

Assessing the efficacy of targeted therapy using circulating epithelial tumor cells (CETC): the example of SERM therapy monitoring as a unique tool to individualize therapy

Katharina Pachmann · Oumar Camara ·
Annika Kohlhase · Carola Rabenstein ·
Torsten Kroll · Ingo B. Runnebaum · Klaus Hoeffken

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Abstract

Purpose In malignant tumors, predictive markers have been developed with respect to targeted therapies. One of the first targeted therapies was the hormone-blocking treatment of tumors of the male and female reproductive system. A typical therapy in breast cancer is the use of the selective estrogen receptor modulator, tamoxifen. However, only some of the patients, positive for the target molecules, respond to the selected therapy. It would, therefore, be highly desirable to have a tool to promptly assess the therapeutic efficacy of the applied agent in the individual patient.

Methods Longitudinal observation of CETC provides a unique tool for monitoring therapy response. About 178 patients with breast cancer were followed prospectively during hormone therapy, requiring only 1 ml of peripheral blood, using a fluorochrome-labeled antibody against surface-epithelial antigen. Image analysis allowed CETC numbers to be calculated in relation to blood volume and monitoring over the entire course of treatment.

Results A more than tenfold increase in CETC during therapy was a strong indicator of looming relapse ($P = 0.0001$ hazard ratio 5.5; 95% confidence interval

1,297–23,626), and a Cox regression analysis of age, tumor size, receptor expression, nodal status and previous treatment resulted in a regression model, in which CETC behavior was the parameter with the highest independent correlation to relapse-free survival.

Conclusions The change in the number of CETC (increase or decrease) may, in the future, be used to guide therapy in order to change to other available treatment options in good time.

Keywords Breast cancer · Hormone therapy · Circulating epithelial tumor cells

Purpose

Malignant tumors of the male or female reproductive system, especially of the prostate in men and of the breast in women, are among the most frequent malignancies, accounting for about 310,000 deaths per year in the developed world. However, in most cases, patients do not die from the primary tumor but from metastases seeded from the tumor into vital organs. This has led to the assumption that cells are released from the primary tumor, which then migrate via the blood to distant loci where they can settle and re-grow. In breast cancer, adjuvant chemotherapy protocols have been developed aimed at eliminating these cells. Indeed, adjuvant chemotherapy has retained its relevance after 30 years of observation (Bonadonna et al. 2005), indicating that these remnant cells present after breast surgery are the cells that give rise to later metastases. Several purification and enrichment methods have been used to search for such cells in bone marrow and blood, and the debate continues as to the “true” number of such cells and micrometastases. All of these methods, however, have

K. Pachmann (✉) · A. Kohlhase · T. Kroll · K. Hoeffken
Department of Experimental Hematology and Oncology,
Clinic for Internal Medicine II, Friedrich Schiller University Jena,
Erlanger Allee 101, 07747 Jena, Germany
e-mail: Katharina.pachmann@med.uni-jena.de

O. Camara · I. B. Runnebaum
Women's Hospital,
Friedrich Schiller University Jena, Jena, Germany

C. Rabenstein
Transfusion Centre Bayreuth, Bayreuth, Germany

major drawbacks: density gradient purification with interphase recovery and several washing steps may lead to a significant loss of relevant cells (Fleisher and Marti 2001) and magnetic bead enrichment as used by the CellSearch system may lead to massive cell destruction, leaving mainly cell debris (Comans et al. 2010). Omitting all enrichment procedures, we have developed a method termed MAINTRAC[®], with which we were able to analyze the presence of viable cells in different stages of disease and their response to the applied therapies. The commercially available Cellsearch method, which probably due to the above-mentioned massive cell loss, detects >1 viable cells only in about 10% of patients with primary breast cancer (Bidard et al. 2010), whereas this was true in 3% of normal donors (Miller et al. 2010). We were able to detect CETC in 92% of all patients with primary breast cancer and in 3% of healthy donors (Pachmann et al. 2005).

