Detection of Circulating Tumor Cells in Breast Cancer Patients: Prognostic Predictive Role

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Abstract

A determination of circulating tumor cell (CTC) effectiveness for prediction of progression-free survival (PFS) and overall survival (OS) was conducted as an adjunct to standard treatment of care in breast cancer management. Between November 2008 and March 2009, 22 metastatic and 12 early stage breast carcinoma patients, admitted to Ankara Oncology Training and Research Hospital, were included in this prospective trial. Patients’ characteristics, treatment schedules and survival data were evaluated. CTC was detected twice by CellSearch method before and 9-12 weeks after the initiation of chemotherapy. A cut-off value equal or greater than 5 cells per 7.5 ml blood sample was considered positive. All patients were female. Median ages were 48.0 (range: 29-65) and 52.5 (range: 35-66) in early stage and metastatic subgroups, respectively. CTC was positive in 3 (13.6%) patients before chemotherapy and 6 (27.3%) patients during chemotherapy in the metastatic subgroup whereas positive in only one patient in the early stage subgroup before and during chemotherapy. The median follow-up was 22.0 (range: 21-23) and 19.0 (range: 5-23) months in the early stage and metastatic groups, respectively. In the metastatic group, both median PFS and OS were significantly shorter in any time CTC positive patients compared to CTC negative patients (PFS: 4.0 vs 14.0 months, Log-Rank p=0.013; and OS: 8.0 months vs 20.5 months, Log-Rank p<0.001). OS was affected from multiple visceral metastatic sites (p=0.055) and higher grade (p=0.044) besides CTC positivity (log rank p<0.001). Radiological response of chemotherapy was also correlated with better survival (p<0.001). As a result, CTC positivity was confirmed as a prospective marker even in a small patient population, in this single center study. Measurement of CTC by CellSearch method in metastatic breast carcinoma cases may allow indications of early risk of relapse or death with even as few as two measurements during a chemotherapy program, but this finding should be confirmed with prospective trials in larger study populations.

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reliable and reproducible method than conventional
also concluded that CTC detection was more sensitive,
metastatic breast cancer patients (Budd et al., 2006). They
to detect CTC was 0.7 % in a randomised study of 138
The difference between operators were also reported
study of metastatic breast cancer (Hayes et al., 2006).

Recent study with 236 metastatic breast cancer,
claimed that nomograms which relied on CTC counts as
a continuous covariate, induced the use of web based tool
for estimating survival, supporting treatment plan and
clinical trial stratification in first line MBC (Giordano et
al., 2012). The presence and detection of CTC is rarer in
early breast cancer. Larger study populations and longer
follow up time is needed to show clinical benefit of CTC
detection. Thus prospective randomised study data is
limited especially in early breast cancer. Ongoing Treat
CTC trial combines the prognostic information of CTC in
adjvant setting and the promise of adjuvant trastuzumab
given to HER2-negative patients in past studies (Bidard
et al., 2012).

CellSearch (Veridex, LLC, Raritan, NJ) is a widely
used semi-automatic commercial system that relies on
immunomagnetic capture of CTCs using epithelial cell
adhesion molecule (EpCAM) which is expressed on the
surface of epithelial malignancies, followed by positive
selection with cytokeratin and negative selection of
leukocytes. This method has been approved by US Food
and Drug Association (FDA) and European Union (CE) for
detection of CTCs in breast, colon and prostate cancer but
results of prospective randomised studies are awaited for
routine use as a standard of care if this detection yielded
progression free survival (PFS) and overall survival (OS)
(Harris et al., 2007; Bidard et al., 2012).

