Circulating tumor cells (CTCs) in metastatic breast cancer (MBC): prognosis, drug resistance and phenotypic characterization

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Background: The expression of ATP-binding cassette transporters on circulating tumor cells (CTCs) is predictive of response to chemotherapy in cancer patients. We tested the hypothesis that drug-resistant CTCs might have predictive value in metastatic breast cancer (MBC) and possibly retain stem-like properties.

Patients and methods: CTCs obtained from 42 MBC patients were evaluated for multidrug-resistance-related proteins (MRPs), aldehyde dehydrogenase 1 (ALDH1), estrogen receptor α (ERα) and human epidermal growth factor receptor 2 (HER2/neu). Primary objective was to evaluate the prognostic and predictive value of CTCs profile. Secondary end points were the level of concordance in ERα and HER2/neu status between primary tumors and CTCs and the correlation in CTCs between ALDH1, drug resistance profile and number of MRPs.

Results: A difference in progression-free survival (PFS) was found between CTCs-positive and CTCs-negative patients. PFS was shorter in patients with a ‘drug resistance’ CTCs profile and in patients whose CTCs expressed two or more MRPs. No correlation was found between tumor characteristics and ALDH1. ALDH1 correlated to negative ERα and positive HER2/neu status in CTCs. The correlation between the number of MRPs expressed in CTCs and ALDH1 was statistically significant.

Conclusion: In MBC, the presence of CTCs expressing MRPs and ALDH1 is predictive of response to chemotherapy.

Key words: breast cancer, cancer stem cells, circulating tumor cells, drug resistance

Introduction

Regardless of therapeutic advances made over the past decades, the prognosis for breast cancer patients depends on the occurrence of metastases that still represent an incurable condition. Thus, considerable efforts focused on the identification of new prognostic and predictive markers, to improve the stratification of patients, monitoring therapy response and identification of therapeutic targets.

Recently, the attention has been focused on the identification of detectable epithelial cells [circulating tumor cells (CTCs)] in peripheral blood of patients with solid tumors, providing a proof-of-principle for the early primary cancer cell dissemination through the vascular network, recently hypothesized [1, 2]. Currently, the results of prospective studies demonstrated the independent prognostic value of CTCs in metastatic breast cancer (MBC) patients [3–9]. Furthermore, recent evidences suggest that the finding of CTCs in course of therapies owns a reliable prognostic value and is considered a predictive tool for response to treatments in MBC [10].

Besides enumeration of CTCs, there is interest in the characterization of their genes and/or proteins expression profiles. In a previous study, we identified a drug resistance profile on CTCs from epithelial cancers through the evaluation of drug transporters belonging to ATP-binding cassette family, named multidrug-resistance-related proteins (MRPs) [11]. These transporters mediate the extrusion of chemotherapics out of cancer cells in a specific manner. The expression of MRPs on CTC was found predictive of resistance to chemotherapy, independently of tumor type and stage of disease [12].

These data agree with recent investigations describing the heterogeneity of breast cancer and suggesting that the limited effectiveness of most anticancer therapies is associated to a specific subset of cancer cells identified as cancer stem cells...
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(CSCs), already advocated to explain the concept of minimal residual disease [13].

In this study, we aimed to confirm the prognostic and predictive value of CTCs drug resistance profile in MBC patients, further improving our knowledge on biological characteristics of these cells. We explored specific stemness features and tested the hypothesis that drug-resistant CTCs might retain stem-like properties. Finally, we analyzed on CTCs some classical molecular prognostic factors in order to determine whether a molecular portrait of CTCs might reliably predict therapeutic resistance in MBC.

patients and methods

study design

From January 2007 to February 2009 we conducted a prospective study. Forty-two patients with MBC were enrolled to evaluate prognostic and predictive value of CTCs molecular profile.

The study was approved by the local ethical board and all patients provided written informed consent. Primary objective of the trial was to evaluate the prognostic and predictive value of CTCs molecular profile in patients with MBC about to start a new therapy.

Secondary end points were

- level of concordance in estrogen receptor α (ERα) and human epidermal growth factor receptor 2 (HER2/neu) status between primary tumors and their corresponding CTCs;
- correlation in CTCs between the expression of aldehyde dehydrogenase 1 (ALDH1), as a stemness marker, and either a drug-resistant molecular profile and the number of MRPs expressed.