It is hypothesized that all CETC from the primary tumor are rapidly eliminated from the blood (Molloy and van't Veer 2008) and that subsequently detected tumor cells must be continuously spread from occult micrometastatic loci (Meng et al. 2004). We have, however, shown that CETC are present in patients with cancer already before treatment (Camara et al. 2007), but additional cells can be disseminated by surgery (Camara et al. 2006). Part of these cells, after surgery, are rapidly eliminated (Camara et al. 2006) but remnant cells can be present in the blood at constant numbers over long times (Pachmann 2005), indicating that they are only rarely completely eliminated and can survive and re-circulate in peripheral blood.

CETC can be reduced or eliminated by systemic chemotherapy, and this correlates with a good prognosis, indicating of the role of these cells play in metastasis formation (Lobodasch et al. 2007). A reappearance or increase in CETC already during chemotherapy, possibly also through mobilization from remote loci, is associated with poor relapse-free survival (Pachmann et al. 2008). These re-increasing CETC obviously have a high capacity to resettle again when released at the end of chemotherapy, thereby giving them an advantage for re-growth into measurable metastases.

Monitoring the response of CETC with this method, we were able to confirm results for individual patients at the single-cell level (Pachmann et al. 2008) that had previously only been statistically determined in large patient populations, such as the observation that adjuvant systemic chemotherapy treatment is not necessary or not sufficient in all patients with breast cancer (van der Hage et al. 2007) and that estrogen receptor-negative (ER⁻) patients respond better to chemotherapy than estrogen receptor-positive (ER⁺) patients (Camara et al. 2007). In this report, we have extended our research to investigate the impact of the addition of hormones-blocking ER on CETC.

Androgens in male and estrogens in female patients can stimulate the growth of tumor cells carrying the respective receptors in the primary tumor as well as the metastases and thus contribute to a fatal outcome. Agents that bind to these receptors but that do not activate have been shown capable of inhibiting the growth of receptor-positive tumors, providing the first known targeted therapy. Such hormone-blocking treatment has contributed to improving outcome in patients with hormone receptor-positive tumors (Heel et al. 1978; Senn 1968). It is not known whether anti-androgens and tamoxifen can also exert influence on the remnant cells circulating in the blood after complete removal of the primary tumor. Obviously, such cells can persist for extended periods in the host, and this can lead to the formation of distant metastases even up to 20 years afterward (Meng et al. 2004).

A high proportion of patients with breast cancer have estrogen receptor-positive tumors, especially in the elderly group over 50 years. Hormone treatment has empirically been observed to exert beneficiary results (Bonnetterre 1992) only after a long, continuous treatment. This indicates that such a treatment may also influence tumor cells in the circulation that have not settled yet. However, some patients may not benefit from tamoxifen therapy due to an enzyme defect in the tamoxifen metabolism (Gaston and Kolesar 2008), and approximately 60% of patients with ER-positive breast cancer who initially respond to the selective estrogen receptor modulator tamoxifen will develop acquired resistance. On the other hand, patients are sometimes difficult to convince that it is necessary to comply (Owusu et al. 2008), particularly if adverse symptoms are prevalent (Fink et al. 2004) and immediate treatment success is not measurable for the individual patient. In the present report, we show that monitoring of CETC during hormone treatment is possible and can contribute to better determining the response to hormone-blocking agents and the efficacy of therapy.

Methods

During the recruitment interval from 2001 to 2006 in our institution, all patients with primary breast cancer scheduled for SERM treatment after neoadjuvant or adjuvant therapy or without prior chemotherapy, in total 178 sequential patients, who gave their informed consent, were included. They were prospectively analyzed for CETC up to 5 years of treatment with tamoxifen or, if relapse occurred earlier, until relapse. Most patients were analyzed for their CETC already during chemotherapy, but are included into the present evaluation only at the start of treatment with tamoxifen.

