and metastatic disease, respectively. The median percentage of apoptotic CTCs per patient was 53.6% (range: 0%–100%) in patients with early and 0% (range: 0%–100%) in patients with metastatic disease (Fig. 2A, II). Moreover, 47.4% of the total analyzed CTCs were caspase-positive in patients with early breast cancer compared with 8.1% in metastatic patients ($P = 0.0001$; Fig. 2A, III).

Apoptotic cells presented a variation in their morphology. Few of them could have a totally degraded nucleus with dots all around the cytoplasm or sometimes cell fragments were obvious in the slides. In the current study, only CTCs with intact nucleus were included to avoid potential artifacts. The activated caspases were mainly sited in the cytoplasm in CTCs of a breast cancer patient as shown in Fig. 2B, whereas the observed nearby PBMCs in patients' cytospins were not apoptotic, as shown in Fig. 2B and C supporting our previous conclusion that apoptosis in CTCs is an *in vivo* phenomenon and not induced *ex vivo* by the experimental procedure.

M30-positive CTCs in early and metastatic breast cancer patients. All patients were also evaluated for the presence of apoptotic CTCs using M30 antibody. Staining of MCF7 cells with the M30 antibody revealed both apoptotic cells (green) along with nonapoptotic (red) cells (Fig. 1B). The intracellular distribution of M30 antibody in CTCs of a patient with breast cancer is shown in Fig. 2C.

A total of 338 and 156 apoptotic [CK (+)/M30(+)] CTCs could be detected in patients with early and metastatic breast cancer, respectively. Apoptotic CTCs were observed in 93% and 52% of patients with early and metastatic disease, respectively ($P = 0.001$; Fig. 2A, I). Exclusively, CK(+)/M30(+) CTCs were observed in 31% and 22.2% of patients with early and metastatic breast

Table 1. Patient characteristics

Early breast cancer		Metastatic breast cancer	
No. of patients enrolled	29	No. of patients enrolled	27
Age, y		Age, y	
Median, range	58 (26–76)	Median, range	59 (36–75)
	N (%)		N (%)
ECOG PS		ECOG PS	
0	28 (96.6)	0	10 (37)
1	1 (3.4)	1	17 (63)
Histology		Histology	
Ductal	24 (82.8)	Ductal	22 (81.4)
Lobular	3 (10.3)	Lobular	4 (14.8)
Other	2 (6.9)	Other	1 (3.7)
Menopausal status		Menopausal status	
Premenopausal	11 (37.9)	Premenopausal	7 (25.9)
Postmenopausal	18 (62.1)	Postmenopausal	19 (70.4)
		UN	1 (3.7)
Hormone receptor status		Hormone receptor status	
ER-positive/PR-positive	13 (44.8)	ER-positive/PR-negative	12 (44.5)
ER-positive/PR-negative	6 (20.7)	ER-positive/PR-negative	6 (22.2)
ER-negative/PR-positive	1 (3.4)	ER-negative/PR-positive	2 (7.4)
ER-negative/PR-negative	9 (31.0)	ER-negative/PR-negative	5 (18.5)
		UN	2 (7.4)
HER2 Status		HER2 Status	
Positive	6 (20.7)	Positive	11 (40.8)
Negative	22 (75.9)	Negative	13 (48.1)
UN	1 (3.4)	UN	3 (11.1)
Tumor size		No. of organs affected	
T1	9 (31)	1	5 (18.6)
T2	16 (55.2)	2	3 (11.1)
T3	2 (6.9)	3	7 (25.9)
UN	2 (6.9)	≥4	8 (29.6)
		UN	4 (14.8)
Grade		Adjuvant chemotherapy	
I	2 (6.9)	Yes	15 (55.6)
II	11 (37.9)	No	10 (37.0)
III	14 (48.3)	UN	2 (7.4)
UN	2 (6.9)		
Positive nodes		Visceral/nonvisceral disease	
N0	14 (48.3)	Nonvisceral	10 (37)
N1	10 (34.5)	Visceral	2 (7.4)
N2	2 (6.9)	Visceral and nonvisceral	12 (44.5)
N3	3 (10.3)	UN	3 (11.1)

Abbreviations: ECOG PS, Eastern Cooperative Oncology Group performance status; UN, unknown.

cancer, respectively. Moreover, 76.6% of the total analyzed CTCs were CK(+)/M30(+) in patients with early breast cancer compared with 32.1% in the group of patients with metastatic breast cancer ($P = 0.002$; Fig. 2A, III). The median percentage of apoptotic CTCs per patient was 80% (range: 0%–100%) in patients with early and 15% (range: 0%–100%) in patients with metastatic disease (Fig. 2A, II). However, 69.0% of early and 77.8% of metastatic patients also had detectable nonapoptotic CTCs.