It was reported in 177 metastatic breast cancer who
had CTC≥5 detected by Cellsearch method at baseline,
had less PFS and OS compared to patients of whom
baseline CTC<5 (7 months vs 3 months and 22 months
vs 10 months, respectively) (Cristofanilli et al., 2004).
They also claimed that the presence of high levels of
CTC 3-5 weeks after the initiation of chemotherapy
good treatment failure (Cristofanilli et al., 2004).
Reproducibility of CellSearch method was reported
between 80-82% and the incidence of CTC as 70% in a
study of 92 metastatic breast cancer patients (Riethdorf et
al., 2007). Statistically shorter PFS and OS were reported
in patients who had basal CTC≥5 than CTC<5 at the time
of 14th week of treatment in a prospective randomised
study of metastatic breast cancer (Hayes et al., 2006).
The difference between operators were also reported
to detect CTC was 0.7 % in a randomised study of 138
metastatic breast cancer patients (Budd et al., 2006). They
also concluded that CTC detection was more sensitive,
reliable and reproducible method than conventional
radiological methods for the determination of survival in
earlier treatment time (Budd et al., 2006). It was reported
that CTC count was a significant predictive factor for
overall survival (OS) in all immunohistochemically
defined molecular subtypes (Munzone et al., 2012). In a
larger study, 468 MBC patients were divided into three
subgroups based on immunohistochemical staining of the
primary tumor: hormone receptor positive, Her2-
positive and triple negative groups. This study confirmed
independent prognostic value of CTC detection but
lack to show difference between primary tumor-based
molecular subgroups and impact of CTC status on survival
(Wallwiener et al., 2013). Circulating cell-free DNA
carrying tumor-specific alterations (circulating tumor
DNA) has been investigated and compared with cancer
antigen 15-3 (CA 15-3) and circulating tumor cells in a
recent study (Dawson et al., 2013). They concluded that
circulating tumor DNA levels showed a greater dynamic
range, and greater correlation with changes in tumor
burden, than did CA 15-3 or circulating tumor cells.
Among the measures tested, circulating tumor DNA
provided the earliest measure of treatment response in
10 of 19 women (53%) (Dawson et al., 2013). There was
also evidence that CTC in breast cancer exhibit dynamic
changes in epithelial and mesenchymal composition and
reversible shifts between these cell fates accompanied
each cycle response to therapy and disease progression
in a recent study (Yu et al., 2013).

Detection of CTC may give knowledge about
microscopic disease and this in turn yielded prognostic
cue of the disease state. The detection of CTCs may
be involved in the staging of metastatic breast cancer
patients. Better classification of high risk patients later
may be resulted in better use of targeted therapies finally
causing improvement in personalized treatment. By the
detection of CTCs and markers on CTCs may lead to cure
of metastatic breast cancer in the future. The effectiveness
of CTC detection on PFS and OS in breast cancer
management as an adjunct to standard care of treatment
was evaluated in the presenting study.

Materials and Methods

Between November 2008 and March 2009, 22
metastatic and 12 early stage breast carcinoma patients,
admitted to Ankara Oncology Training and Research
Hospital and gave informed consent to participate,
were included in this prospective trial. This study was
approved by the local ethical committee of the hospital.
Patients’ characteristics, treatment schedules and survival
data were evaluated. Physical examination, staging and
routine radiological assessment were done regularly for
the evaluation of response without any intervention.
CTC was detected by CellSearch method before and
9-12 weeks after the initiation of chemotherapy. Study
was not supported by any firm or foundation, CTC kits
were donated to Ankara Oncology Training and Research
Hospital for demonstration of CellSearch Method from
the local distributor were readily used.

Patients were included in this study if they were Eastern
Cooperative Oncology Group (ECOG) performance status

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was between 0-2. Metastatic patients and non-metastatic patients who started a new chemotherapy regimen entered this study. Staging was done by TNM system (Edge et al., 2010). Chemotherapy responses were evaluated by using revised response evaluation criteria in solid tumours (RECIST) version 1.1 (complete response, partial response, stable disease and progression) (Eisenhauer et al., 2009). CTC was detected by CellSearch method before and 9-12 weeks after the initiation of chemotherapy. For processing, a 7.5 ml venous blood sample was dropped to 10 ml ‘Cellsave Tube’ (Veridex LLC, Raritan, NJ). For isolation and counting of CTCs, CellSearch System (Veridex LLC, Raritan, NJ, USA) was used which was defined before (Cristofanilli et al., 2004). Results were expressed as number of cells per 7.5 ml blood sample (Cristofanilli et al., 2004). Isolation and counting of CTC was done by an independent operator without knowing patients data as defined before previous studies (Kagan et al., 2002). Cut-off value equal or greater than 5 cells per 7.5 ml blood sample was considered to be ctc positive as defined before (Kagan et al., 2002). CTC results were not used as a part of staging or treatment decision plan throughout the entire study.