About 1 ml of anti-coagulated peripheral blood was obtained, according to ethics committee approval, and analyzed using the previously described microfluorimetric method, where assay method stability of the sample and reproducibility are extensively described (Pachmann et al. 2005). In short, in order to compensate for shipping delays, samples were subjected to red blood cell lysis at day 2 after blood drawing (with usually 95% viability) using 10 ml of erythrocyte lysis solution (Qiagen, Hilden, Germany) for 10 min in the cold, spun down at 700 g and re-diluted in 1 ml of PBS. About 10 μ l of fluorescein isothiocyanate (FITC)-conjugated mouse anti-human epithelial antibody (HEA) (Milteny, Bergisch Gladbach Germany) and 1 μ l of phycoerythrin (PE)-labeled anti-CD45 were added to 100 μ l of cell suspension, incubated for 15 min in the dark and readjusted to 1 ml, and 20 μ l of this suspension was used for measuring epithelial antigen-positive cells.

A defined volume of the cell suspension was applied to a defined area either on adhesion slides (Menzel Gläser, Braunschweig, Germany) or into wells of Elisa plates; the adherent cells were measured either using a Laser Scanning Cytometer (LSC[®] Compucyte Corporation, Cambridge, MA, USA) and collecting the FITC-HEA and the PE-CD45 fluorescence using a photomultiplier (PMT) or using image analysis in the ScanR (Olympus, Munich, Germany). Values are displayed in scatter grams and histograms. Both approaches enable the user to locate cells contained within the positive population for visual examination and to take photos and fluoromicrographs. Figure 1a depicts an example of the procedure. In Fig. 1b, viability of the cells was visually detected and verified by propidium iodide (PI) staining (entering exclusively dying cells), looking for nuclear PI stain and exclusive surface EpCAM staining. Most patients have been followed for their CETC numbers during adjuvant or neoadjuvant treatment, if necessary. During the subsequent maintenance therapy, CETCs were analyzed at each visit, if possible at intervals of 3 months and later at more extended intervals. This allowed longitudinal follow-up of the CETCs for each patient. Patients were categorized according to their behavior into those with a more than tenfold increase or decrease over a period of 2 years or if relapse occurred before this time. Patients with changes less than tenfold were grouped as those with no significant change. The median relapse-free survival in the groups with decreasing CETCs was not reached, whereas the median relapse-free survival time for the patients with increasing CETCs was about 4 years. If a level of significance of $P = 0.001$ with a power of 0.8 was to be detected, the sample size was calculated to require 35 patients in each group, which was exceeded in both the group with decreasing and increasing CETCs. Statistical analyses of Kaplan–Meier relapse-free survival and the Cox regression analysis for the confounding variables

tumor size, lymph node status, HER2/neu status, hormone receptor status and previous chemotherapy were performed using the SPSS program, version 16.1.

Results

The characteristics of all patients are shown in Table 1. They were similar to the patients' characteristics in large studies such as the Breast International Group 1–98 trial (Doughty 2008) with respect to age, tumor size, nodal status and estrogen receptor positivity and frequency of relapses. One hundred and seventeen out of 178 patients (66%) had previously received neoadjuvant or adjuvant chemotherapy. Patients with confirmed HER2/neu positivity were treated with trastuzumab for 1 year. As previously reported (Pachmann et al. 2008), most patients even after adjuvant systemic chemotherapy still had circulating tumor cells detectable and all 178 patients received tamoxifen for hormone blockade. The median of CETC numbers before hormone treatment was 3,200/ml (200–51,200/ml, 5–95% confidence interval). For 29 (16%) patients, only single analyses of CETC values were available during tamoxifen therapy, leaving 149 patients evaluable for CETC changes during hormone-blocking maintenance treatment. About three patients with primary metastasized breast cancer were excluded. Cell numbers decreased in 66 (45%) and marginally changed in 23 (16%) patients. A more than tenfold increase in cell numbers in spite of systemic hormone blockade occurred in 58 (39%) patients. Typical courses of cell numbers from three individual patients each are shown in Fig. 2a and b. In contrast to the sharp, more than tenfold decrease or increase in CETC numbers seen during the weeks of chemotherapy (left negative axis in Fig. 2a, b), the response to hormone therapy was a slow stepwise reduction (right positive axis) over a period of up to 4 years of monitoring.