In healthy volunteers, no CK-positive cells (CTCs) were observed with both methods while apoptotic PBMCs were observed in low frequency in the samples.

Expression of Ki67 in CTCs of early and metastatic breast cancer patients

As nonapoptotic CTCs were observed in both early and advanced breast cancer, we further investigated the

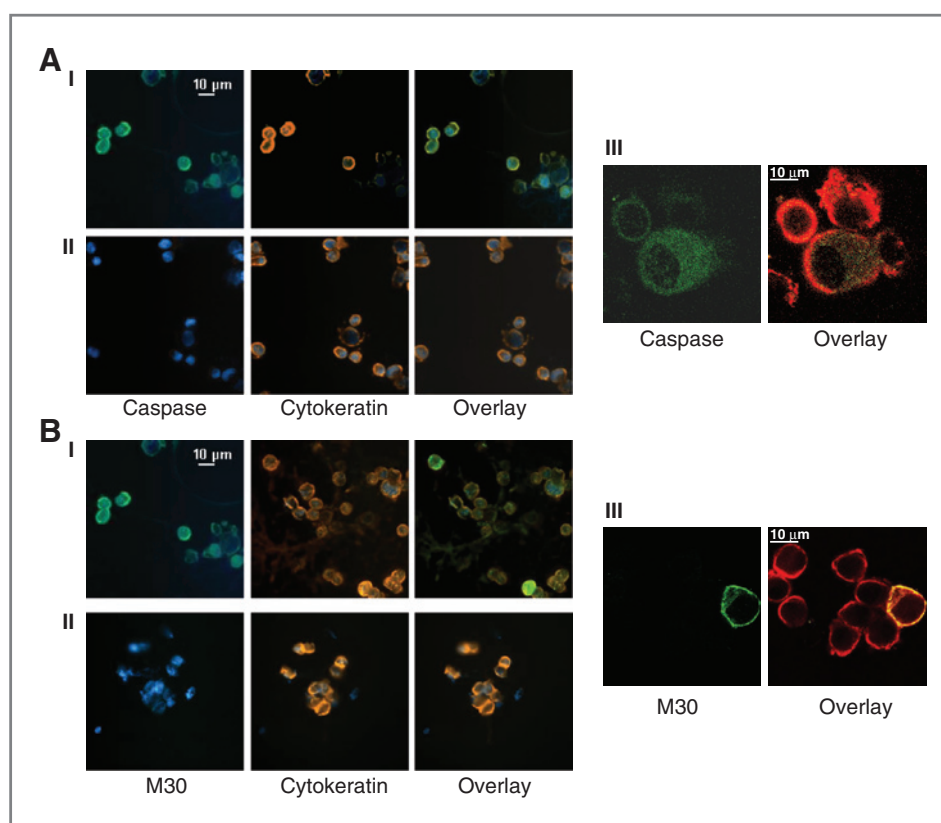


Figure 1. Expression of M30 and activated caspases in control MCF7 cells treated with 2 $\mu\text{mol/L}$ staurosporine. A, representative ARIOL system images ($\times 40$) from a cytospin of MCF7 treated with 2 $\mu\text{mol/L}$ staurosporine and stained with pancytokeratin (A45-B/B3)/Alexa 555 (red) and FLICA reagent (green; I). II, representative ARIOL system images ($\times 40$) from a cytospin of MCF7 not treated with staurosporine and stained with pancytokeratin (A45-B/B3)/Alexa 555 (red) and FLICA reagent (green). III, confocal laser scanning image of MCF7 cells treated with 2 $\mu\text{mol/L}$ staurosporine and stained with pancytokeratin (A45-B/B3)/Alexa 555 (red) and FLICA reagent (green). B, representative ARIOL system images ($\times 40$) from a cytospin of MCF7 treated with 2 $\mu\text{mol/L}$ staurosporine and stained with pancytokeratin (A45-B/B3)/Alexa 555 (red) and M30 antibody (green; I). II, representative ARIOL system images ($\times 40$) from a cytospin of MCF7 not treated with staurosporine and stained with pancytokeratin (A45-B/B3)/Alexa 555 (red) and M30 antibody (green). III, confocal laser scanning image of MCF7 cells treated with 2 $\mu\text{mol/L}$ staurosporine and stained with pancytokeratin (A45-B/B3)/Alexa 555 (red) and M30 antibody (green).