Statistics
Overall survival (OS) was calculated from the date of diagnosis and death for any reason or the date of last contact. Progression free survival (PFS) was calculated from the date of first treatment until disease progression. Cut-off value equal or greater than 5 was considered to be ctc positive as defined before. Fisher’s exact test was used to compare patient characteristics, CTC distribution and tumor factors between the populations. The survival of the patients was estimated using the Kaplan-Meier method. Long-rank test was used to compare and analyse the survival data. The determination of independent prognostic factors influencing survival was performed by Cox proportional hazard model. The 95% confidence interval was calculated for all hazard ratios (HRs) in Cox regression analysis. A p value less than 0.05 was considered to be statistically significant. For statistical analysis, SPSS for Windows, version 15.0 software (SPSS Inc, Chicago, Illionis, USA) was used.

Results
Patients’ characteristics
Between November 2008 and March 2009, 22 metastatic and 12 early stage breast carcinoma patients, admitted to Ankara.Oncology Training and Research Hospital and gave informed consent to participate, were included in this prospective trial. Patients’ characteristics, treatment schedules and survival data were evaluated. Physical examination and routine radiological assessment were done regularly for the evaluation of response. CTC was detected twice by CellSearch method before and 9 to 12 weeks after the initiation of chemotherapy.

All patients were female. Median age was 48.0 (range: 29-65) and 52.5 (range: 35-66) in early stage and metastatic groups, respectively (Table 1). Treatment schedules and first site of metastasis was shown in Table 1.

CTC was positive (≥5) in 3 (13.6%) patients before chemotherapy and 6 (27.3%) patients at anytime during chemotherapy in the metastatic group whereas CTC was positive in only one patient in early stage group before and during chemotherapy (Table 3). Clinical and radiological

| Characteristics                  | Metastatic Patients | Early-stage Patients |
|----------------------------------|---------------------|----------------------|
| n (%)                            | n (%)               |
| Menopausal Status                |                     |                      |
| Premenopausal                    | 15 (68.2)           | 7 (58.3)             |
| Postmenopausal                   | 7 (31.8)            | 5 (41.7)             |
| Family History                   |                     |                      |
| Present                          | 4 (18.2)            | 1 (8.3)              |
| Absent                           | 18 (81.8)           | 11 (91.7)            |
| Breast Operation                 |                     |                      |
| Present                          | 19 (86.4)           | 12 (100)             |
| Absent                           | 3 (13.6)            | 0 (0.0)              |
| Stage at Diagnosis               |                     |                      |
| I-II                             | 10 (45.5)           | 7 (58.3)             |
| III-IV                           | 12 (54.5)           | 5 (41.7)             |
| Grade                            |                     |                      |
| Unknown                          | 9 (40.9)            | 1 (8.3)              |
| II                               | 4 (18.2)            | 5 (41.7)             |
| III                              | 9 (40.9)            | 6 (50.0)             |
| Estrogen Receptor                |                     |                      |
| Positive                         | 12 (54.5)           | 6 (50.0)             |
| Negative                         | 10 (45.5)           | 6 (50.0)             |
| Progesterone Receptor            |                     |                      |
| Positive                         | 12 (54.5)           | 7 (58.3)             |
| Negative                         | 10 (45.5)           | 5 (41.7)             |
| c-erbB2 Status                   |                     |                      |
| Positive                         | 11 (50.0)           | 7 (58.3)             |
| Negative                         | 11 (50.0)           | 5 (41.7)             |