In total, 43/175 (25%) relapses occurred during treatment with tamoxifen (8 local and 35 distant metastases), for which CETC monitoring was available in 28/147 (19%), indicating that the population with repeated CETC analysis was representative for the whole patient population; 2 relapses occurred among the 66 cases with decreasing CETC (3%), 3 of the 23 cases with marginal changes (13%) and 21 of the 58 evaluable cases with increasing CETC during tamoxifen treatment (36%). Patients with lacking information from a HER2/neu analysis and the few patients for whom hormone receptor determination was not available were well balanced between patients in complete remission and those in relapse.

The two patients who suffered relapse in spite of decreasing CETC had an extremely high increase in numbers of CETC during surgery. Kaplan–Meier relapse-free

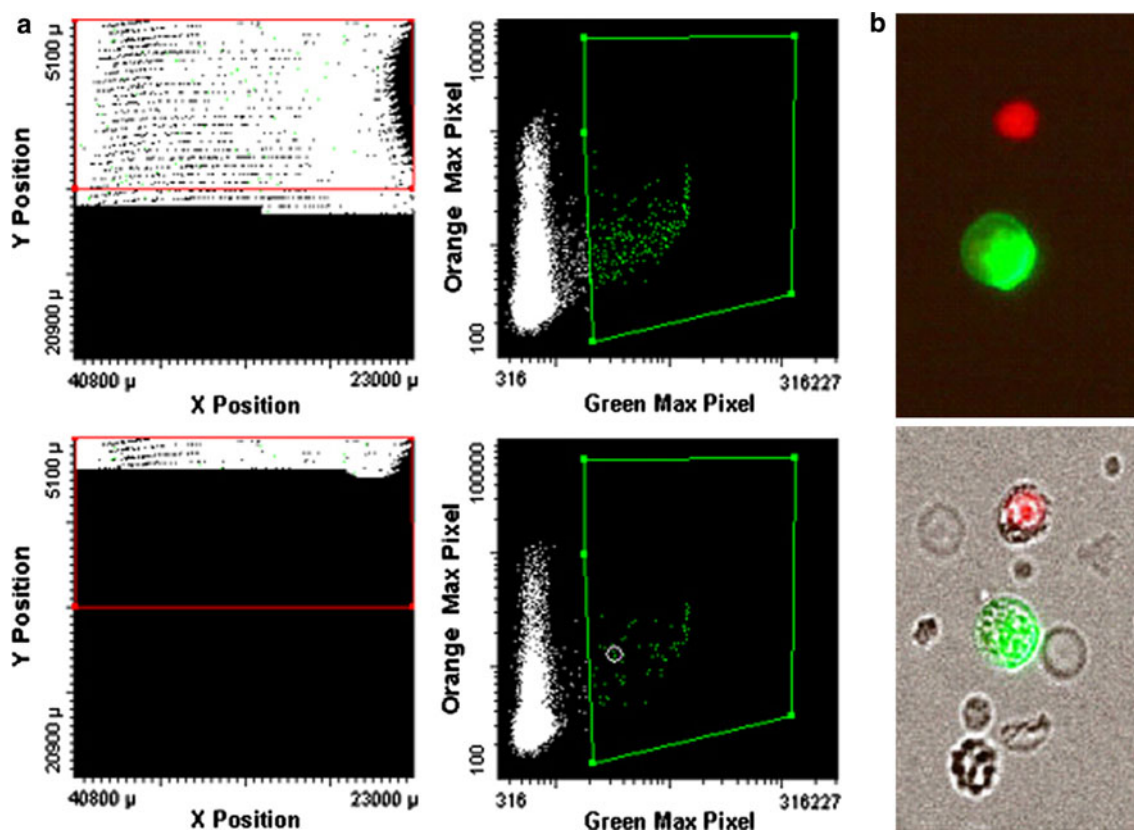


Fig. 1 **a** Example of the procedure used for analysis of epithelial cells in the LSC. The microscope scans a defined area on a slide, to which a defined volume of cell suspension is applied. The majority of cells are normal blood cells, showing only CD45 fluorescence. Positively stained green fluorescing cells are gated in the *green window*. Cells in

this window can be localized again, viewed, photographed and reanalyzed. **b** Shows a typical picture of a tumor cell detectable in the Scan R by its *green fluorescing cap*, accompanied by two dead (PI-positive) normal blood cells. In transmitted light, more live (PI-negative) blood cells are visible

survival curves are shown in Fig. 3. Patients with increasing numbers of CETC during tamoxifen treatment had a significantly poorer relapse-free survival than patients with decreasing CETC ($P < 0.0001$ hazard ratio = 5.5; 95% confidence interval 1,297–23,626) during the subsequent up to 5½ years (CR: median 850 days, minimum 9 days, maximum 3,155 days, relapses: median 723 days, minimum 175 days, maximum 8,278 days) of observation independent of the previous neoadjuvant or adjuvant treatments. Patients with stable or slightly undulating CETC had an intermediate risk. If compared for known risk factors, there was no difference