Inclusion criteria were measurable MBC, commencement of a new systemic therapy without limits to number of previous therapies, and Eastern Cooperative Oncology Group performance status score of zero to two. Prior adjuvant and/or metastatic treatments were both permitted. Before starting a new treatment, patients underwent baseline blood sampling for CTCs evaluation and standard imaging studies. All CTCs samples were collected on the first day of treatment. Reassessment of disease status was conducted with the same techniques used at baseline every 9–12 weeks, depending on treatment type and schedule. Standard RECIST criteria were used to determine patients’ responses to treatment [14].

From primary tumor samples, prognostic markers including ERα, progesterone receptor, Ki-67, grading of tumor differentiation (G) and HER2/neu determined by FISH or chromogenic in situ hybridization were retrieved from the pathology report. All these biomarkers were evaluated by immunohistochemistry. Although patients were enrolled in different institutions (Division of Medical Oncology, Sapienza University of Rome, and Division of Medical Oncology, Hospital ‘S. Giovanni Evangelista’, Tivoli, Italy), all CTCs and prognostic markers analysis were carried out in a single institution.

CTC isolation and molecular profile

Peripheral blood (15 ml) was obtained with informed consent from each patient. Blood samples were collected at baseline before starting any systemic therapy. To avoid contamination with epithelial cells from the skin, blood samples were obtained at the middle of vein puncture after the first 5 ml of blood was discarded.

CTCs were isolated by CELLection™ Dynabeads® coated with the monoclonal antibody toward the human epithelial cell adhesion molecule (EpCAM), as previously described [12]. Tumor cells were eluted and subjected to RNA extraction and complementary DNA (cDNA) synthesis. For each blood drawing, the RNA yield was 1–2 µg. The quality of RNA preparations was determined by absorbance at 260 and 280 nm (ratio 260 : 280 = 2). In order to verify the integrity of extracted RNAs, 5 µl of each cDNA was amplified in PCR buffer containing 25 pmol each of upstream and downstream glicerdehyde-3-phosphate dehydrogenase (GAPDH) primers as housekeeping gene and 1.25 units of Platinum Taq polymerase (Life Technologies, Carlsbad, CA). To avoid illegitimate transcription from mononuclear cells, cDNAs from EpCAM-positive cells were subjected to PCR amplifications for CD45, used as a marker of leukocytes, and cytokeratins 8 and 20 (CK8 and CK20), used as markers of epithelial cells. CTCs were defined as all EpCAM-positive cells negative for CD45 expression but expressing CK8 and CK20.

Each sample found positive for CTCs presence was then evaluated for the expression of a multidrug resistance genes panel, as previously reported [12] (Figure 1B). On the basis of densitometric analysis of the amplification bands obtained by normalization with the GAPDH internal controls, we had two categories of CTC profile: (i) sensitivity (ratio MRP : GAPDH ≤ 1) and (ii) resistance (ratio MRP : GAPDH > 1).

On the basis of MRP : GAPDH expression ratios on CTCs, we traced for each patient a molecular profile of sensitivity to all standard chemotherapeutic drugs used in the treatment of MBC. At a median follow-up time of 24 months, the chemotherapy regimen adopted in each patient and the correspondent drug sensitivity profile were matched and evaluated. In this case, due to the presence of more than one drug used in each patient, we considered ‘sensitive’ patients with CTC sensitivity to any of the drugs utilized and ‘resistant’ those patients with a resistance profile to all drugs.

Furthermore, to better characterize the molecular profile of CTCs, we investigated the expression of ERα and HER2/neu to be compared with that of primary tumors and ALDH1 as a marker of breast cancer stemness. Amplifications were carried out on a Techne Progene (Cambridge, UK) amplifier. Amplification products were loaded on 2% agarose gel. Primer sequences used in PCR reaction and amplification conditions, not previously described, are listed in Figure 1C.

statistical analysis

Statistical analysis was carried out with BMDP statistical software, version 7 (Statistical Solutions, Saugus, MA) and SPSS (Chicago, IL, version 15.00 for Windows).