| Metastatic Patients (n=22) | Non-Metastatic Patients (n=12) |
|---------------------------|-------------------------------|
| Adjuvant Chemotherapy     |                               |
| Present                   | 16 (72.7)                     | 12 (100)              |
| Absent                    | 6 (27.3)                      | 0 (0.0)               |
| Type of Adjuvant Chemotherapy |                         |
| Antracycline based         | 10 (45.5)                     | 6 (50.0)              |
| Taxane-trastuzumab         | 6 (27.3)                      | 6 (50.0)              |
| Adjuvant Chemotherapy Cycle |                       |
| 3-4                       | 9 (40.9)                      | 3 (25.0)              |
| 6-9                       | 13 (59.1)                     | 9 (75.0)              |
| Adjuvant Hormonotherapy    |                               |
| Present                   | 11 (50.0)                     | 9 (75.0)              |
| Absent                    | 11 (50.0)                     | 3 (25.0)              |
| Adjuvant Radiotherapy      |                               |
| Present                   | 9 (40.9)                      | 9 (75.0)              |
| Absent                    | 13 (59.1)                     | 3 (25.0)              |
| Metastatic Site (s)        |                               |
| Local-Regional             | 7 (31.8)                      | -                     |
| Multiple/Visceral Metastatic Sites |         |
| Palliative Chemotherapy    |                               |
| 1st Line                   | 10 (45.5)                     | -                     |
| 2nd Line                   | 8 (36.4)                      | -                     |
| 3rd Line                   | 4 (18.2)                      | -                     |
| Palliative Chemotherapy Type |                             |
| Antracycline               | 4 (18.2)                      | -                     |
| Taxane-trastuzumab         | 5 (22.7)                      | -                     |
| Capectabine-platin-taxane  | 4 (18.2)                      | -                     |
| Platin-gemcitabine. etoposide | 5 (22.7)                |
| Other                      | 4 (18.2)                      | -                     |
treatment response evaluation results were shown in Table 3.

**Survival analysis**
The median follow-up was 22.0 (range: 21-23) and 19.0 (range: 5-23) months in the early stage and metastatic groups, respectively.

In the metastatic group, according to initial CTC measurements, both median PFS and OS were significantly shorter in initial (before chemotherapy) CTC positive patients compared to initial CTC negative patients (PFS: 3.0 vs 13.0 months, Log-Rank p<0.001; and OS: 6.0 months vs. 19 months, Log-Rank p<0.001, Figure 1 and 3).

In metastatic group both PFS and OS were affected from multiple visceral metastatic sites (p=0.002 and p=0.055 respectively, table-4), higher grade (p=0.015 and p=0.044 respectively, table-4), initial CTC positivity (log rank p<0.001 and p<0.001 respectively, Table 4) and anytime CTC positivity (p=0.013 and p<0.001 respectively) and also radiological response of chemotherapy during chemotherapy cycles (both p<0.001, Table 4).

Both PFS and OS were not different according to stage of the tumor, ER, PR and CerbB2 status, age, ECOG performance status, adjuvant chemotherapy, radiotherapy or hormonotherapy. Since data about the grade of pathological specimens of the 9 patients were missing, correlation between grade and PFS or OS, was not considered to be clinically significant. Because of the

**Table 3. Treatment Responses of Study Population According to Clinical, Radiological and Circulating Tumor Cell Change**

|                   | Metastatic Patients | Non-Metastatic Patients |
|-------------------|---------------------|-------------------------|
|                   | (n=22)              | (n=12)                  |
| ECOG Status 1     | 21 (95.5)           | 12 (100.0)              |
| ECOG Status 2     | 1 (4.5)             | 0 (0.0)                 |
| Outcome at the end of study |                  |                         |
| Exitus            | 12 (54.5)           | 0 (0.0)                 |
| Alive             | 10 (45.5)           | 12 (100.0)              |
| Progression       | 18 (81.8)           | 1 (8.3)                 |
| Absent            | 4 (18.2)            | 11 (91.7)               |
| CTC Status (Measured at any time ) |               |                         |
| Positive (≥5)     | 6 (27.3)            | 1 (8.3)                 |
| Negative (<5)     | 16 (72.7)           | 11 (91.7)               |
| CTC Change        |                     |                         |
| Negative to Negative | 16 (72.7)           | 11 (91.7)               |
| Positive to Positive | 3 (13.6)            | 0 (0.0)                 |
| Negative to Positive | 3 (13.6)            | 0 (0.0)                 |
| Positive to Negative | 0 (0.0)             | 1 (8.3)                 |
| Radiological Evaluation (Mid-term) |              |                         |
| Stabile Disease   | 11 (50.0)           | No Recurrence           |
| Regression        | 6 (27.3)            | No Recurrence           |
| Partial Regression| 4 (18.2)            | No Recurrence           |
| Progression       | 1 (4.5)             | 1 (8.3)                 |

