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Cytokeratin-19 mRNA-positive circulating tumor cells during follow-up of patients with operable breast cancer: prognostic relevance for late relapse

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Abstract

Background: The detection of cytokeratin-19 (CK-19) mRNA-positive circulating tumor cells (CTCs) before and/or after adjuvant chemotherapy in patients with operable breast cancer is associated with poor clinical outcomes. Reliable prognostic markers for late disease relapse are not available. In this study we investigated the value of CTC detection during the first five years of follow-up in predicting late disease relapse.

Methods: Blood was analyzed from 312 women with operable breast cancer who had not experienced disease relapse during the first two years of follow-up. A real-time reverse transcriptase polymerase chain reaction (RT-PCR) for CK-19 mRNA was used to detect CTCs three months after the completion of adjuvant chemotherapy and every six months thereafter for a follow-up period of five years.

Results: Eighty patients (25.6% of the study population) remained CTC free throughout the five year period. A change in CTC status was observed in 133 patients (42.6%); 64 patients (20.5%) with initially CK-19 mRNA-positive CTCs during the first 24 months turned CTC-negative afterwards while 69 (22.1%) who were initially CTC-negative became CTC-positive. Ninety-nine patients (31.7%) remained persistently CK-19 mRNA-positive. After a median follow-up period of 107 months (range: 38-161 months), the persistently CTC-positive patients with either hormonal receptor positive or negative tumors, had a higher risk of late-disease relapse compared to the persistently CTC-negative patients (36.4% versus 11.2%, $P<0.001$). Multivariate analysis revealed that persistently CTC-positive patients also had a shorter disease-free ($P=0.001$) and overall survival ($P=0.001$).

Conclusion: Persistent detection of CK-19 mRNA-positive CTCs during the first five years of follow-up is associated with an increased risk of late relapse and death in patients with operable breast cancer and indicates the presence of chemo- and hormonotherapy-resistant residual disease. This prognostic evaluation may be useful when deciding on subsequent adjuvant systemic therapy.

Introduction

Invasive breast cancer is the most common malignancy in women, accounting for 28 percent of new cancer cases and 15 percent of cancer deaths [1]. Due to declining mortality rates that are attributable mostly to the use of screening mammography and effective adjuvant therapy, more women nowadays survive their breast cancer [2]. Since metastatic disease is considered incurable, the early recognition and treatment of potentially still curable minimal residual disease is one of the major goals of care of breast cancer survivors and requires the in depth understanding of relapse patterns.

Depending on the specific breast cancer type, the majority of recurrences occur during years 2 to 5 [3], although they can occur earlier or much later [4-5]. Especially, for women with hormone receptor-positive disease, more than one-half of all recurrences and deaths occur beyond five years from diagnosis [5-6]. To date no tool is available for monitoring the effect of adjuvant treatment and in most cases the recurrence risk is calculated based on previous statistical analyses [7]. Therefore, with existing methods prediction of the risk of relapse for the individual patient is limited.

Disseminated tumor cells (DTCs) in bone marrow [8-9] and circulating tumor cells (CTCs) in peripheral blood [10-11] of patients with operable breast cancer have been shown to be independent adverse prognostic factors for disease recurrence and disease-related death. Immunocytochemistry using antibodies against proteins that are expressed on epithelial but not on mesenchymal cells is widely used for the detection of DTCs and CTCs. However, the detection of mRNA transcripts for specific epithelial markers by using reverse transcriptase polymerase chain reaction (RT-PCR) and, more recently, the quantitative real-time RT-PCR (QPCR) seems to have higher diagnostic sensitivity [12]. The major advantage of RNA-based approaches is related to the rapid degradation of RNA released from cells in the blood by RNAses;

therefore, the origin of detectable in blood RNA transcripts is considered to be viable cells. Cytokeratin-19 (CK-19), a cytoskeletal component present in normal and cancerous epithelial cells, has been extensively used for the detection of breast cancer cells in mesenchymal tissues and seems to be the most sensitive and reliable tumor marker in both patients with operable and metastatic breast cancer [13,14].

Several studies have shown the prognostic significance of CK-19 mRNA-positive CTCs in patients with operable breast cancer [10,11,15-17]. However, all these studies have investigated the prognostic value of CTCs at initial diagnosis and before the initiation and/or following the completion of adjuvant chemotherapy. Only a few reports exist concerning the clinical relevance of DTCs, but none for CTCs, during the surveillance period after the completion of adjuvant chemotherapy [18,19]. The unfavorable clinical outcome of patients with detectable isolated tumor cells in bone marrow was shown in the latter studies [18,19]. Given that DTCs and CTCs are theoretically the primary targets of adjuvant treatments, their fate after systemic therapy could be a potential useful marker permitting a direct and individualized assessment of treatment efficacy and a more accurate estimation of the risk of relapse.

In the present study, we sought to evaluate the clinical relevance of CK-19 mRNA-positive CTCs detected by a quantitative real-time RT-PCR assay at different time points during the follow up period after the completion of adjuvant chemotherapy in patients with operable breast cancer. We hypothesized that patients presenting detectable CK-19 mRNA-positive cells during follow up despite the administration of adjuvant therapy could be at an increased risk of late disease relapse (defined as relapse at least two years after the end of adjuvant chemotherapy) and death.