between relapsing patients and patients in complete continuous remission with respect to age, HER2/neu status, estrogen receptor status or adjuvant or neoadjuvant treatment. But patients with a relapse had a tendency to have larger tumors ($P = 0.45$) and had a significantly higher frequency of positive lymph nodes ($P = 0.005$). Patients who did not receive chemotherapy due to good prognosis had a tendency ($P = 0.12$) to have fewer relapses (Table 2). However, in a multivariate Cox regression model, the increase in CETC turned out to be the most significant independent prognostic factor (Table 2).

The behavior of the CETC during the tamoxifen treatment (decrease and marginal changes or increase) was also highly predictive for the subsequent outcome of 45 patients who were scheduled to receive aromatase inhibitors (AI) either due to relapse (17 (38%)) or due to the new treatment strategy of switching from tamoxifen to an AI (28 (62%)) (Fig. 4). Patients who had already shown an increase in cell numbers during tamoxifen treatment had a poorer relapse-free survival during treatment with AI than patients who had responded to tamoxifen therapy with a decrease in CETC ($P = 0.004$). However, there was a clear progression and relapse-free survival advantage in patients receiving AI after tamoxifen also in our patient sample as will be shown in a following paper.

Conclusions

Although in patients with breast cancer the risk of relapse is higher in the hormone receptor-negative (HR-) group between years 3 and 4, the hazard of recurrence for HR- and HR+ patients subsequently crosses, and beyond 5 years it

Table 1 Characteristics of all 175 patients with respect to age, tumor size, lymph nodes, HER2/neu status, hormone receptor, complete remission/relapse/progress, chemotherapy

Total patients	175
Mean age	55
Tumor size	
T1	91 (52%)
T2>	67 (38%)
n.a.	17 (10%)
Lymph node	
N0	103 (59%)
N1>	56 (32%)
n.a.	16 (9%)
Her2/neu status	
Negative	83 (48%)
Positive	42 (24%)
n.a.	50 (28%)
Hormone receptor	
Negative	9 (5%)
Positive	155 (89%)
n.a.	11 (6%)
Relapse/CR	
Relapse	43 (25%)
CR	132 (75%)
n.a.	0 (0%)
Chemotherapy	
Adj	73 (42%)
Neo	41 (23%)
None	46 (26%)
n.a.	15 (9%)