Progression-free survival (PFS) was defined as the time elapsed between the date of blood sampling, corresponding to the start of treatment, and the date of clinical disease progression or death for any cause.

Kaplan–Meier product-limit method was used to correlate PFS with CTCs presence, CTCs chemoresistance profile and number of different MRPs expressed on CTCs. Different prognostic groups were compared using the log-rank test. A P value of <0.05 was considered statistically significant.

To define the level of concordance between the characteristics of primary tumors and corresponding CTCs, the χ² test was used.

To assess the correlation between the expression of ALDH1 and classical prognostic factors (ERα and HER2/neu) in CTCs, the Pearson’s correlation test was used. The correlation was considered significant when P value was <0.05.

results

patient characteristics

A total of 42 cancer patients were enrolled. The median age of the patients was 52.6 years (range 33–76 years).

All the 42 patients were affected by stage IV breast cancer; 15 patients had no visceral metastatic sites. Twenty-five of all
tumors were positive for ERα, while 21 were positive for HER2/neu; none of all patients had a well-differentiated (G1) tumor, while 23 patients had a moderately differentiated (G2) and 19 patients had a poorly differentiated (G3) tumor. Out of 42 patients, 35 had a >10% Ki-67.

Clinical and biological characteristics of the study population are listed in Table 1.

All patients received chemotherapy for metastatic disease. The median follow-up period was 24 months. The results of the imaging studies used to evaluate the clinical response to treatment documented a complete response (CR) in 1 of 42 patients (2%), a partial response (PR) in 6 of 42 (14%), a stable disease (SD) in 16 of 42 (38%) and a progressive disease (PD) in 19 of 42 (45%).

prognostic and predictive value of CTCs molecular profile

For each patient, a molecular portrait of CTCs has been traced according to MRPs, ERα, HER2/neu and ALDH1 expression (Table 2). In Figure 1, an exemplificative panel of PCR amplifications is shown. Of 42 patients, 28 (67%) were found positive for the presence of CTCs (CD45+/CK8+/CK20+). A statistically significant difference in PFS was found between CTCs-positive and CTCs-negative group of patients (9.2 versus 16.3 months; $P = 0.023$) (Figure 2).

Further RT-PCR assays were carried out on the 28 samples with detectable CTCs. We investigated the expression of MRP transporters, specific for chemotherapeutic drugs administered to each patient. On the basis of this assay, we classified patients in two separate groups, 9 with a ‘drug sensitivity’ and 19 with a ‘drug resistance’ signature. Patients identified as resistant on the basis of MRP expression had a significant shorter PFS than patients defined as sensitive (5.4 versus 19.5 months; $P = 0.000$) (Figure 2B). Positive predictive value and negative predictive value were 78.94% and 100%, respectively.

Of the 19 patients with a drug resistance CTCs molecular profile, 15 had PD at 24 months and 4 achieved a PR or an SD. Interestingly, the nine patients with a drug sensitivity CTCs molecular profile had PR or SD, with one patient achieving CR.

To better define the prognostic and predictive relevance of the CTCs molecular portrait, we carried out a quantitative analysis evaluating the number of different MRPs expressed in each sample. The expression of four MRPs was detected in CTCs from 7 of 28 (25%) patients; the expression of three MRPs was found in CTCs from 8 of 28 (29%) patients; two and one MRPs were found expressed in 4 of 28 (14%) and 5 of 28...
Table 1. Characteristics of MBC patients

<table>
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<tr>
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<td>Progression of disease</td>
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<td>CTC+ (CD45−/CD8+/CD20+)</td>
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<td>67</td>
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MBC, metastatic breast cancer; HER2/neu, human epidermal growth factor receptor 2; ER, estrogen receptor; CTC, circulating tumor cell; CK, cytokeratin.

Table 2. Characteristics of CTC in MBC patients

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<tr>
<td>Patients with CTC+</td>
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<tr>
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<td>Resistant profile</td>
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<tr>
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<td>16/28</td>
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<tr>
<td>HER2/neu</td>
<td>13/28</td>
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<td>9/28</td>
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<tr>
<td>2–4</td>
<td>19/28</td>
</tr>
<tr>
<td>ALDH1+</td>
<td>17/28</td>
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</table>

CTC, circulating tumor cell; MBC, metastatic breast cancer; ER, estrogen receptor; HER2/neu, human epidermal growth factor receptor 2; MRP, multidrug-resistance-related protein; ALDH1, aldehyde dehydrogenase 1.