Materials and methods

Patients and clinical samples

We conducted a retrospective analysis of prospectively collected data in the context of an ongoing longitudinal study that has been previously reported [17]. Women with operable breast cancer (stage I to III) who were under surveillance and had not experienced disease relapse during the first two years of follow up, were eligible for this study. All patients had received adjuvant chemotherapy mostly in the context of research protocols of the Hellenic Oncology Research Group. After completion of adjuvant chemotherapy, patients received adjuvant radiotherapy and hormonal therapy when indicated according to their individual disease characteristics. There were no subgroups of patients who received only adjuvant hormone therapy or no adjuvant systemic therapy at all.

Patients' follow-up consisted of pertinent medical history and physical examination, with laboratory and imaging studies restricted as indicated, every 3 months for the first 2 years, every 6 months for the next 3 years and yearly thereafter. All treating physicians were completely unaware of the CK-19mRNA results for their individual patients and all follow up laboratory and imaging studies to detect disease relapse were performed independently of the CK-19mRNA results. All patients signed an informed consent to participate in the study, which was approved by the Ethics and Scientific Committees of our Institution.

Cytokeratin-19 mRNA-positive CTCs were monitored at specific time points after the completion of adjuvant chemotherapy for a 5-year follow up period. The first blood sample was obtained 3 months after the end of chemotherapy and subsequent samples were obtained every 6 months thereafter during the 5-year follow up.

Patients were classified into four groups based on their CTCs' status during the first two years and the subsequent three years of follow up (as persistently negative, persistently-positive, negative turn to positive and the opposite). At least one CK-19 mRNA-positive blood sample in the corresponding period of time was required for classifying the patient in the CTC-positive group. On the other hand, if all the collected blood samples were negative for CK-19 mRNA, the patient was characterized as CTC-negative. Using this definition, the patients were classified in the "persistently CTC-negative" group, if there were no positive blood samples for CK-19 mRNA throughout the 5-year follow up period. On the other hand, the "persistently CTC-positive" patients had at least one positive blood sample for CK-19 mRNA in the first 2 years and at least another positive one in the subsequent 3 years of follow up. Accordingly, patients in the "CTC-negative turn to positive" group had no positive samples in the first two years, but at least one positive sample in the next 3 years. The opposite was true for the "CTC-positive turn to negative" group.

Blood samples and Real-Time RT-PCR for CK-19 mRNA

Twenty milliliters (mL) of peripheral blood in EDTA was collected at each visit. To avoid contamination with epithelial cells from the skin, all blood samples were obtained at the middle of vein puncture after the first 5 mL of blood was discarded. Peripheral blood mononuclear cells were obtained by gradient density centrifugation using Ficoll-Hypaque [10]. Total RNA isolation was carried out with the use of Trizol LS reagent (Gibco, Life Sciences, BRL, Grand Island, NY) according to the manufacturer's instructions. The isolated RNA was dissolved in diethylpyrocarbonate-treated water and stored at −80°C until used. RNA concentration was determined by absorbance reading at 260 nm with the Hitachi UV-VIS (U-2000) spectophotometer

(Tokyo, Japan). The integrity was tested by PCR amplification of the β-actin housekeeping gene. As positive and negative controls we use RNA samples prepared from the MCF-7 breast cancer and ARH-77 leukemic cell lines respectively.

Reverse transcription of RNA was carried out with the Thermoscript RT-PCR system (Invitrogen, Paisley, UK). Complementary DNA (cDNA) was synthesized according to the manufacturer's instructions. The qPCR assays for the detection of CK-19 mRNA-positive cells, the used primers and the details of the cycling protocol have been previously described [20]. Briefly, 2 μl of cDNA were placed into a 8 μl reaction volume containing 1 μl of the sense primer CK-19- for (3 mM), 1 μl of the antisense primer CK-19-do (3 mM), 2.4 μl of the LightCycler Fast Start DNA Master Hybridization Probes reagent (10× concentration), 1 μl of the probe CK-19-FL (3 mM) and 1 μl of the probe CK-19-LC (3 mM). [15] The quality of cDNAs was evaluated by real-time PCR for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase.

The presence of >0.6 MCF-7 cell equivalents/5μg of total RNA was considered a positive result, according to the previously reported analytic detection limit of our assay [20]. Using this cutoff, only two of 89 (2.2%) female healthy blood donors were positive for CK-19 mRNA-positive cells while, none of nine women with benign breast disease (fibroadenomas) had positive blood samples [20]. The high specificity of the method was made possible by avoiding contamination of skin epithelial cells during venipuncture, as well as by carefully designing the primers and hybridization probes used, so that amplification of known CK-19 pseudogenes and genomic DNA would be avoided [20].

Statistical analysis

Disease-free survival (DFS), defined as the time from study entry until the day of the first evidence of disease recurrence and overall survival (OS) defined as the time from study entry to death were the main dependent variables of the study. The data-cut-off date was the July 20th 2010. Kaplan-Meier curves for DFS and OS were compared using the log-rank test to provide a univariate assessment of the prognostic value of selected clinical risk factors. Clinicopathologic factors known to be associated with prognosis, such as menopausal status (premenopausal *vs* postmenopausal), tumor size (T1 *vs* T2-3), number of axillary lymph nodes involved (0-3 *vs* ≥4), histological grade (1or 2 *vs* 3), estrogen receptor (ER) status (negative *vs* positive), progesterone receptor (PR) status (negative *vs* positive) and HER-2/*neu* status (negative *vs* positive) were tested in univariate analysis. Variables that were found to be significant at the univariate screen were then entered in a stepwise multivariate Cox proportional hazards regression model to identify those with independent prognostic information. Entry into and removal from the model were set at 5% and 10%, respectively. All statistical tests were performed at the 5% level of significance. SPSS version 13 (SPSS Inc, Chicago, IL) statistical software was used for the analysis. This report is written according to the reporting recommendations for tumor marker prognostic studies (REMARK criteria) [21].