n.a. not analyzed, *CR* complete remission, *adj* adjuvant, *neo* neoadjuvant

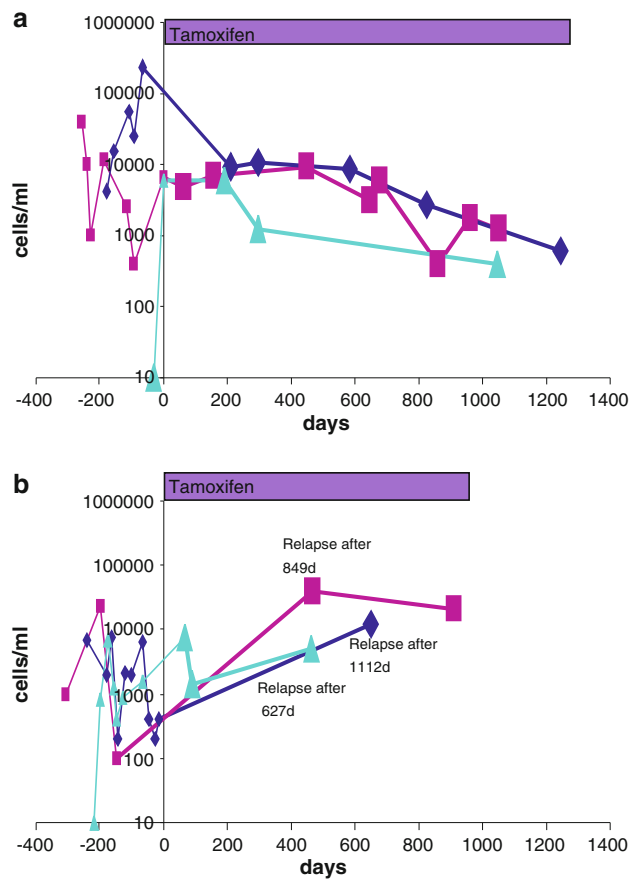


Fig. 2 a and b Typical changes in cell numbers in three individual patients each (increase and decrease during treatment with tamoxifen)

is actually higher for HR+ patients (Saphner et al. 1996). This means that, despite optimized treatment, eventually a high proportion of patients, even in the HR+ group with a relatively good prognosis, suffer relapse. Hormone receptor-blocking treatment, without or after previous chemotherapy, has proven to be advantageous over surgery alone in hormone-sensitive breast cancer, with tamoxifen being the gold standard in hormone therapy (EBCTCG 1998), and is regarded as one of the first targeted therapies improving outcome with or without previous chemotherapy. Tamoxifen has been assumed to block the growth-stimulating effect of estrogen causing cells to stay in the G1 phase of the cell cycle (Osborne 1998), whereas AI work by inhibiting the formation of estrogen (Hind et al. 2007). Hormone treatment may also prevent tumor cells from settling (Epstein 2005). Tamoxifen is relatively well tolerated and cost effective. This has prompted studies comparing different times of tamoxifen administration followed by AI (Rabaglio et al. 2007; Mamounas et al. 2008; Kaufmann et al. 2007), and it is not yet clear how long either hormone therapy should be given for optimal results (Ingle et al. 2006). Long-term tamoxifen therapy is also