proliferation status of CTCs. The detection of Ki67 positivity in MCF-7 cells and in CTCs of a breast cancer patient is shown in Fig. 3A, I and II. A total of 183 and 171 CK(+) CTCs were detected in patients with early and metastatic breast cancer, respectively. Double-stained CK(+)/Ki67(+) CTCs were observed in 51.7% and 44.0% of patients with early and metastatic breast cancer, respectively ($P = 0.443$; Fig. 3B). Exclusively CK(+)/Ki67(+) CTCs were identified in one patient with early disease and in 2 with metastatic breast cancer. Moreover, 24.9% and 27.0% of the total CTCs detected in patients with early and metastatic breast cancer were CK(+)/Ki67(+) (Fig. 3B). Three of 4 patients with early breast cancer who finally relapsed displayed detectable CK(+)/Ki67(+) CTCs at the baseline sample. Furthermore, 2 patients, who consequently passed away, had Ki67-positive CTCs in their blood.

In healthy volunteers, no CK-positive cells (CTCs) were observed, whereas spontaneously proliferative PBMCs were observed in the cytopins.

Evaluation of apoptosis and proliferation in CTCs before and after adjuvant chemotherapy

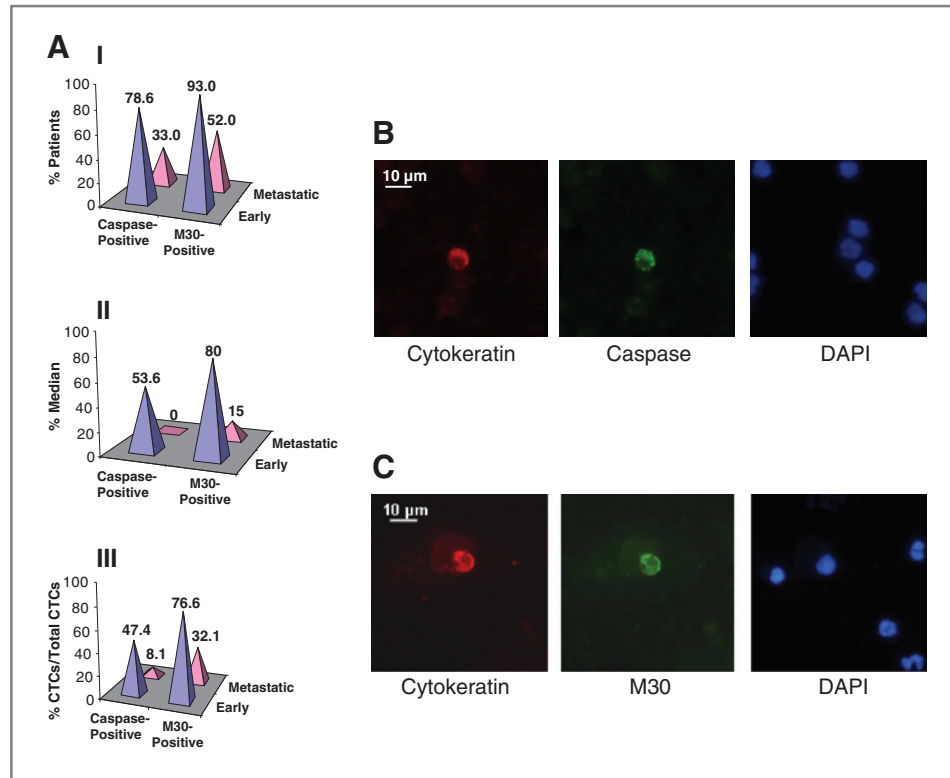
The effect of adjuvant chemotherapy on the apoptotic and proliferative status of CTCs was analyzed in 10 patients with early breast cancer for whom samples were available both before and after the completion of chemotherapy.

Apoptotic status. Apoptosis was evaluated using both the polycaspase detection kit and M30 antibody. The absolute number of CTCs for all the examined molecules was reduced after chemotherapy in all but 4 patients (Supplementary Fig. S1A).

The absolute number of apoptotic CTCs, as detected with the polycaspase detection kit, was reduced in 7 of 10 patients postchemotherapy (Supplementary Fig. S1B); however, in 4 patients, nonapoptotic CTCs were still detectable posttreatment (Table 2).

