Apoptotic Circulating Tumor Cells (CTCs) in early and metastatic breast cancer patients

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Abstract:

The detection of circulating tumor cells (CTCs) in breast cancer is strongly associated with disease relapse. Since it is unclear if all CTCs are capable to generate metastasis, we investigated their apoptotic and proliferative status in 56 CTC-positive (29 early and 27 metastatic) breast cancer (BC) patients. Double staining immunofluorescence (IF) experiments were performed in peripheral blood mononuclear cells (PBMC) cytospins, 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 to early breast cancer patients (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 to 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/patient and the number of proliferating CTCs (63.9% vs 30%). In conclusion apoptotic CTCs could be detected in patients with BC irrespectively of their clinical status, though the incidence of detection is higher in early compared to metastatic patients. The detection of CTCs which survive despite adjuvant therapy implies that CTC elimination should be attempted using agents targeting their distinctive molecular characteristics.
Introduction

Circulating tumor cells (CTCs) are increasingly considered as a “liquid biopsy” that allows the assessment of tumour changes over time simply by repeated blood draws (1). The presence of CTCs in the blood of breast cancer patients has emerged as a poor prognostic indicator (2-4). Indeed, metastasis is associated with the presence of disseminated (DTCs) or circulating (CTCs) tumour 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 tumour 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 biological characteristics, as well as on the microenvironment at the secondary site (10). Only rare subsets of cells finally succeed in establishing a cross-talk with stromal cells in secondary organs which promote tumour-cell survival, angiogenesis and metastatic outgrowth.

Characterizing the viability of CTCs in each patient might be important in order to improve prognostication and individualize treatment. Thus, the presence of exclusively apoptotic CTCs in a patient may represent a favourable 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 et al (11) evaluating the presence of apoptotic and non-apoptotic CTCs in metastatic prostate cancer reported that the number of apoptotic or intact CTCs differs among patients. Similarly, both apoptotic and non-apoptotic CTCs could be detected in a
patient with metastatic breast cancer (MBC) (12). Viable CTCs and DTCs were also evaluated in ovarian cancer patients where it was shown that patients with viable DTCs after treatment had significantly reduced progression-free survival (13). The detection of DTCs in breast cancer patients 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, serological cell death biomarkers such as M30 and M65 have been detected in the serum of prostate, colorectal and lung cancer patients 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 which are involved in survival pathways are expressed in CTCs from early and metastatic breast cancer patients (19, 20). There are also conflicting studies concerning the proliferation status of these cells. Muller et al 2005 (21), reported that CTCs from breast cancer patients do not express Ki67. However other studies in prostate and small cell lung patients revealed Ki67 expression in many patients’ CTCs but in different frequencies per patient (22, 23).

The aims of the current study was 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 cytopsin preparation

A total of 56 patients with early (n=29) and metastatic (n=27) breast cancer with detectable CK-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 have at least two CTCs per 10⁶ PBMCs.

In addition in order to evaluate CTCs before and after chemotherapy in a cohort of ten patients we have obtained blood before and after the end of adjuvant chemotherapy whenever this occurred; samples were obtained after the 8th chemotherapy dose-dense cycle in six out of 10 patients (corresponding to a 4-month treatment), after the 6th cycle in three and after the 4th chemotherapy cycles in one patients, 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 were discarded. These precautions were undertaken in order 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.

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient (d=1,077gr/ml) centrifugation at 1800rpm for 30min. Aliquots of 500,000 PBMCs were cyto-centrifuged at 2000rpm for 2min on glass slides. Cytospins were
dried and stored at –80°C until use. At least two slides with a total of \(5 \times 10^5\) PBMCs were analyzed for each examined molecule in individual patients.

**Cell cultures**

MCF7 and SKBR3 breast cancer cells (obtained from ATCC; 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 cytospins according to the procedure followed for patients’ samples and they were used as controls for apoptotic and Ki67 staining experiments, respectively.