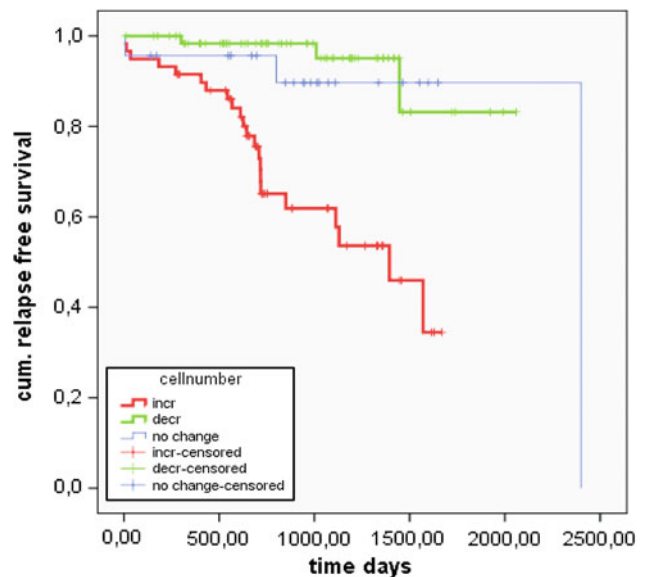


Fig. 3 Kaplan–Meier relapse-free survival curves of patients with increasing (red line) and decreasing (green line) CETC numbers during tamoxifen treatment ($P < 0.001$ hazard ratio = 5.5). Patients with no change in cell numbers (thin blue line)

Table 2 Differences between relapsing patients and patients in complete continuous remission with respect to age, tumor size, lymph nodes, HER2/neu status, hormone receptor and chemotherapy

	CR	Rel	P
Number of patients	132 (75%)	43 (25%)	
Mean age	51	53	ns
Tumor size			
T1	71 (54%)	20 (46%)	0.45 ns
T2>	49 (37%)	18 (42%)	
n.a.	12 (9%)	5 (12%)	
Node			
N0	87 (66%)	16 (37%)	0.005
N1>	33 (25%)	23 (54%)	
n.a.	12 (9%)	4 (9%)	
Her2/neu status			
Negative	62 (47%)	21 (49%)	ns
Positive	31 (23%)	11 (25.5%)	
n.a.	39 (30%)	11 (25.5%)	
Hormone receptor			
Negative	8 (6%)	1 (2%)	ns
Positive	115 (87%)	40 (93%)	
n.a.	9 (7%)	2 (5%)	
Chemotherapy			
Adj	51 (39%)	22 (51%)	ns
Neo	31 (23%)	10 (23%)	
None	38 (29%)	8 (19%)	0.12 ns
n.a.	12 (9%)	3 (7%)	
Patients with CETC analysis	121 (82%)	26 (18%)	
Decrease	64 (52%)	2 (11%)	<0.0001
Increase	37 (30%)	21 (81%)	
No change	20 (16%)	3 (8%)	

CR complete remission, Rel relapse, adj adjuvant, neo neoadjuvant, n.a. not analyzed

associated with a variety of adverse effects, including an increased risk of endometrial cancer and thromboembolic events. It would, therefore, be desirable to define earlier and more accurately the subgroup of patients who benefit from this treatment and those not or no longer benefiting from tamoxifen in order to provide them with the best therapy in good time.

In the present report, we have extended CETC monitoring, the value of which was established during neoadjuvant and adjuvant chemotherapy, to adjuvant hormone therapy. In contrast to the rapid response of CETC to chemotherapy, during tamoxifen therapy, we observed a slow, continuous change in CETC numbers over several years decreasing or increasing. This is compatible with the results of cell culture analysis, where cells are driven into the G0/G1 phase of the cell cycle by tamoxifen, rather than cell killing (Osborne 1998).

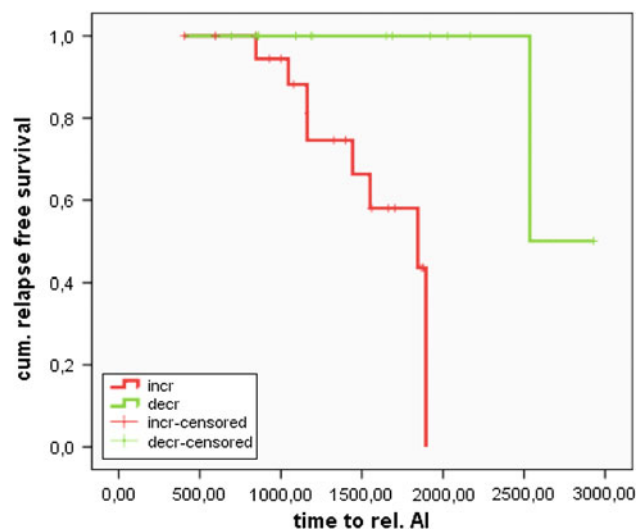


Fig. 4 Kaplan–Meier relapse-free survival curves of patients during treatment with aromatase inhibitors who had had increasing (red line) or decreasing (green line) CETC numbers during tamoxifen treatment, all patients ($P = 0.004$)

