Gene expression signatures in circulating tumor cells correlate with response to therapy in metastatic breast cancer

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Comment on: Bredemeier M, Edimiris P, Mach P, *et al.* Gene Expression Signatures in Circulating Tumor Cells Correlate with Response to Therapy in Metastatic Breast Cancer. Clin Chem 2017;63:1585-93.

Received: 09 January 2018; Accepted: 01 March 2018; Published: 10 April 2018.
doi: 10.21037/jlpm.2018.03.01
View this article at: http://dx.doi.org/10.21037/jlpm.2018.03.01

Breast cancer is the most common cancer among women worldwide (1). Despite the development of new therapeutic strategies, metastatic breast cancer (MBC) remains an incurable disease characterized by a high clinical variability, partly linked to the molecular heterogeneity of both the primary tumor and its metastases (2). This heterogeneity can be the result of the intrinsic cancer-related genomic instability and/or be induced by therapies (3). The biological discordance between primary tumor and metastases is usually paralleled by changes in the genomic/transcriptional landscape of the different tumor sites determining resistance to ongoing therapies and/or acquired sensitivity to new therapies.

For this reason, circulating tumor cells (CTC) are considered a special window to study the evolution of the metastatic disease, allowing, in primis, the possibility to monitor the tumor load in the peripheral blood (4,5). Indeed, it has been definitely demonstrated the prognostic (overall survival and progression free survival) and predictive value of both the absolute number of circulating tumor cells before the beginning of a new therapeutic line as well as the change in CTC number upon therapy (6-8).

However, the clinical utility of CTC, i.e., their ability to improve the patient outcome by guiding therapy, remains to be demonstrated and, after the disappointing results of the SWOG500 trial (9), it is now under investigation in numerous prospective, randomized, multicenter clinical trials (e.g., STIC CTC METABREAST, DETECT V, and COMETI P2) (5).

In the meantime, scientists are exploring the advantage of going beyond the simple CTC enumeration by characterizing CTC from a molecular point of view. The purpose is to use CTC as a surrogate for the conventional tissue biopsy, to non-invasively evaluate the cancer genomic/transcriptomic/proteomic landscape and its evolution during treatment, in order to early detect drug-resistance and, possibly, predict new therapeutic targets (5).

The paper of Bredemeier *et al.* fits into these lines of research and aims to correlate the gene expression profile of CTC with the response to therapy (10).

Specifically, it evaluates the expression of 46 genes in CTC samples, enriched by Adna test, of 45 patients affected by MBC, enrolled before starting of a new therapeutic line, at the time of disease progression (PD). CTC were assessed at three time points: before the beginning of the new therapy (T0), and at two subsequent follow-ups (T1 and T2), about 8–12 weeks from each other. At the first follow-up (T1), patients were divided into responders (R) and non-responders (NR) depending on the presence of disease progression, as assessed by RECIST criteria. At T2 patients were classified as overall responder (OR) and overall non-responder (ONR) if the progression status were unchanged with respect to T1. Late responders and late non-responders, that is NR patients at T1 evaluated as responders in T2 and R patients at T1 undergoing progression in T2, were not further assessed in the study.

The method employed by Bredemeier *et al.* to assess CTC involved an initial immunomagnetic enrichment in CTC expressing the epithelial marker, EpCAM, EGFR and HER2, followed by mRNA isolation. mRNAs were

Table 1 Name and description of the 14 genes differentially expressed between CTC-positive and CTC-negative samples (10)

Gene ID	Description
KRT19	Keratin 19
CD24	CD24
PGR	Progesteron receptor erb-b2 receptor tyrosine kinase 2
EGFR	Epidermal growth factor receptor
EPCAM	Epithelial cell adhesion molecule
ERBB2	Erb-b2 receptor tyrosine kinase 2
ALDH1A1	Aldehyde dehydrogenase 1 family member A1
TWIST1	Twist family bHLH transcription factor 1
PLAU	Plasminogen activator, urokinase
CTSD	Cathepsin D
GZMM	Granzyme M
KIT	KIT proto-oncogene receptor tyrosine kinase
FLT1	Fms related tyrosine kinase 1
MKI67	Marker of proliferation Ki-67

CTC, circulating tumor cells.

then pre-amplified and high-throughput analyzed using a multiplex quantitative real-time PCR (RT-qPCR) targeting 46 genes cancer-related genes, including genes related to breast cancer, stemness and epithelial-to mesenchymal transition (EMT).