MCF7 mammary adenocarcinoma cells were incubated with 2μM staurosporin for 2h in order to be engaged to apoptosis (24). MCF7 cells were cultured in 1:1 (v/v) Dulbecco’s Modified Eagle Medium (DMEM)/Ham’s F12 medium (GIBCO-BRL Co, MD, USA) supplemented with 10% foetal bovine serum (FBS) (GIBCO-BRL), 2mM L-glutamine (GIBCO-BRL), 30mM NaHCO\(_3\), 16ng/ml insulin and 50mg/ml penicilline/streptomycin (GIBCO-BRL). SKBR3 cells were cultured in RPMI supplemented with 10% FBS. Cells were maintained in a humidified atmosphere of 5% CO\(_2\) in air. Subcultivation was performed with 0.25% trypsin and 5mM EDTA (GIBCO-BRL). All experiments were performed during the logarithmic growth phase. Twenty to 24 hours prior to the experiments, cells were transferred in serum-free medium containing only L-glutamine, NaHCO\(_3\) and penicillin/streptomycin. After incubation, cells were centrifuged on cytospins 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, Carlsbad, CA, USA). The kit utilizes 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, Mannheim, Germany) CTCs. The M30 antibody binds to the respective neo-epitope exposed after caspase cleavage of human cytokeratin (18). For either method, cytospins were evaluated using a confocal laser scanning microscope module (Leica Lasertechnik, Heidelberg, Germany) and the Ariol microscopy system (Genetix, UK), an automated system for CTC identification (27, 28).

a) Detection of apoptotic CTCs using the Polycaspase detection kit

Cytospins from patients PBMCs’ were washed with PBS and incubated with 0.5x FLICA reagent for 1h. 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, Germany) followed by the secondary anti-mouse antibody Alexa 555 (Invitrogen). The pancytokeratin A45-B/B3 antibody was used as marker for epithelial cells. The cyto-morphological criteria proposed by Meng et al (29) (i.e. high nuclear/cytoplasmic ratio, larger cells than white blood cells etc) were used in order to characterize a CK-positive cell as a CTC. Cells were also stained with DAPI conjugated with antifade (Invitrogen).

b) Detection apoptotic M30-positive CTCs
Cytospins from the same cohort of patients were fixed with cold aceton:methanol 9:1 (v/v) for 20min 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 1h. 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 2μM staurosporin (Merck, Darmstadt, Germany);

ii) two negative controls: a) untreated MCF7 cells stained with M30 antibody or FLICA reagent; b) cells treated with staurosporin 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, Cambridge, UK) or CD45 (Santa Cruz, Santa Cruz, CA, USA) 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 early breast cancer patients (median: 2.5 CTCs/per patient; range: 1-27) and 505 CTCs (median: 3 CTCs/patient; range: 1-40) in MBC patients. CTC counts per patient correspond to CTCs/5x10^5 PBMCs which is approximately included into 0.5 ml of blood. After a median follow-up period of 54.5 months (range, 42-94), four (14%) of the early breast cancer patients presented a distant relapse.

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

MCF7 control cells with and without staurosporin were analyzed with Ariols system (Figure 1A, B) and confocal laser scanning microscopy (Figure 1A (III), 1B (III)). Control experiments have shown that in slides without staurosporin (Figure 1A (II), 1B(II)) the percentage of apoptotic MCF7 cells was less than 1% while after 2h treatment with staurosporin 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 (Figure 1A(I), 1B(I)). In addition, the patients’ PBMCs nearby were not apoptotic (figures 2B, 2C) indicating that the observed apoptosis in CTCs is not induced ex vivo by the experimental procedure.
a) Polycaspace 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 to patients with metastatic disease (78.6% vs 33%, respectively; p=0.001) (Figure 2A, I). Exclusively apoptotic CTCs were observed in 42.9% vs 7.4% of early and metastatic disease, respectively. The median percentage of apoptotic CTCs/patient was 53.6% (range: 0%-100%) in patients with early and 0% (range: 0%-100%) in patients with metastatic disease (Figure 2A, II). Moreover, 47.4% of the total analyzed CTCs were caspase-positive in patients with early breast cancer compared to 8.1% in metastatic patients (p=0.0001) (Figure 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, in order to avoid potential artifacts. The activated caspases mainly sited in the cytoplasm in CTCs of a breast cancer patient as it is demonstrated in Figure 2B while the observed nearby PBMCs in patients’ cytospins were not apoptotic, as it is shown in figures 2B, 2C supporting our previous conclusion that apoptosis in CTCs is an in vivo phenomenon and not induced ex vivo by the experimental procedure.