**Table 4. Factors Related to Progression Free Survival and Overall Survival**

| Variables                        | P Value  |
|----------------------------------|----------|
| Progression Free Survival        |          |
| Initial CTC (≥5 vs <5)           | <0.001   |
| CTC at anytime (≥5 vs <5)        | 0.013    |
| Metastasis Site (Locoregional vs Multiple Site) | 0.002    |
| Grade (Grade II vs III)          | 0.015    |
| Radiological Response (Regression vs Progression) | <0.001    |
| Overall Survival                 |          |
| Initial CTC (≥5 vs <5)           | <0.001   |
| CTC at anytime (≥5 vs <5)        | <0.001   |
| Metastasis Site (Locoregional vs Multiple Visceral Metastatic Sites) | 0.055    |
| Grade (Grade II vs III)          | 0.044    |
| Radiological Response (Regression vs Progression) | <0.001    |

**Figure 1. Kaplan-Meier Survival Curves Showing Progression Free Survival in Metastatic Breast Cancer Patients According.**

A) **Initial CTC Status (positive vs negative)**: Ordinate (Y) represents cumulative progression free survival and axis (X) represents time (months). Progression Free Survival of patients (dotted line) who were initially CTC positive (CTC≥5) was shorter than patients (straight line) who were initially CTC negative (CTC<5). (Progression free survival 3.0 months vs 13.0 months; Log-Rank p<0.001).

B) **CTC Status at Any Time of the Study (positive vs negative)**: Ordinate (Y) represents cumulative progression free survival and axis (X) represents time (months). Progression Free Survival of patients (dotted line) who were CTC positive at any time of the study (CTC≥5) was shorter than patients (straight line) who were initially CTC negative (CTC<5). Progression free survival 4.0 months vs 14.0 months; Log-Rank p=0.013
limited number of patients in adjuvant treatment group, survival analysis was not performed, at all.

Discussion

The incidence of CTC in breast cancer was reported to be 12-50% in early setting and 25-80% in advanced and metastatic settings depending on the methods used in different clinical studies (Riethdorf S et al., 2008; Franken et al., 2012). In our study CTC was positive (≥5) in 3 (13.6%) patients before chemotherapy and 6 (27.3%) patients during chemotherapy in the metastatic group whereas CTC was positive in only one patient in early stage group before and at anytime during chemotherapy. There was only one recurrence in the adjuvant patient group, that gave an impression that this patient group had unintendedly low risk for recurrence. However, this was a speculative result because of small number of patients and relatively short follow-up time in the adjuvant setting. It was recommended that more than a single cut-off point for positivity of CTC should be used for better risk classification especially in adjuvant trials because there has been no defined threshold point for CTC positivity in this group (Tibbe et al., 2007). It was reported that as CTC levels increased as a continuous variable, there was a non linear risk of death in a study of 80 metastatic breast cancer patients (Botteri et al., 2010).

In the metastatic group in our study, both median PFS and OS were significantly shorter in CTC positive patients compared to CTC negative patients according to initial or anytime CTC measurements. It was previously reported similar results in a randomised prospective multicenter study in 177 metastatic breast cancer (Cristofanilli et al., 2004). In a subgroup analysis of 83 metastatic breast cancer who had received first line chemotherapy further showed that OS was shorter in basal CTC positive and control CTC positive women at the 4th week of treatment than CTC negative women (Cristofanilli et al., 2005). They also concluded that CTC was an independent prognostic factor for prediction of progression free survival and overall survival (Cristofanilli et al., 2005). Similar randomised study confirmed this results by detecting CTC positivity at baseline (before chemotherapy) and at the 14th week of chemotherapy (Hayes et al., 2006). Researchers claimed that CTC was correlated better with hematogenous dissemination rather than locally invasive disease (Nakagawa et al., 2007). They also believed that non metastatic breast cancer cases who was CTC positive, had greater risk of early distant metastasis than CTC negative breast cancers of same stage (Nakagawa et al., 2007). Prognostic significance of CTCs in metastatic breast cancer patients also confirmed in 185 newly diagnosed cases retrospectively (Dawood et al., 2008). CTC positivity (CTC≥5) was found to be an independent parameter and relative risk of death was 3.64 (CI: 2.11-6.30) (Dawood et al., 2008). They proposed that CTC positivity should be involved in staging of breast cancer and high risk patients were a candidate for clinical trials of selected targeted therapies in order to eliminate CTCs (Dawood et al., 2008). Recently, CTC detection compared with serum tumor markers in a large prospective trial in first line chemotherapy for 267 MBC patients (Pierga et al., 2012). They confirmed that threshold of CTC≥5 was statistically significant for PFS and OS on multivariate analysis independently from serum tumor marker (Pierga et al., 2012).