In parallel experiments using immunostaining with the M30 antibody, CK(+)/M30(+) apoptotic CTCs were

Figure 2. Expression of M30 and activated caspases in CTCs of patients with early and metastatic breast cancer. A, I, quantification of the incidence of double-stained CTCs in patients with 29 early and 27 metastatic breast cancers. II, quantification of the percentage of median expression per patient for each examined molecule. B, representative ARIOL system images ($\times 40$) from a cytospin of a patient double-stained with monoclonal pancytokeratin (A45-B/B3)/Alexa 555 (red) antibodies and FLICA reagent (green). C, representative ARIOL system images ($\times 40$) from a cytospin of a patient double-stained with monoclonal pancytokeratin (A45-B/B3)/Alexa 555 (red) and M30 FITC-conjugated antibodies (green).



reduced in 6 of 10 patients (Supplementary Fig. S1C), whereas 6 patients still had detectable M30-negative CTCs postchemotherapy (Table 2). In addition, the apoptotic index in CTCs, calculated as the percentage of apoptotic

CTCs among the total CTCs detected, altered from 38.3% prechemotherapy to 30% postchemotherapy using the polycaspase kit and from 56.6% to 28.6% using M30 staining.

Figure 3. Ki67 expression in CTCs of patients with breast cancer. A, representative ARIOL system images ($\times 40$) of control MCF7 cells stained with monoclonal pancytokeratin (A45-B/B3)/Alexa 555 (red) and Ki67 FITC-conjugated antibodies (green; I). II, representative ARIOL system images ($\times 60$) from a cytospin of a patient double-stained with monoclonal pancytokeratin (A45-B/B3)/Alexa 555 (red) and Ki67 FITC-conjugated antibodies (green). B, left, quantification of the incidence of CTCs double-stained with pancytokeratin and Ki67 antibodies in patients with 29 early and 27 metastatic breast cancer. Right, quantification of percentage of double-positive CTCs/total CTCs expressing Ki67 antigen detected in all patients.

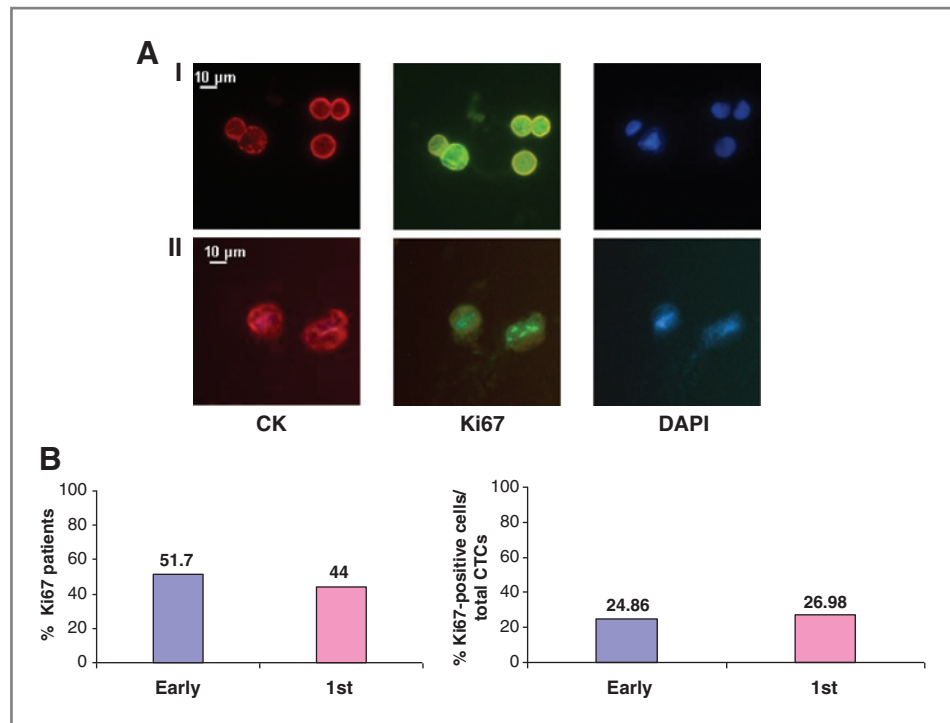


Table 2. Apoptotic and nonapoptotic CTCs/ 5×10^5 PBMCs in individual patients before and after adjuvant treatment