Results

Patients' characteristics

A total of 455 consecutive patients with diagnosis of operable breast cancer treated and followed at the Department of Medical Oncology of the University Hospital of Heraklion between January 1997 and December 2004 were screened for eligibility for

this study. Four hundred twelve (91%) patients belong to the same cohort of patients that was used to evaluate the prognostic significance of CK-19 mRNA-positive CTCs detection before initiation and/or after completion of adjuvant chemotherapy [17].

One hundred forty-three patients were excluded for reasons listed in Figure 1 and 312 were included in the study. Patients' characteristics at the time of primary diagnosis in relation to CTCs' status during follow-up are summarized in Table 1. The persistent detection of CTCs during follow-up did not correlate with patient's and/or tumor's characteristics, such as age (p=0.197), menopausal status (p=0.372), tumor size (p=0.637), lymph node status (p=0.082), histopathological grade (p=0.746) and hormone receptor status (p=0.156). There was a difference in the type of adjuvant chemotherapy administered, with more patients in the persistently positive group having received anthracycline-based regimens (p=0.011).

Detection of CK-19 mRNA-Positive Cells during follow up

Cytokeratin-19 mRNA-positive cells were detected in the blood of 232 patients (74.4%) at any time point during the 5-year follow up period, while 80 patients (25.6%) remained CTC-free throughout the same period (persistently negative). More specifically, 99 patients (31.7%) had persistently detectable CK-19 mRNA-positive cells both during the first two and the subsequent three years of follow up (persistently-positive group). A change in CK-19 mRNA status was observed in almost half of patients (133 patients or 42.6%). Of those, 64 patients (20.5%) with initially detectable CK-19 mRNA-positive cells during the first 24 months turned CTC-negative afterwards (positive turn to negative group), while 69 patients (22.1%) who were initially CTC-negative became CTC-positive afterwards (negative turn to positive group) (Figure 1).

Detection of CK-19 mRNA-Positive Cells and Clinical Outcome

Disease recurrence. After a median follow-up period of 107 months (range: 38-161 months), 63 patients (20.2%) had developed a distant (n= 56; 88.8%) or locoregional recurrence (n=7; 11.2%) (Table 2). Compared to the persistently negative patients, only the group of CK-19 mRNA-persistently positive patients had a significant higher risk of disease relapse (36.4% versus 11.2%; Fisher's exact test, $p<0.001$). In fact, risk of disease recurrence was the highest in patients with persistently-positive CTCs (36.4% versus 7.8%; $p< 0.001$ and 36.4% versus 18.8%; $p=0.016$ compared to positive turn to negative and negative turn to positive groups, respectively) (Table 2).

The 5-year DFS rates were 82.5% versus 92.7% for persistently-positive versus persistently-negative patients, respectively. As illustrated in Figure 2A, persistently positive patients had a significantly shorter DFS than the persistently negative patients ($p < 0.001$). Although no group has as yet reached the median DFS, there was a progressive decrease in the DFS of the four groups of patients according to the detection of CK-19 mRNA-positive CTCs during the 5 years of follow up (Figure 2A).

Survival. Forty-one patients (13.1%) died during follow up as a result of disease progression. Twenty-four (58.5%) and five (12.2%) of these deaths occurred in the persistently-positive and persistently-negative groups, respectively (Fisher's exact test; $p=0.001$; Table 2). The 10-year overall survival rates were 81.4% for persistently-positive versus 96.7% for persistently-negative patients. Estimated median overall survival was significantly shorter for persistently positive compared to persistently negative patients ($p=0.013$). Similar to DFS, there was a progressive decrease in the OS of the four groups of patients according to the detection of CK-19 mRNA-positive CTCs during the 5 years of follow up (Figure 2B).

Subgroup analysis based on cumulative number of positive tests pre-chemotherapy CTC status and hormone receptors status.

Since patients underwent serial blood draws for the assessment of CK-19 mRNA-positive CTCs, we analyzed our data to address the question whether the cumulative number of positive tests matter. Among patients with positive tests 38.7% (during the first two years of follow up), 24.4% (during the subsequent 3 years) and 57.3% (during all the 5 years) had two or more positive test results (Additional file 1: supplementary table S1). No difference was found in disease-free survival between the groups with different cumulative number of positive tests, probably due to the small number of patients and events in each group (Additional file 1: supplementary figure S1).

Given the prognostic role of the CTCs' detection before the administration of adjuvant chemotherapy has already been reported [11], we investigated whether it could offer additional prognostic information to that of the serial measurements of CTCs during follow up. For this purpose, we reviewed the pre-chemotherapy CTCs' status of the patients included in this analysis (Additional file 1: supplementary table S2). No difference was found in the detection rate of the CK-19 mRNA-positive CTCs between the four groups (Pearson chi-square, p=0.320). Interestingly, the persistently positive patients with detectable CK-19 mRNA-positive CTCs before the administration of adjuvant chemotherapy had shorter DFS but not OS compared to the patients of the same group who tested negative for pre-chemotherapy CK-19 mRNA-positive CTCs (Additional file 1: supplementary figure S2).

Finally, a subgroup analysis was performed according to hormone receptor status. Interestingly, the persistently positive patients with either hormone receptor positive or negative tumors had a significantly higher relapse rate (table 3), risk of

death and shorter DFS than the persistently negative patients [(p= 0.039 and p=0.004 for persistently positive vs persistently negative patients with ER/PR negative and ER and/or PR positive tumors respectively) (figures 3a and 3b)]. However, the overall survival was shorter only for the persistently positive patients with ER/PR negative tumors (p=0.035, figures 3c and 3d).