For a deeper understanding of CTCs molecular features, we tested them for the expression of ERα and HER2/neu. Among the 28 CTCs-positive patients, 13 were classified as HER2/neu positive and 15 as HER2/neu negative, while 16 were classified as ERα positive and 12 as ERα negative.

The correlation between ERα and HER2/neu status in CTCs and in the corresponding primary tumor was also tested. In our series, we found 10 of 28 discordant cases (35.7%).

Among patients with ERα-positive primary tumors, 10 of 16 (62.5%) had ERα-negative CTCs. Among those with ERα-negative primary tumors, none had detectable ERα-positive CTCs. Eighteen patients (18 of 28) were found concordant for ERα status between CTCs and corresponding primary tumors (64.2%).

Among patients with HER2/neu-positive primary tumors, 4 of 13 (30.7%) had HER2/neu-negative CTCs, while 6 of 13 (46.1%) patients with HER2/neu-negative primary tumors developed HER2/neu-positive CTCs. Eighteen of 28 patients were found concordant for HER2/neu status (64.2%), equally distributed in double positive and double negative.

Furthermore, we evaluated the expression of ALDH1 in CTCs, as a marker of breast cancer stemness. The expression of ALDH1 was further correlated either to primary tumor prognostic factors, including G, Ki67, ERα and HER2/neu, or to the expression of ERα and HER2/neu in CTCs. No significant correlations were found between primary tumor characteristics and ALDH1 expression. ALDH1 statistically correlated with ERα and HER2/neu status in CTCs. Particularly, a major proportion of ALDH1-positive CTCs were ERα negative and HER2/neu positive (Table 3).

Moreover, of the 17 patients with ALDH1-positive CTCs, 13 of 17 (76.47%) had a drug resistance, while 4 of 17 (23.5%) had a drug sensitivity profile. Ten patients with ALDH1-negative CTCs were found homogeneous for drug resistance and drug sensitivity profiles.

The correlation between the number of MRPs expressed in CTCs and ALDH1 was found statistically significant ($P = 0.000$), as shown in Table 4.

Finally, the difference in PFS between patients with ALDH1-positive and -negative CTCs was found not statistically significant ($P = 0.188$) (Figure 2D).

Discussion

The presence of five or more CTCs per 7.5 ml of blood, before treatment and at the first follow-up visit, has been demonstrated as an independent predictor of PFS and overall survival in MBC patients, with a prognostic power independent of and either equivalent or superior to tumor burden and disease phenotype [3, 8]. In addition, the serial assessment of CTCs levels over the course of treatment has shown to provide a reliable estimate of clinical outcome and to represent a more precise indicator of treatment response compared with current radiographic methods [4, 5, 15]. Particularly, the count of CTCs has been shown to be superior in prognostic value to the metabolic imaging as assessed by 2-[fluorine-18]fluoro-2-deoxy-d-glucose/computed tomography [8].
Such studies have highlighted the need of a molecular characterization of CTCs, beyond simple enumeration, that would provide critical information in distinguishing subpopulations of CTCs with different biological properties. Such information may affect the choice and be useful to monitor the efficacy of systemic therapies and overall the clinical outcome of patients [10].

In a recent report, we identified a drug resistance profile of CTCs, predictive of response to chemotherapy, independently of tumor type and stage of disease. Of note, the major drawback of the study was the heterogeneity of population, consistently with the pivotal nature of the work. By contrast, in the present study we evaluated the predictive value of a drug resistance profile in a population of patients homogeneous for tumor type and stage of disease through the expression of selected drug efflux pumps.