Pts	CTCs before chemotherapy with polycaspase kit		CTCs after chemotherapy with polycaspase kit		CTCs before chemotherapy with M30 antibody		CTCs after chemotherapy with M30 antibody	
	A45+Caspase+	A45+Caspase-	A45+Caspase+	A45+Caspase-	A45+M30+	A45+M30-	A45+M30+	A45+M30-
1	1	1	0	2	5	1	0	1
2	3	0	0	0	2	0	0	2
3	3	0	0	1	1	0	4	0
4	2	2	0	1	2	1	2	0
5	1	1	0	0	6	1	2	3
6	2	1	1	0	1	0	1	0
7	0	3	1	4	1	0	1	3
8	0	2	0	0	6	1	0	0
9	1	0	0	0	6	1	1	15
10	1	0	0	0	2	0	1	2
Total	14	9	2	8	28	5	12	26

Proliferation status. Six of 10 patients had detectable CK(+)/Ki67(+) CTCs before the initiation of adjuvant chemotherapy; in 4 of them, no Ki67-expressing CTCs were detected posttreatment (Table 3). The proliferative fraction among the total population of CTCs detected pre- and postchemotherapy decreased from 63.9% to 30%, respectively. The absolute change in the numbers of proliferative CTCs is depicted in Supplementary Fig. S1D.

Discussion

CTCs hold significant prognostic information for patients with early and metastatic breast cancer due to their involvement in the initiation of distant metastases (2–4, 30, 31). Several lines of evidence suggest that the population of CTCs is composed of biologically heterogeneous cells,

providing a possible explanation for the fact that a proportion of patients with early disease will not finally relapse despite the presence of CTCs in their blood. Indeed, it seems that only a minority of CTCs have the potential to generate metastases, whereas most of them will die in the bloodstream (7, 19, 20, 28, 32). Thus, it is important to characterize individual patient's CTCs to have an insight into their fate during the metastatic process.

We have recently reported that activated kinases, participating in cell survival and proliferation such as AKT and PI3K are expressed in CTCs of patients with early and metastatic breast cancer (20). However, a significant heterogeneity of this expression was observed not only in metastatic but mainly in early stages of the disease (7). In the current study, the apoptotic status of CTCs detected in patients with both early and metastatic breast cancer was evaluated to investigate whether apoptosis is related to disease stage and thus to better define their biologic relevance. The apoptotic status in CTCs was analyzed using two different methods that identify sequential phases of the apoptotic procedure. Specifically, the expression of the M30 epitope of cytokeratin is the result of the activation of caspases (polycaspase kit).

In the present study, 56 patients with breast cancer with detectable CTCs were enrolled. Using the polycaspase detection kit, apoptotic CTCs were observed both in patients with early and metastatic disease but their detection was significantly more frequent in the early disease setting (78.6% vs. 33%, $P = 0.001$). To the best of our knowledge, there are no data in the literature concerning the expression of apoptotic markers in CTCs of patients with early breast cancer. Similar rates of CTCs undergoing apoptosis in advanced breast cancer have been observed in a study by Mehes and colleagues (33), where 37.5% of CK-positive patients presented with apoptotic CTCs which were characterized according to an "inclusion type" cytokeratin staining pattern and nuclear condensation.

Table 3. Ki67-positive and -negative CTCs/ 5×10^5 PBMCs in individual patients before and after adjuvant treatment

Pts	CTCs before chemotherapy		CTCs after chemotherapy	
	Ki67+CK+	Ki67-CK+	Ki67+CK+	Ki67-CK+
1	0	1	7	5
2	1	1	0	1
3	1	1	0	1
4	1	1	1	0
5	0	1	0	5
6	0	3	0	3
7	1	4	1	4
8	0	3	0	0
9	16	10	0	2
10	1	2	0	0
Total	21	27	9	21

Further evaluation using the M30 antibody confirmed the data obtained with the polycaspase detection kit by revealing that the percentage of patients with early disease who had detectable apoptotic CTCs in their blood is significantly higher compared with patients with metastatic disease. Specifically, CTCs undergoing apoptosis were detected in 93% of patients with early disease and in 52% of patients with metastatic disease ($P = 0.001$). This observation is in accordance with a previous study based on cell and nuclear morphology, showing that less intensively stained, probably apoptotic, CTCs were determined more frequently in early compared with advanced disease (34).

Even though the above methods (polycaspase detection kit and M30 antibody) revealed that apoptosis is statistically higher in CTCs of early compared with metastatic patients, there are discrepancies concerning the percentages of apoptotic cells. These differences can be explained by the fact that each assay evaluated different sequential steps in the apoptotic procedure: the first one characterizes the activation of caspases, whereas the second identifies a neo-epitope of cytokeratin 18 created after caspase cleavage.