b) 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 non-apoptotic (red) cells (Figure 1B). The intracellular distribution
of M30 antibody in CTCs of a patient with breast cancer patient is showed in Figure 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) (Figure 2A, I). Exclusively CK (+)/M30 (+) CTCs were observed in 31% and 22.2% of early and metastatic breast cancer patients, respectively. Moreover, 76.6% of the total analyzed CTCs were CK (+)/M30 (+) in patients with early breast cancer compared to 32.1% in the group of patients with MBC (p=0.002) (Figure 2A, III). The median percentage of apoptotic CTCs/patient was 80% (range: 0%-100%) in patients with early and 15% (range: 0%-100%) in patients with metastatic disease (Figure 2A, II). However, 69.0% of early and 77.8% of metastatic patients also had detectable non-apoptotic 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:**

Since non-apoptotic CTCs were observed in both early and advanced breast cancer we further investigated the proliferation status of CTCs. The detection of Ki67 positivity in MCF-7 cells (I) and in CTCs of a breast cancer patient (II) is demonstrated in Figure 3A. A total of 183 and 171 CK (+) CTCs were detected in early and metastatic breast cancer patients, respectively. Double stained CK(+)/Ki67(+) CTCs were observed in 51.7% and 44.0% of early and metastatic breast cancer patients, respectively (p=0.443) (Figure 3B). Exclusively CK (+)/Ki67 (+) CTCs were identified in one patient with early disease and in two with MBC. Moreover, 24.9%
and 27.0% of the total CTCs detected in patients with early and metastatic breast cancer were CK (+)/Ki67 (+) (Figure 3B). Three out of four early breast cancer patients who finally relapsed displayed detectable CK (+)/Ki67 (+) CTCs at the baseline sample. Furthermore two patients, who consequently passed away, had Ki67-positive CTCs in their blood.

In healthy volunteers no CK-positive cells (CTCs) were observed while spontaneously proliferative PBMCs were observed in the cytospin.

**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 early breast cancer patients for whom samples were available both before and after the completion of chemotherapy.

**i) 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 four patients (Figure 1A Supplementary).

The absolute number of apoptotic CTCs, as detected with the polycaspase detection kit, was reduced in seven out of 10 patients post-chemotherapy (Figure 1B Supplementary); however, in four patients non-apoptotic CTCs were still detectable post-treatment (Table 2).

In parallel experiments using immunostaining with the M30 antibody, CK (+)/M30(+) apoptotic CTCs were reduced in six out of 10 patients (Figure 1C Supplementary), whereas six patients still had detectable M30-negative CTCs post-chemotherapy (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% pre-chemotherapy to 30% post-chemotherapy using the polycaspase kit and from 56.6% to 28.6% using M30 staining.

ii) Proliferation status: Six out of ten patients had detectable CK (+)/Ki67 (+) CTCs before the initiation of adjuvant chemotherapy; in four of them, no Ki67 expressing CTCs were detected post-treatment (Table 3). The proliferative fraction among the total population of CTCs detected pre- and post-chemotherapy decreased from 63.9% to 30%, respectively. The absolute change in the numbers of proliferative CTCs is depicted in supplementary Figure 1D.
Discussion

Circulating tumor cells hold significant prognostic information for early and metastatic breast cancer patients 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 comprised 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 in order 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 PI-3K are expressed in CTCs of early and metastatic breast cancer patients (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 both early and metastatic breast cancer patients was evaluated in order to investigate whether apoptosis is related to disease stage and thus to better define their biological 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 breast cancer patients 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 early breast cancer patients. Similar rates of CTCs undergoing apoptosis in advanced breast cancer have been observed in a study by Mehes et al (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.

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 to 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 to 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 to 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 neoepitope 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, non-apoptotic 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 demonstrated in previous studies reporting on the expression of M30 in CTCs isolated from prostate and breast cancer patients (11, 12, 35). It should be mentioned that due to the pilot nature of the study and to the relative small number of patients, the only statistical correlation with clinical parameters (p<0.024) was between the ER status of the primary tumor and polycaspase expression in CTCs in metastatic breast cancer patients. This implies that ER-positive patients trends to have more apoptotic CTCs in their blood.