Univariate and Multivariate Analysis

Persistent detection of CK-19 mRNA-positive CTCs during follow up after the completion of adjuvant chemotherapy, tumor size greater than 2.0 cm, more than three involved axillary lymph nodes and postmenopausal status were significantly associated with reduced DFS and OS in univariate analysis (Table 4). Multivariate analysis revealed that persistent detection of CK-19 mRNA-positive CTCs, tumor size and more than three involved axillary lymph nodes were independent prognostic factors for shorter DFS and OS (Table 5).

Discussion

We provide, to our knowledge, the first clear evidence of a strong correlation between detection of CK-19 mRNA-positive CTCs during follow up and increased risk of late disease relapse and death in patients with either hormonal receptor positive or negative operable breast cancer. These findings support the role of CTCs' monitoring as an adjunct to standard clinical and radiographic methods in the evaluation of disease status during follow up.

Although the prognostic role of DTCs for disease relapse and death in early breast cancer is clearly documented [8], the assessment of tumor cells in peripheral blood is an easier, more broadly applicable than bone marrow aspirates and certainly

more appropriate for repeated testing. Our group has previously reported that the detection of CK-19 mRNA-positive CTCs in the blood of patients with node-negative operable breast cancer before the initiation of any systemic treatment was an independent prognostic factor associated with an increased risk of disease recurrence [11]. More recently, we demonstrated that patients' risk of relapse could be distinguished based on the response of their CTCs to adjuvant chemotherapy [17] and we reviewed our experience regarding the prognostic role of CK-19 positive CTCs in operable breast cancer [22]. Other investigators have shown that longitudinal monitoring of CTCs was superior to a single test analysis, and a more than 10-fold increase in the CTCs' numbers towards the end of therapy is highly predictive for early relapse [23].

The present study was designed to investigate the prognostic value of CTCs' detection during follow up in predicting the risk of late disease relapse. Accordingly, patients who experienced disease relapse during the first two years from diagnosis were excluded from this analysis and the median follow up period for the studied patients was extended to 107 months. The changes in CK-19 mRNA-positive CTCs status were thus analyzed in 312 patients and four groups were distinguished. The first group included patients without detectable CTCs throughout the follow up period only 11.2% of whom experienced disease relapse. The second group included patients with CK-19 mRNA-positive CTCs early during the first two years. These patients had similar relapse risk to the persistently negative patients and might indeed have derived a benefit from adjuvant therapy. In the third group patients with detectable CTCs after, but not during the first two years were included. The relapse risk for these patients was almost 50% higher compared to the risk of persistently negative patients, perhaps due to the growth of therapy-resistant residual disease, which could not be

detected early on by our method. Finally, in the forth group patients with CK-19 mRNA-positive CTCs throughout the follow up period were included. One third of them experienced disease relapse, while one out of four patients died. This persistently positive group was by far the group with the highest relapse risk.

Almost 40% of the "positive at any time" patients had detectable CK-19 mRNA-positive CTCs only in one single sample. This observation could theoretically be attributed at least in part to false positive results. However, given the very low false positive rate of our assay (approximately 2%) this occurrence should be rather limited.

Our results could be explained by the hypothesis that drug-resistant cancer cell clones generated during tumor evolution constitute the re-emerging dominant tumor cell population and may start proliferating under the selective pressure of drug exposure. The high probability of subsequent disease relapse indicates that these resistant cells have a proliferative and survival advantage. This hypothesis seems to be supported by the observation (unpublished data) that the vast majority of CTCs detected during the follow up period from CK-19 mRNA persistently positive patients who had experienced disease relapse, did not express the M30 antigen which is a neo-epitope expressed only after caspase cleavage of cytokeratin 18 during early apoptosis [24-25].

An algorithm for the optimal timing of CTCs' detection during follow up is currently lacking. Our findings suggest that serial assessments every six months for up to five years represent an acceptable timetable. Furthermore, for hormone receptor positive patients, in whom half of the recurrences occur beyond five years, extension of the CTCs' serial assessments for even longer might be reasonable. These patients

may represent the group which could derive benefit from extended adjuvant treatment approach or switch to another agent.

The qRT-PCR used in our study is not the only available assay for CTCs' detection. A semi-automated approach, the CellSearch® system (Veridex, USA), which has been approved by the Food and Drug Administration for monitoring CTCs in metastatic breast cancer setting, has gained considerable attention [26,27]. The prognostic relevance of CTCs' detection in peripheral blood of operable breast cancer patients, using the CellSearch® system, has been evaluated in the SUCCESS trial. According to the most updated results, detection of at least one CTC in 23 mL of peripheral blood after surgical resection of the primary tumor and before the start of adjuvant systemic treatment was an independent predictor for worse DFS and OS in multivariate analysis [28].The prognostic significance of CTCs detection before and/or after the completion of adjuvant chemotherapy [17], as well as the high specificity/sensitivity represent some advantages of the qPCR assay compared to the CellSearch®. On the other hand, the ability for direct enumeration, morphological analysis and isolation of CTCs for further analysis are important advantages of the latter [29].