Samples were defined as CTC positive for the presence of at least one of the epithelial markers *EpCAM*, *MUC1*, *KRT19* or *ERBB2*. Although the expression of these 4 markers were variable among patients, underlining the already described heterogeneity of CTC, most of the CTC-positive samples co-expressed two of the listed markers. Considering all the different time points, 75% of samples were found positive for the presence of CTC and this frequency was higher than that reported in literature.

At the baseline (T0), CTC were found in 58% of the patients. At both T1 and T2 the fraction of CTC-positive samples was higher in NR (73%) and ONR (75%) than in R (42%) and OR (38%), respectively, thus confirming data already present in the literature showing the predictive value of CTC in MBC (6-8).

Analyzing the differences in the gene expression profile of samples CTC-positive and CTC-negative, independently from response to therapy and time-points, authors identified a 14-gene signature differentiating the two groups, which included (*Table 1*): breast cancer genes (*KRT19*, *CD24*, *PGR*, *EGFR*, *EPCAM*, and *ERBB2)*, stem cell markers (*ALDH1A1*), markers related to EMT and metastases (*TWIST1*, *PLAU*, *CTSD* and *GZMM*), receptor tyrosine kinases (*KIT* and *FLT1*), as well as the proliferation *MKI67* gene. Eight of these genes (*EGFR*, *GZMM*, *FLT1*, *PGR*, *PLAU*, *KIT*, *MKI67*, and *TWIST1*) were also differentially expressed between patients that resulted to be always CTC-positive or CTC-negative, at all the time points analyzed, respectively.

Finally, authors tried to evaluate differences in the gene expression profile of blood samples depending on response to therapy. At T1, R and NR significantly differed in the expression of KRT19 and ADAM17. While the first one was strictly related to the presence of CTC, the second marker was independent from CTC status. At T2, CTC-positive and CTC-negative samples differed in the expression of *KRT19*, *EPCAM*, *CDH1*, and *SCGB2A2*. Considering instead the differences between OR and ONR, only ABCC1 and KRT19 were differentially expressed, independently from CTC expression, while all the other markers were strictly related to the presence of CTC.

To summarize the findings, a larger fraction of drug-responder patients, with respect to non-responders, presented CTC and, among the studied genes, KRT19, encoding for keratin 19, was related to both CTC presence and drug-resistance. Additionally, independently from CTC presence, ADAM17 (ADAM metallopeptidase domain 17) and ABCC1 (ATP binding cassette subfamily C member 1) were differentially expressed between R/NR and OR/ONR patients, respectively.

Four are the major point to discuss here.

The first one regards the method chosen to detect CTC. As well known, CTC are extremely rare cells, e.g., a single tumor cell in a background of millions to billions of blood cells (11), and their detection requires approaches with high analytical sensitivity and specificity (4). Since no single definition of CTC and no single CTC biomarker have been identified (12), current methods employ several strategies, which include selection on biophysical or metabolic properties as well as on more 'specific' biological features, such as tumor cell surface marker expression (11). However, the only FDA approved method to detect and enumerate CTC in MBC patients is CellSearch. It is based on the staining of EpCAM enriched blood samples by the nuclear dye DAPI and antibodies recognizing CD45 and Cytokeratin 8/18/19. CTC are then defined as DAPI+/EpCAM+/Cytokeratins 8/18/19+/CD45− cells. This method exclusively allows the enumeration of epithelial CTC, while it could miss mesenchymal CTC not expressing anymore EpCAM. Additionally, the molecular characterization of CTC, although possible even at single cell level, requires a subsequent sorting of the cells. Here, authors adopted a commercialized assay to enrich samples in CTC by magnetic beads functionalized with cocktails of antibodies specific to breast cancer. The CTC detection and characterization is then conducted through a sensitive analysis of breast cancer-associated gene by reverse transcription and RT-qPCR (13). Therefore, even the Adna test select CTC on the basis of epithelial markers, and, as CellSearch, can miss CTC not expressing epithelial markers. Interestingly, in the analyzed patients, about one third of NR and ONR did not present CTC. Whether the absence of CTC was authentic or due to the inability of the assay to detect CTC (e.g., mesenchymal CTC) remains to be determined. Regarding the choice of using RT-qPCR to analyze Adna test- enriched samples, it presents some advantages, such as the possibility to analyze and quantify, with high sensitivity, during the same reaction, many different genes (14). However, there are also disadvantages related to the fact that RNA samples are evaluated and this requires the use of high quality RNA an accurate choice of target genes and control samples to avoid either false negative or false positive results (14). Furthermore, the detection of or tumor specific mRNAs by RT-qPCR requires preferentially viable CTC in order to maintain the integrity of the genetic material.