Patients with increasing numbers of CETC under tamoxifen therapy clearly had a significantly higher (5.5-fold) risk for relapse than those with decreasing CETC. Although it was not the aim of this work to provide an additional prognostic marker, the increase in CETC during tamoxifen therapy turned out to be the most significant independent prognostic factor in our patient sample which, although small, was distributed in prognostic factors similar to other larger studies. Rather, we aimed at developing a diagnostic tool for therapy monitoring and, we were indeed able to show that, in the absence of overt metastases, an increase in CETC during tamoxifen treatment is the earliest indicator of therapy resistance.

Patients with relapse in spite of decreasing CETC had already had a massive leachate of tumor cells during surgery. Such cells may have had the opportunity to settle and grow into metastases already before the start of tamoxifen therapy. In these patients with peripherally circulating cells still responsive to therapy, the metastases obviously did not respond to tamoxifen, probably due to resistance mechanisms developing in the metastases. If it turns out that one of the main actions of tamoxifen is to prevent circulating tumor cells from settling, then it would be meaningful to apply it early during treatment. Unfortunately, a direct comparison between the cells in the metastases and the circulating cells was not performed; this will be the aim of further research.

Most of the relapses occurred in patients with increasing CETC despite tamoxifen. Therefore, this method makes it possible to identify patients at high risk for progression early in the course of the disease and

quickly identify an inactive treatment regimen without requiring lengthy studies using survival endpoints. Apart from mechanisms postulated as being responsible for tamoxifen resistance (EBCTCG 1998; Osborne 1998; Hind et al. 2007; Jordan 2008), tamoxifen discontinuation may also play a role, particularly in elderly patients (Gaston and Kolesar 2008). Therefore, at times we find decreasing CETC numbers followed by a re-increase that may indicate tamoxifen resistance, but may also be due to failed compliance to tamoxifen. Gauging the behavior of their CETC might help to convince patients to take a medication that otherwise seems to have no immediate positive effect and eventually also help to decide when unnecessary medication can be terminated.

The increase in CETC during tamoxifen therapy was, however, also a highly significant predictor of relapse during subsequent treatment with AI. Some patients after relapse during tamoxifen can be kept in long-lasting progression-free status with AI. Some tamoxifen-resistant patients, possibly those with CYP2D6 deficiency may even be rescued by treatment with AI. Some of our patients were only switched to AI after developing metastases. It will be one of the main advantages of this new tool of CETC quantification that treatment results might be additionally improved if patients are switched to a better working treatment before the manifestation of metastases, and a switch to other treatment options available should be taken into consideration already at this point in time. CETC monitoring during hormone therapy would, thus, help in the decision of the appropriate time for changing therapies and help to increase the compliance of hormone therapy.

With many issues regarding the adjuvant use of hormone treatment remaining unsettled, such as the optimal duration of treatment or the optimal sequencing of tamoxifen with AI, CETC monitoring will, for the first time, provide a tool applicable to the individual patient to better control and tailor hormone therapy according to the patients' requirements, benefiting not only patients in complete remission but also patients who have already suffered relapse (Cianfrocca and Gradishar 2005).

This method could also contribute to trials in male androgen-dependent tumors (Souhami et al. 2009), giving reliable data, in order to tailor adjuvant endocrine treatment to the needs of the individual patient.

Conflict of interest We declare that we have no conflict of interest.

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