Our study has some potential limitations that should be taken into account when considering the results. This is a single institution study and the analysis was performed in one laboratory. Therefore, before the establishment of our assay as a clinically relevant test, sample analysis must be performed in several laboratories and stability during shipment must be demonstrated. Also, despite the fact that our assay has been validated in multiple patients' cohorts and data analyses [10, 11, 14, 15, 17], neither survival advantage, nor improvement in quality of life have been demonstrated in a prospective randomized trial. In this regard, the SWOG and the

Breast Cancer Intergroup of North America have initiated a prospective trial in metastatic setting to test whether patients with elevated CTCs' count (using the CellSearch® system) after one cycle of first-line chemotherapy will be benefit from switch to a different chemotherapeutic regimen (SWOG protocol S0500). However, for patients with operable breast cancer the lower CTCs' detection rate post chemotherapy makes this strategy far more challenging [28, 30].

Additionally, the patients of our study received various types of adjuvant therapy based on available clinical and disease data at the time of enrolment. This heterogeneity may be a confounding variable, but the similarity between our findings on relapses (20.2%) and those published by the Early Breast Cancer Trialists' Collaborative Group is encouraging [6]. Finally, the cellular heterogeneity of CTCs was not analysed using the qRT-PCR detection method. This is very important since various studies have already confirmed that CTCs present significant genetic and phenotypic heterogeneity [31] which could explain why not all patients who have detectable CTCs actually experience disease relapse while some patients relapse although they do not present detectable CTCs.

Conclusions

Our data support a prognostic role and potential clinical utility of monitoring CTCs in conjunction with standard surveillance strategies in the follow up of patients with operable breast cancer. Given their independent unfavorable prognostic value for reduced DFS and OS, the detection of CTCs after therapy could be considered as indirect evidence for the presence of chemotherapy and hormonal therapy resistant disease. Analyzing a 20-mL blood sample at various time points during follow up

might, therefore, enable clinicians to assess the efficacy of administered adjuvant therapy, limit patient exposure to ineffective agents with unnecessary toxicity, assist in the identification of patients who are most likely to benefit from clinical trials of novel therapeutics and perhaps making eradication of cancer cells more feasible, when the tumor burden is still low and before the appearance of clinically overt metastases. Since only one third of patients with persistent CK-19 mRNA-positive CTCs experience disease relapse, additional prognostic markers are needed to better define those patients who indeed might benefit from novel extended adjuvant therapies. These hypotheses can be addressed only in the context of well-designed, adequately powered, prospective, randomized clinical studies. In this way, definitive proof will be provided as to whether monitoring of CTCs can be used to improve clinical outcome in patients with operable breast cancer.

Abbreviations

cDNA: complementary Deoxyribonucleic acid, CK-19: Cytokeratin-19, CTCs: circulating tumor cells, DFS: disease-free survival, DNA: Deoxyribonucleic acid, DTCs: Disseminated tumor cells, ER: estrogen receptor, mL: milliliters, mRNA: messenger Ribonucleic acid, OS: overall survival, PR: progesterone receptor, RT-PCR: real-time reverse transcription-polymerase chain reaction

Competing interests

The author(s) declare that they have no competing interests'.

Authors' contributions

ES: participated in the design of the study, collected the data, analyzed the results and drafted the manuscript. MP, S. Apostolaki, GK: performed the laboratory work. KK, AK, S. Agelaki: tool care of the patients, AX: performed the statistical analysis. VG, DM: conceived and supervised the study and reviewed the manuscript. All authors have read and approved the final manuscript.

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Table 1: Patients' characteristics at diagnosis according to CK-19 mRNA-positive cells during follow up.

Characteristics	**All Patients**		**CK-19 mRNA-Persistently Negative**		**CK-19 mRNA-Persistently Positive**		
	N	**%**	**N**	**%**	**N**	**%**	
Patients enrolled	312		80	25.6	99	31.7	
Age median (range)	54 (26-77)		51.5 (26-75)		54 (28-75)		Mann-Whitney p=0.197
Menopausal Status							
Pre-menopausal	145	46.5	41	51.3	44	44.4	
Post-menopausal	167	53.5	39	48.8	55	55.6	p=0.372
Tumor Size							
T1	116	37.2	30	37.5	33	33.3	
T2	174	55.8	43	53.8	59	59.6	T2/T3 vs T1
T3	22	7.1	7	8.8	7	7.1	p=0.637
Lymph Nodes							
N0	107	34.3	33	41.3	28	28.3	
N1-3	119	38.1	29	36.3	35	35.4	
N>3	86	27.6	18	22.5	36	36.4	p=0.082
Histology Grade							
1 / 2	159	51.0	39	48.8	56	56.6	
3	119	38.1	28	35.0	36	36.4	
lobular	34	10.9	13	16.3	7	7.1	p=0.746
HR							
ER(-)/PR(-)	70	22.4	24	30.0	22	22.2	
Other	190	60.9	39	48.8	60	60.6	
Unknown	52	16.7	17	21.3	17	17.2	p=0.156
HR and Her-2							
ER(-)/PR(-)/HER-2(-)	52	16.7	19	23.8	16	16.2	
Other	255	65.1	41	51.3	66	66.7	
Unknowm	57	18.3	20	25.0	17	17.2	p=0.116
Radiation therapy							
No	24	7.7	7	8.8	9	9.1	
Yes	289	92.3	73	91.2	90	90.9	p=0.937
Hormonotherapy							
No	23	7.4	10	12.5	5	5.1	
Yes	289	92.6	70	87.5	94	94.9	p=0.103
Type of Hormonotherapy							
No Hormonotherapy	23	7.4	10	12.5	5	5.1	
AIs	33	10.6	10	12.5	11	11.1	
T	50	16.0	10	12.5	19	19.2	
AIs & T	57	18.3	9	11.3	22	22.2	
LHRH	32	10.3	11	13.8	8	8.1	
LHRH + T or AIs	117	37.5	30	37.5	34	34.3	p=0.119
Chemotherapy							
CMF	30	9.6	11	13.8	4	4.0	
FEC	149	47.8	31	38.8	57	57.6	
T/EC	133	42.6	38	47.5	38	38.4	p=0.011

Abbreviations: HR, Hormone Receptor; AIs, Aromatase inhibitors; T, Tamoxifen; LHRH, Luteinizing-hormone-releasing hormone; CMF, Cyclophosphamide-

Methotraxate-Fluorouracil; FEC, Fluorouracil-Epirubicin- Cyclophosphamide; T/EC, Taxane/Epirubicin-Cyclophosphamide.