The second one regards the evaluation of CTC heterogeneity. This latter can be evaluated at single cell level or globally, and taking into consideration genomic alterations, as well as gene or protein expression. Authors decided not to evaluate the heterogeneity at single cell level, but to explore the global expression of 46 genes, including those related to stemness and epithelial-to-mesenchymal transition (EMT), frequently shown as associated with drug resistance (5). Indeed, in these years several attempts have been made to identify, by RT-qPCR, a gene signature predictive of response to therapy (14). For example, Mostert *et al.* identified a 16-gene signature able to predict a rapid drug-treatment failure (15), while, in luminal patients, Reijm *et al.*, identified an 8-gene signature predictor of good/poor outcome to first-line aromatase inhibitors (16). In the paper of Bredemeier *et al.*, although CTC-positive samples as well as ONR were frequently characterized by a variable expression of stemness and EMT markers, no predictive value of these markers was demonstrated, perhaps for the small number of recruited patients. Instead, *KRT19* was markedly present in CTC positive patients and highly expressed in OR/ONR patients, independently from CTC status. Similarly, Georgoulias *et al.* demonstrated that the detection of high levels of *KRT19* both before and after chemotherapy was associated with a significantly decreased overall survival in MBC patients (17).

The third attractive point is the detection of genes differentially expressed between R/NR and OR/ONR patients, independently from CTC detection. This approach would open the possibility to overcome the limitations related to CTC detection. Interestingly, ADAM17 is a membrane-bound protease that sheds the extracellular domain of various receptors or its ligands from the cell membrane, thus activating downstream signaling transduction pathways; its role in breast cancer, including cell proliferation, invasion, and drug resistance is known (18). ABCC1, instead, is a transporter associated with multi-drug resistance to cancer chemotherapy in many tumors including breast cancer (19). Similarly, it would be interesting to understand if there are differences in the CTC profile of R and NR patients. Of course, this requires and increased number of enrolled patients.

The fourth point is the fact that the MBC patients analyzed in the paper are not selected on the basis of specific molecular subtype or drug regimen, and since the idea is to specifically correlate CTC phenotype and drug-response, it could be even more informative to restrict the analysis to specific and homogeneous clinical groups.

In conclusion, although promising, the data presented in the paper of Bredemeier *et al.* might be considered preliminary and, as suggested by authors, they must be confirmed in largest and/or better-defined case studies. Increasing the number of patients enrolled will allow: (I) to better understand if there are differences in the gene expression profile of CTC between R and NR as well as OR and ONR patients. In the present study, such differences were not assessed; (II) to include in the study LR and LNR patients, excluded from this study; (III) to establish if the transcriptional phenotype of CTC depends on key clinicopathological characteristics of the tumor, including molecular type, metastatic sites, and therapeutic regimen administered; (IV) to better clarify the role played by markers able to predict drug response independently from CTC presence.

Acknowledgments

D Cesselli is supported by the AIRC grant IG2017_20443: dissecting the heterogeneity of circulating tumor cells in metastatic breast cancer patients to predict clinical outcome.

Footnote

Provenance and Peer Review: This article was commissioned and reviewed by the Section Editor Dr. Dao-Jun Hu (Department of Clinical Laboratory, Xin Hua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Chongming Branch, Shanghai, China).

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/jlpm.2018.03.01). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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doi: 10.21037/jlpm.2018.03.01
Cite this article as: Bulfoni M, Beltrami AP, Cesselli D. Gene expression signatures in circulating tumor cells correlate with response to therapy in metastatic breast cancer. J Lab Precis Med 2018;3:33.