Our results confirm the prognostic value of CTCs presence in MBC patients, as demonstrated by the significantly shorter PFS observed in CTCs-positive compared with CTCs-negative patients. Furthermore, the predictive value of CTCs drug resistance profile, according to MRPs expression, is confirmed in MBC, similarly to what we previously described in other epithelial malignancies. Indeed, we demonstrated in this population of patients that MRP overexpression on CTCs is predictive of poor response to specific chemotherapy regimens. In addition, we report for the first time that the number of MRPs expressed on CTC is predictive of poor response to treatment and significantly associated to shorter PFS, suggesting the possible applicability of a new quantitative approach.

Our study is innovative for three reasons: (i) we did not include patients treated with biological or endocrine therapies but only patients receiving cytotoxic chemotherapy. This choice allows to better eliminate any confounding factor and provide additional validity to our data; (ii) we identified a CTCs drug resistance profile specific for each type of drug used in MBC setting; (iii) we provided preliminary evidence that expression of MRPs could serve as an additional functional assay for CSCs. In fact, recent evidences demonstrated that the presence of ALDH1-positive cancer cells may represent a significant predictor of resistance to chemotherapy. Consistently with this hypothesis, we found a correlation between ALDH1 expression in CTCs and drug resistance profile, as well as between ALDH1 expression and number of MRPs expressed. Moreover, it has been suggested that human mammary epithelial cells with increased ALDH1 activity have stem/progenitor cell properties and retain the essential property of self-protection through the enhanced activity of multidrug resistance transporters, which

Figure 2. Kaplan–Meier curves of progression-free survival (PFS) according to baseline levels of circulating tumor cells (CTCs) (A); drug resistance profile of CTCs (B); number of different multidrug-resistance-related proteins (MRPs) expressed from CTCs (C) and difference in PFS between patients with aldehyde dehydrogenase 1 (ALDH1)-positive versus -negative CTCs (D).
breast tumors are characterized by a biologically aggressive...patient...ALDH1-positive CTCs may help selecting a more aggressive subpopulation of CTCs, theoretically including a fraction of putative CSCs resistant to chemotherapy [17–19]. Of note, a significant correlation was found between ALDH1 positivity in CTCs and the expression of ERz and HER2/neu in the same cells but not in the corresponding primary tumor. These data support the idea that defining a precise molecular portrait of CTCs, rather than of the primary tumor, might reliably predict therapeutic resistance.

We did not expect the lack of statistical significance in PFS between patients with CTCs positive and negative for ALDH1 expression. We hypothesize that this result may be ascribed to the small number of patients enrolled. These results were consistent with the previous reports [19, 20].

Finally, comparing molecular prognostic factors expressed in CTCs and in the corresponding primary tumor, we found a lack of concordance in HER2/neu expression in almost 40% of patients, similarly to the previous literature data [21]. The clinical utility of anti-HER2/neu therapies in patients with HER2/neu-negative primary tumors and HER2/neu-positive CTCs is currently under investigation.

To date, few studies have compared the expression of ERz in the primary tumors and CTCs in MBC, it has been shown that CTCs were more likely to be ERz-negative and PR-negative compared with the primary tumor. Conversely, more studies have compared ERz status between primary tumors and disseminated tumor cells, with similar results [22, 23]. Our findings are in agreement with this observation, since we found phenotype [16]. These observations seem to be consistent with the hypothesis that the expression of ALDH1 may help selecting a more aggressive subpopulation of CTCs, theoretically including a fraction of putative CSCs resistant to chemotherapy [17–19]. Of note, a significant correlation was found between ALDH1 positivity in CTCs and the expression of ERz and HER2/neu in the same cells but not in the corresponding primary tumor. These data support the idea that defining a precise molecular portrait of CTCs, rather than of the primary tumor, might reliably predict therapeutic resistance.

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the loss of ERα from primary tumors to CTCs in ~60% of patients, while we did not find ERα-positive CTCs from ERα-negative primary tumors. This could be a plausible explanation of the failure to endocrine therapy observed in a subset of hormone-receptor-positive patients.

In conclusion, we believe we have developed a method that allow sophisticated detection and characterization of CTCs and provide additional information beyond the simple enumeration. The evaluation of biomarkers that would help define chemoresistance or select appropriate targeted therapies is a major strength of this technology and could further contribute to a more personalized therapeutic approach in MBC.

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**disclosure**

The authors have declared no conflicts of interest.

**references**