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Original Articles

Gene expression profiles of circulating tumor cells versus primary tumors in metastatic breast cancer

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ABSTRACT

Before using circulating tumor cells (CTCs) as liquid biopsy, insight into molecular discrepancies between CTCs and primary tumors is essential. We characterized CellSearch-enriched CTCs from 62 metastatic breast cancer (MBC) patients with ≥5 CTCs starting first-line systemic treatment. Expression levels of 35 tumor-associated, CTC-specific genes, including *ESR1*, coding for the estrogen receptor (ER), were measured by reverse transcription quantitative polymerase chain reaction and correlated to corresponding primary tumors. In 30 patients (48%), gene expression profiles of 35 genes were discrepant between CTCs and the primary tumor, but this had no prognostic consequences. In 15 patients (24%), the expression of ER was discrepant. Patients with ER-negative primary tumors and ER-positive CTCs had a longer median TTS compared to those with concordantly ER-negative CTCs (8.5 versus 2.1 months, $P = 0.05$). From seven patients, an axillary lymph node metastasis was available. In two patients, the CTC profiles better resembled the lymph node metastasis than the primary tumor. Our findings suggest that molecular discordances between CTCs and primary tumors frequently occur, but that this bears no prognostic consequences. Alterations in ER-status between primary tumors and CTCs might have prognostic implications.

Introduction

Over the past decade, the concept of tumor heterogeneity between primary tumors and metastases has increasingly been acknowledged. Under the influence of time and treatment, tumor cell characteristics, including the expression of treatment targets such as the estrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2) in breast cancer, can vary between the primary tumor and distant metastatic sites [1–6]. Besides intertumor or temporal heterogeneity, even cell clones within one tumor site can differ in characteristics, giving rise to intratumor or spatial heterogeneity. Tumor heterogeneity may form the basis of treatment resistance and is therefore important to take into account in treatment decision-making.

Nevertheless, the choice for palliative treatments in metastatic breast cancer (MBC) is still generally based on primary tumor characteristics. Although a re-evaluation of ER and HER2 expression on a tumor tissue biopsy at the time of metastatic disease is recommended in guidelines [7], this is frequently omitted as obtaining tissue from metastases can be challenging or even impossible. Therefore, better and more patient-friendly tools are urgently needed to analyze characteristics of metastases before start of and repetitively during treatment.

Abbreviations: CTC, circulating tumor cells; EMT, epithelial-to-mesenchymal transition; ER, estrogen receptor; FFPE, formalin-fixed paraffin embedded; HER2, human epidermal growth factor receptor 2; LNM, lymph node metastasis; MBC, metastatic breast cancer; mRNA, messenger ribonucleic acid; OS, overall survival; PT, primary tumor; RT-qPCR, reverse transcription quantitative polymerase chain reaction; TTS, time-to-treatment switch.

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Analysis of circulating tumor cells (CTCs) might be an attractive means to assess the characteristics of metastases. Being present in the peripheral blood, CTCs can easily be obtained through a venipuncture and as such form a promising alternative for biopsies from metastatic lesions [8,9]. However, before we can fully appreciate the potential clinical value of CTC characterization, we need to learn more about the biology and to what extent CTCs – as suggested representation of metastatic cells – differ in their characteristics from primary tumors. In this study we used the CellSearch System (Janssen Diagnostics, Raritan, NJ) to isolate CTCs from MBC patients followed by gene expression profiling of 35 epithelial, tumor-associated, and CTC-specific genes [10]. The main objective of this study was to compare the overall molecular CTC profile to the corresponding primary tumor profile and to assess the proportion of patients with discordant molecular make-up. A profile from an axillary lymph node metastasis taken at the time of primary tumor resection was also available for comparison in a subset of patients. The expression of ER in CTCs and discordances with the primary tumor were investigated separately. Additionally, we explored the prognostic significance of observed discrepancies between primary tumor and CTC profiles.

Materials and methods

Wherever possible, the data are reported conform to the reporting recommendations for tumor marker prognostic studies (REMARK [11]). A study flowchart is presented in Fig. 1.

Patients

We retrospectively selected patients from an clinical trial enrolling MBC patients starting first-line systemic treatment, either endocrine or chemotherapy according to the physician's decision [10,12]. Blood for enumeration and characterization of CTCs was drawn before start of systemic treatment. Clinical data were collected from patient charts. All patients with a CTC count ≥5/7.5 mL blood who were included in the clinical trial between February 2008 and February 2012 were selected for the current study. Patients were recruited from six hospitals in the Rotterdam region. The Erasmus MC and local Institutional Review Boards approved the study (METC 06–248). All patients provided written informed consent.