Table 2: Incidence of disease recurrence and deaths according to the detection of CK-19 mRNA-positive circulating tumor cells.

CK-19 mRNA	No of patients	Relapses			Deaths		
		Yes N (%)	No N (%)	Fisher's Exact test, p	Dead N (%)	Alive N (%)	Fisher's Exact test, p
Persistently Positive	99	36 (36.4)	63 (63.6)	p<0.001	24 (24.2)	75 (75.8)	p=0.001
Persistently Negative	80	9 (11.2)	71 (88.8)		5(6.3)	75 (93.8)	
Positive Turn to negative	64	5 (7.8)	59 (92.2)	p<0.001 versus persistently positive	3 (4.7)	61 (95.3)	p=0.001 versus persistently positive
Negative Turn to positive	69	13 (18.8)	56 (81.2)	p=0.016 versus persistently positive	9 (13.0)	60 (87.0)	p=0.079 versus persistently positive

While also,
p = .248 for relapse of turn to positive versus persistently negative
p = .172 for deaths of turn to positive versus persistently negative

Abbreviation: CK-19, cytokeratine-19

Table 3: Incidence of disease recurrence and deaths according to the detection of CK-19 mRNA-positive circulating tumor cells and the hormonal receptor status of the primary tumor.

HR Status	CK-19 mRNA	No of patients	Relapses			Deaths		
			Yes N (%)	No N (%)	Fisher's Exact test, p	Dead N (%)	Alive N (%)	Fisher's Exact test, p
ER(-)/PR(-)	Persistently Positive	22	9 (40.1)	13(59.9)	p=0.044	8 (34.4)	16 (63.6)	p=0.009
	Persistently Negative	24	3 (12.5)	21 (87.5)		1(4.1)	24 (95.9)	
ER(+) and/or PR(+)	Persistently Positive	60	26 (43.3)	34 (46.7)	p<0.001	15 (25)	45 (75)	p=0.007
	Persistently Negative	39	4 (10.2)	35 (89.2)		2 (5.1)	37 (94.9)	

Abbreviations: CK-19, cytokeratine-19; HR, hormonal receptor; ER, estrogens receptor; PR, progesterone receptor

Table 4: Univariate Analysis (unadjusted relative risks) for Disease-Free and Overall Survival.

	DFS			Overall Survival		
	HR	**95% CI**	**p-value**	**HR**	**95% CI**	**p-value**
Menopausal Status						
Post vs Pre	1.815	1.075-3.064	**0.026**	1.985	1.027-3.836	**0.041**
Tumor Status						
T2/T3 vs T1	2.401	1.304-4.419	**0.005**	4.359	1.710-11.114	**0.002**
Nodes						
N>3 vs N0-3	2.331	1.418-3.832	**0.001**	2.819	1.527-5.207	**0.001**
Histology Grade						
3 vs 1 / 2	1.665	0.989-2.805	0.055	1.240	0.644-2.390	0.520
HR						
ER(-)/PR(-) vs Other	0.826	0.454-1.504	0.533	1.182	0.585-2.387	0.641
Triple Negative						
ER(-)/PR(-)/HER-2 vs Other	0.874	0.454-1.681	0.686	1.075	0.492-2.348	0.856
Hormonotherapy						
No vs Yes	1.618	0.696-3.759	0.264	1.439	0.442-4.687	0.546
Chemotherapy						
CMF (ref)		0.589-3.812	0.339		0.352-2.976	0.911
FEC	1.498		0.396	1.024		0.966
T/EC	1.034	0.389-2.745	0.947	1.176	0.388-3.564	0.775
CK-19 at 5 years FU						
Persistently Negative	0.300	0.144-0.623	0.001	0.318	0.121-0.836	0.020
Turn to Negative	0.201	0.079-0.513	0.001	0.260	0.078-0.868	0.028
Turn to Positive	0.498	0.264-0.939	0.031	0.582	0.271-1.253	0.167
Persistently Positive (ref)			**<0.001**			**0.025**

Abbreviations: DFS, Disease-free survival; HR, Hormone receptor; CK-19, cytokeratine-19; CMF, Cyclophosphamide-Methotraxate-Fluorouracil; FEC, Fluorouracil-Epirubicin- Cyclophosphamide; T/EC, Taxane/Epirubicin-Cyclophosphamide.

Table 5: Prognostic factors by multivariate analysis for Disease-free and Overall survival.