In our study, in metastatic group both PFS and OS were also affected from multiple visceral metastatic sites (p=0.002 and p=0.055 respectively) and higher grade (p=0.015 and p=0.044 respectively), but not affected from the stage of the tumor, ER, PR and CerbB2 status, age, ECOG performance status, adjuvant chemotherapy, radiotherapy or hormonotherapy. Since grade data of the 9 patients were missing, correlation between grade and PFS and OS, were not considered clinically significant.

Radiological response of chemotherapy during...
chemotherapy cycles was also correlated with better survival in our study (both p<0.001). Similar result was reported in 138 metastatic breast cancer patients (Budd et al., 2006). They found that both positive basal CTC and positive CTC at 4th weeks of study and better correlated with outcome than conventional radiological evaluation at 10th weeks of the study (Budd et al., 2006).

It has not well known how frequently and in which period of chemotherapy cycle was necessary to detect CTCs for prediction of survival benefit of chemotherapy yet. Researchers found a correlation between radiological progression and CTC positive patients (CTC≥5) in a 3rd-5th weeks and 7th-9th weeks of chemotherapy in a heavily pretreated 68 metastatic breast cancer study population (Liu et al., 2009). Others confirmed this results in a 119 metastatic breast cancer patient population in a prospective multicenter study (Nakamura et al., 2010). They found that 7 of 11 (63.6%) of patients, whose CTC level increased 100% after one cycle of chemotherapy, had progressive disease by imaging at first follow up. They concluded that CTCs were highly correlated with imaging results before and after chemotherapy (Nakamura et al., 2010). A meta-analysis of the prognostic value of CTC in breast cancer showed that between January 1990 and January 2012, eligible 49 studies enrolling 6,825 patients were conducted (Zhang et al., 2012). The prognostic value of CTC was significant in both early (DFS: HR 2.86; 95% CI 2.19-3.75; OS: HR 2.78; 95% CI 2.22-3.48) and metastatic breast cancer (DFS: HR 1.78; 95% CI 1.52-2.09; OS: HR 2.33; 95% CI 2.09-2.60). Further subgroup analyses showed that this results were stable irrespective of the CTC detection method and time point of blood withdrawal (Zhang et al., 2012).

In our study, because of limited number of patients in adjuvant treatment group, survival analysis were not performed. Since it is a rarer entity for adjuvant breast cancer patient group there were studies claimed that only a single CTC number might be important (Slade et al., 2009). Even in metastatic cancer patients it was reported that detection and phenotyping of CTC was challenging (Cowmans et al., 2012). A randomized study investigated prognostic effect of CTC in a neoadjuvant trial of 115 locally advanced breast cancer patients (Bidard et al., 2010). They found that there was a correlation between presence of CTC (CTC≥1, detected by CellSearch method) and a shorter time to metastasis (p=0.01) and a worse overall survival (p=0.007) during 36 months follow up (Bidard et al., 2010). Other study confirmed that persistence of disseminated tumor cells after neoadjuvant treatment for locally advanced breast cancer predicts poor survival (Mathiesen et al., 2012).

As a result, CTC positivity was confirmed as a prospective marker even in our small patient population, in this single center study. Measurement of CTC by CellSearch method in metastatic breast carcinoma may be an indicative of early risk of relapse or death and even as less as two times of measurement during the whole chemotherapy program may be enough to have a decision but this finding should be confirmed with prospective trials in larger study populations.

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