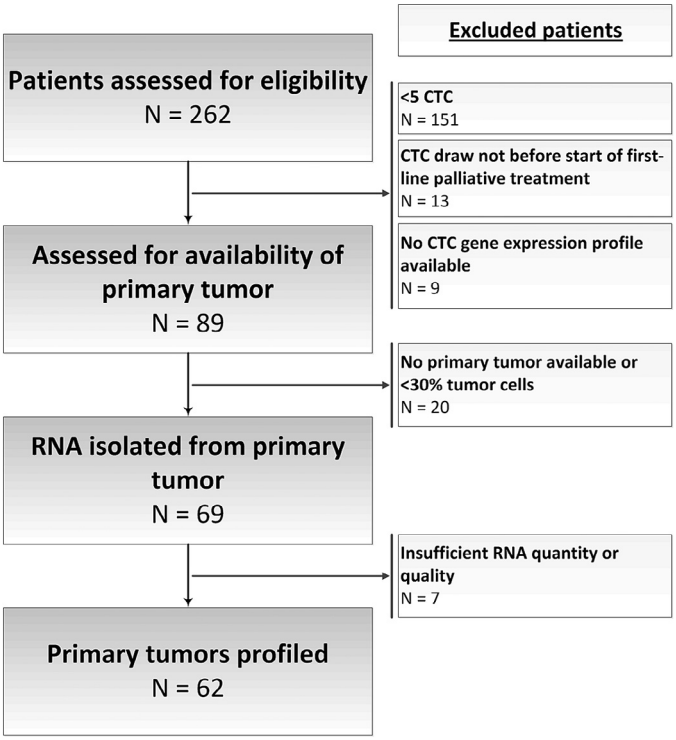

Fig. 1. Study flowchart. In total 262 patients from an ongoing prospective clinical trial were evaluated for eligibility for this study. After excluding patients not meeting our inclusion criteria (right boxes), 62 pairs of CTC and FFPE primary tumor profiles were used for subsequent analyses.

Sample processing

Enumeration and characterization of CTCs and using the CellSearch System and the generation of cDNA, linear preamplification, and reverse transcription quantitative polymerase chain reaction (RT-qPCR; using Taqman Gene Expression Assays; Applied Biosystems, Carlsbad, CA) were performed as described in detail before [10,12].

Archived formalin-fixed paraffin embedded (FFPE) primary tumors and axillary lymph node metastases were collected from pathology laboratories. Only paraffin blocks with ≥30% tumor cells on hematoxylin and eosin slides were selected. Isolation of RNA from FFPE samples was done using the High-Pure RNA Paraffin Kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions. Quantity and quality checks of isolated RNA were performed using the Nanodrop 1000-v.3.7 (Thermo Scientific, Wilmington, USA), the MultiNA Microchip Electrophoresis system (Shimadzu, Kyoto, Japan), and multiplexed RT-qPCR reference genes.

In all CTC and FFPE tumor samples, we measured our previously described panel of 55 epithelial tumor- and CTC-specific genes. These genes have been selected based on literature for involvement in tumorigenesis and/or mutagenesis along with absent or low expression by leukocytes. Consequently, our panel consists of clinically relevant genes that are reliably measurable in ≥5 CTCs by RT-qPCR [10,12]. To confirm similarly good assay performance on CTC and FFPE tumor samples, we compared expression levels between nine paired fresh frozen and FFPE primary tumor samples and only continued with the 20 genes that significantly correlated (Pearson correlation $P > 0.05$; Table 1).

Normalization and statistical analysis

Expression levels of individual genes in CTC and tumor samples were quantified relative to the average C_q of three reference genes (*GUSB*, *HMBS*, and *HPRT1*) using the ΔC_q method [13]. Samples with an average reference gene C_q>26 were considered to be of insufficient RNA quality and excluded from further analysis. To correct for the leukocyte background in the CTC samples, the median ΔC_q of each gene transcript in 31 CellSearch enriched healthy blood donor samples was used as cut-off. All ΔC_q values below this cut-off were considered undetectable. A compare batches (ComBat) normalization was conducted to enable comparison of corresponding profiles and limit technical variations [14–16].

We used a Pearson correlation analysis to compare the overall expression levels of 35 genes in primary tumors to corresponding CTCs. To enable further statistical testing two groups were formed of concordant and discordant profiles, based on all Pearson correlation coefficients of 62 primary tumors × 62 CTC samples. Among these 3844 correlations were 62 corresponding primary tumor/CTC pairs of the same patient and 3782 non-corresponding pairs of different patients. The mean correlation coefficient from corresponding samples from one patient was 0.72, which was significantly higher than the 0.54 from non-corresponding pairs from different patients ($P < 0.0001$; Fig. 2A). The top 10% strongest correlations among all 3844 pairs were arbitrarily chosen as concordant pairs, leading to a cut-off of r = 0.74.

To determine the ER-status of CTCs, we first established an mRNA cut-off value for ER-positivity by comparing *ESR1* expression levels in primary tumors with known ER-status from routine pathological reports. ER-positivity was defined as immunohistochemical staining in >10% of tumor cells. Expression levels of *ESR1* in 61 primary tumors (one tumor's ER-status was unknown) correlated with ER-status from the pathology reports and led to a reliable *ESR1* cut-off in our patient cohort (Fig. 3). All subsequent analyses were based on the *ESR1* expression levels both in the primary tumors and CTCs.

The Datan Framework GenEx Pro package version 5.4.1 software (MultiD Analyses AB, Göteborg, Sweden), SPSS 20.0 (IBM Corporation, Armonk, NY), and R version 3.0.1 (http://www.R-project.org/) were used to analyze gene expression levels. ComBat normalization was done using the Surrogate Variable Analysis package within R. Standard statistical testing was done using SPSS 20.0 (IBM Corporation, Armonk, NY). Differences in continuous variables were tested using Student's t test or non-parametric Mann–Whitney U, depending on the distribution. Categorical variables were tested by chi-square tests. Correlations were tested either by Pearson (gene expression data) or Spearman correlation (CTC count). Clinical outcome was expressed as time-to-treatment switch (TTS: the interval between start of first-line and second-line treatment or death, whichever comes first) and overall survival (OS: the interval between start of first-line treatment and death or last known to be alive). Associations with clinical outcome were visualized in Kaplan–Meier plots and tested by log-rank tests. All statistical tests were two-sided and $P < 0.05$ was considered statistically significant.