It is interesting to note that despite the fact that apoptotic CTCs were more frequently observed in early stages of the disease, nonapoptotic CTCs could also be observed in a considerable percentage of early CK-positive patients (in 68.9% and in 55.2% of patients using M30 and the polycaspase kit, respectively). Moreover, less than half of the patients were identified with exclusively apoptotic cells. The respective percentages in the metastatic disease setting were even lower (22.2% and 7.4% of patients, respectively). The heterogeneity concerning the apoptotic status of CTCs has been also shown in previous studies reporting on the expression of M30 in CTCs isolated from patients with prostate and breast cancer (11, 12, 35). It should be mentioned that due to the pilot nature of the study and the relative small number of patients, the only statistical correlation with clinical parameters ($P < 0.024$) was between the estrogen receptor (ER) status of the primary tumor and polycaspase expression in CTCs in patients with metastatic breast cancer. This implies that ER-positive patients tend to have more apoptotic CTCs in their blood.

Further characterization of nonapoptotic CTCs by double staining experiments using pancytokeratin and Ki67 antibodies showed that 51.7% and 44.0% of patients with early and metastatic breast cancer harbor proliferative CTCs. In addition, the fraction of the total CTCs representing proliferating cells was 24.9% and 27.0% in patients with early and metastatic disease, respectively. Our results are in agreement with other studies reporting Ki67 expression in CTCs. Ki67 expression in these studies was revealed in many patients with prostate and small cell lung cancer, but in different frequencies per patient (22, 23). On the contrary, the only study addressing Ki67 expression in CTCs of patients with breast cancer showed no expression of Ki67 antigen, although this study was

conducted in 9 CTC-positive patients and included only 22 CTCs (21).

The fate of Ki67-expressing CTCs is unknown; nevertheless, their detection could possibly mirror the proliferative status of the disease at the time of blood sampling. Furthermore, Ki67 positivity in the primary tumor is generally accepted as a marker of poor prognosis in early breast cancer (36). Interestingly, 3 of 4 patients with early disease, who subsequently relapsed, had detectable Ki67 CTCs before the initiation of any systemic adjuvant treatment suggesting that Ki67 expression on CTCs could also be associated with poor patient prognosis.

Previous studies have shown that chemotherapy could influence the absolute number of CTCs in patients with early and metastatic breast cancer, although in almost all the studies many patients remained CTC-positive after treatment. Furthermore, the detection of these cells post-chemotherapy is correlated with worse prognosis and with resistance to therapy (4, 5, 18, 37, 38). In the current study, we sought to characterize the apoptotic and proliferative status of CTCs remaining after the completion of adjuvant chemotherapy. The results revealed that adjuvant chemotherapy resulted in CTC elimination (concerning all the examined molecules) in only one patient (Table 3), whereas in another patient, only apoptotic cells could be detected postchemotherapy; conversely, in the remaining 8 patients, both apoptotic and proliferative CTCs were observed. These findings are in line with those reported by Fehm and colleagues (14) who showed that CTCs could be detected in the bone marrow of patients with breast cancer after neoadjuvant treatment; in the same report, apoptotic cells were mainly observed in patients with complete or partial response, whereas patients with progressive disease did not present apoptotic CTCs (14). Our findings are also in line with other studies reporting the persistence of CTCs after adjuvant treatment (18, 38, 39) implying that distinctive molecular characteristics of these cells are responsible for their resistance to therapy.

Conclusions

The data presented here clearly show a significant heterogeneity in the expression of apoptosis and proliferation markers among CTCs. Quantitative differences were observed in the extent of apoptosis in CTCs of patients with early and metastatic breast cancer, suggesting that the viability of CTCs is related to the stage of the disease. In addition, nonapoptotic and/or proliferating CTCs persist after the completion of adjuvant chemotherapy and could be involved in subsequent disease progression. Further investigation is needed to validate whether apoptotic and/or proliferation markers on CTCs could be used for the evaluation of the efficacy of adjuvant treatment and for the selection of patients at risk of relapse, for further treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. Kallergi, D. Mavroudis, S. Agelaki

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Kallergi, S. Agelaki

Writing, review, and/or revision of the manuscript: G. Kallergi, D. Mavroudis, C. Stournaras, V. Georgoulas, S. Agelaki

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