	DFS			Overall Survival		
	HR	**95% CI**	**p-value**	**HR**	**95% CI**	**p-value**
Menopausal Status						
Post vs Pre	1.462	0.858-2.492	**0.163**	1.365	0.794-2.347	**0.260**
Tumor Status						
T2/T3 vs T1	2.187	1.167-4.098	**0.015**	2.135	1.141-3.994	**0.018**
Nodes						
N>3 vs N0-3	1.801	1.080-3.003	**0.024**	2.150	1.109-4.168	**0.023**
CK-19 at 5 years FU						
Persistently Negative	0.328	0.157-0.683	0.003	0.330	0.159-0.688	0.003
Turn to Negative	0.206	0.081-0.526	0.001	0.201	0.079-0.514	0.001
Turn to Positive	0.622	0.327-1.186	0.149	0.587	0.309-1.115	0.104
Persistently Positive (ref)			**0.001**			**0.001**

Abbreviations: DFS, Disease-free survival; ER, Estrogen receptor; PR, Progesteron Receptor; CK-19, cytokeratine-19; CMF, Cyclophosphamide-Methotraxate-Fluorouracil; FEC, Fluorouracil-Epirubicin- Cyclophosphamide; T/EC, Taxane/Epirubicin-Cyclophosphamide

Figures

Figure 1: Study enrollment, reasons for patients' exclusion and patients' classification according to CK-19 mRNA CTCs' status.

Figure 2 (A): Disease-free survival (B): Overall survival according to the detection of CK-19 mRNA-positive circulating tumor cells during the first two years and the subsequent three years of follow up.

Figure 3: Disease-free survival and overall survival according to the detection of CK-19 mRNA-positive circulating tumor cells in patients with hormone receptor negative (a,c) and hormone receptor positive tumors (b,d) during the first two years and the subsequent three years of follow up.

Additional files

Additional file 1

Supplemental material

Word document containing supplementary tables S1 and S2 and supplementary figures S1 and S2

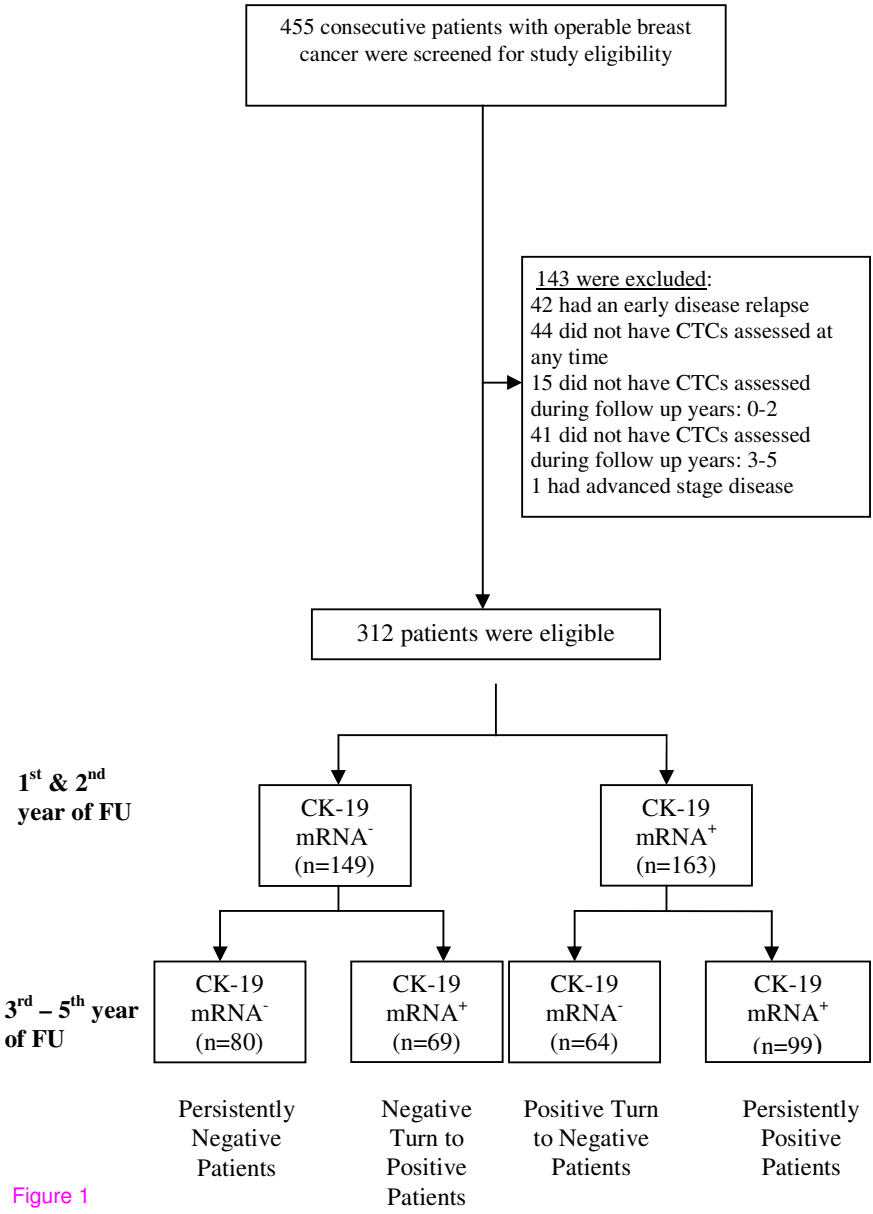
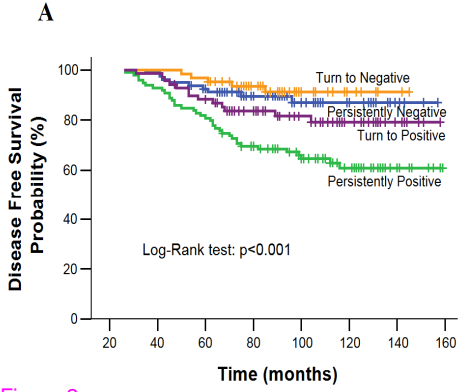