Further characterization of non-apoptotic CTCs by double staining experiments using pan cytokeratin and Ki67 antibodies demonstrated that 51.7% and 44.0% of early and metastatic breast cancer patients harbour 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 prostate and small cell lung cancer patients, but in different frequencies per patient (22, 23). On the contrary the only study addressing Ki67 expression in CTCs of breast cancer patients showed no expression of Ki67 antigen, though 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, three out of four 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 early and metastatic breast cancer patients, though 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 post-chemotherapy; conversely, in the remaining eight patients both apoptotic and proliferative CTCs were observed. These findings are in line with those reported by Fehm et al (14) who showed that DTCs could be detected in the bone marrow of breast cancer patients 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 DTCs (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 demonstrate 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, non-apoptotic 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.
Acknowledgements

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References


Table 1: Patient characteristics

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Table 2: Apoptotic and non-apoptotic CTCs/5*10^5 PBMCs in individual patients before and after adjuvant treatment

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<tr>
<td>Total</td>
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<td>9</td>
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Table 3: Ki67-positive and negative CTCs/5×10⁵ PBMCs in individual patients before and after adjuvant treatment

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<th>CTCs before chemotherapy</th>
<th>CTCs post chemotherapy</th>
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<tr>
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<td>Ki67-CK+</td>
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<tr>
<td>Total</td>
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FIGURE LEGENDS

Figure 1. Expression of M30 and activated caspases in control MCF7 cells treated with 2μM staurosporin.

A (I). Representative ARIOL system images (x40) from a cytospin of MCF7 treated with 2μM staurosporin and stained with pan-cytokeratin (A45-B/B3) /Alexa 555 (red) and FLICA reagent (green)

A(II) Representative ARIOL system images (x40) from a cytospin of MCF7 non-treated with staurosporin and stained with pan-cytokeratin (A45-B/B3) /Alexa 555 (red) and FLICA reagent (green).

A(III) Confocal laser scanning image of MCF7 cells treated with 2μM staurosporin and stained with pan-cytokeratin (A45-B/B3) /Alexa 555 (red) and FLICA reagent (green).

B (I). Representative ARIOL system images (x40) from a cytospin of MCF7 treated with 2μM staurosporin and stained with pan-cytokeratin (A45-B/B3) /Alexa 555 (red) and M30 antibody (green)

B(II) Representative ARIOL system images (x40) from a cytospin of MCF7 not-treated with staurosporin and stained with pan-cytokeratin (A45-B/B3) /Alexa 555 (red) and M30 antibody (green).

B(III) Confocal laser scanning image of MCF7 cells treated with 2μM staurosporin and stained with pan-cytokeratin (A45-B/B3) /Alexa 555 (red) and M30 antibody (green).

Figure 2. Expression of M30 and activated caspases in CTCs of early and metastatic breast cancer patients.
A. (I) Quantification of the incidence of double stained CTCs in 29 early and 27 metastatic breast cancer patients. (II) Quantification of the % median expression per patient for each examined molecule. (III) Quantification of % double positive CTCs/Total CTCs detected in patients for each examined molecule.

B. (I) Representative ARIOL system images (x40) from a cytospin of a patient double stained with monoclonal pancytokeratin (A45-B/B3) /Alexa 555 (red) antibodies and FLICA reagent (green).

(II) Representative ARIOL system images (x40) from a cytospin of a patient double stained with monoclonal pancytokeratin (A45-B/B3) /Alexa 555 (red) and M30 FITC conjugated antibodies (green)

Figure 3. Ki67 expression in CTCs of breast cancer patients

A. (I) Representative ARIOL system images (X40) of control MCF7 cells stained with monoclonal pancytokeratin (A45-B/B3) /Alexa 555 (red) and Ki67 FITC conjugated antibodies (green).

(II) Representative ARIOL system images (x60) from a cytospin of a patient double stained with monoclonal pancytokeratin (A45-B/B3) /Alexa 555 (red) and Ki67 FITC conjugated antibodies (green).

B. (I) Quantification of the incidence of CTCs double stained with pancytokeratin and Ki67 antibodies in 29 early and 27 metastatic breast cancer patients.

(II) Quantification of % double positive CTCs/Total CTCs expressing Ki67 antigen detected in all patients.
Figure 2

A

I

II

III

B

C

10 μm

Cytokeratin  Caspase  DAPI

% patients

% Median

% CTCs/total CTCs

Metastatic

Early

Metastatic

Early

Metastatic

Early

Caspase positive  M30 positive

Caspase positive  M30 positive

Caspase positive  M30 positive
A Figure 3

I

II

10 μm

CK

Ki67

DAPI

B

% Ki67 Patients

Early

1st

51,7

44

% Ki67 positive cells/Total CTCs

Early

1st

24,86

26,98

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Galatea Kallergi, Georgios Konstantinidis, Harris Markomanolaki, et al.

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