455 consecutive patients with operable breast cancer were screened for study eligibility
143 were excluded:
42 had an early disease relapse
44 did not have CTCs assessed at any time
15 did not have CTCs assessed during follow up years: 0-2
41 did not have CTCs assessed during follow up years: 3-5
1 had advanced stage disease
312 patients were eligible
1st & 2nd year of FU
CK-19 mRNA- (n=149)
CK-19 mRNA+ (n=163)
3rd – 5th year of FU
CK-19 mRNA- (n=80)
CK-19 mRNA+ (n=69)
CK-19 mRNA- (n=64)
CK-19 mRNA+ (n=99)
Persistently Negative Patients
Negative Turn to Positive Patients
Positive Turn to Negative Patients
Persistently Positive Patients

Figure 1


A
Disease Free Survival Probability (%)
100
80
60
40
20
0
20
40
60
80
100
120
140
160
Time (months)
Turn to Negative
Persistently Negative
Turn to Positive
Persistently Positive
Log-Rank test: p<0.001

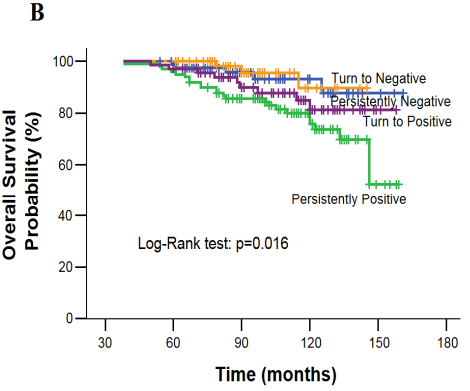

B
Overall Survival Probability (%)
100
80
60
40
20
0
30
60
90
120
150
180
Time (months)
Turn to Negative
Persistently Negative
Turn to Positive
Persistently Positive
Log-Rank test: p=0.016

Figure 2

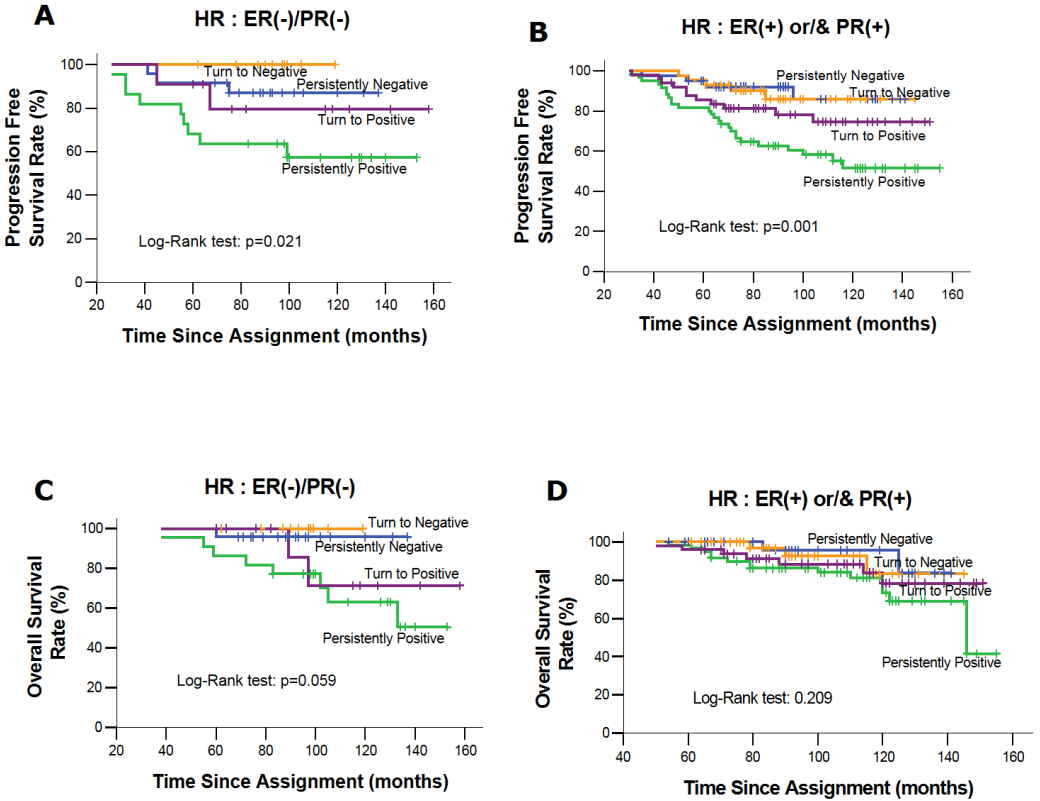

A
HR : ER(-)/PR(-)
Progression Free Survival Rate (%)
Turn to Negative
Persistently Negative
Turn to Positive
Persistently Positive
Log-Rank test: p=0.021
Time Since Assignment (months)
B
HR : ER(+) or/& PR(+)
Progression Free Survival Rate (%)
Persistently Negative
Turn to Negative
Turn to Positive
Persistently Positive
Log-Rank test: p=0.001
Time Since Assignment (months)
C
HR : ER(-)/PR(-)
Overall Survival Rate (%)
Turn to Negative
Persistently Negative
Turn to Positive
Persistently Positive
Log-Rank test: p=0.059
Time Since Assignment (months)
D
HR : ER(+) or/& PR(+)
Overall Survival Rate (%)
Persistently Negative
Turn to Negative
Turn to Positive
Persistently Positive
Log-Rank test: 0.209
Time Since Assignment (months)

Figure 3

Additional files provided with this submission:

Additional file 1: Supplemen_revised Saloustros.doc, 234K
http://breast-cancer-research.com/imedia/1990919838541235/supp1.doc