


Cell-Free DNA Variant Sequencing Using CTC-Depleted Blood for Comprehensive Liquid Biopsy Testing in Metastatic Breast Cancer

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Abstract

Keup and colleagues provide liquid biopsy preliminary results by sequencing variants in circulating tumor cells (CTCs) and cell-free deoxyribonucleic acid (cfDNA) “all from one tube” format, in order to use the same blood sample under the same isolation conditions of both analytes to reach an unbiased comparability and consistency. We appreciated the attempt of the authors to improve technical procedures in liquid biopsy research area, but we wanted to raise several issues related to cfDNA detection, reporting our research experience. This is a feasibility study as the authors analyzed only one sample from a small case series at an advanced line of treatment. In the clinical practice to monitor the disease and predict the treatment response, the analysis should be done at multiple time points. We have previously demonstrated that the quantity and the integrity of the cfDNA are not useful to determine the evolution of early breast cancer (bc), maybe due to the fact that cfDNA is not strictly related to cancer but also to an inflammatory status. Given that a high content of cfDNA could reflect inflammatory processes, we decided to investigate the role of stimulator of interferon gene (STING), an important regulator of cancer cell growth and senescence, in bc tissue in relation to cfDNA. STING biomarker analyzed by immunohistochemistry on tumor tissue could reflect a circulating inflammatory status and needs to be further investigated, not only on CTCs but also on cfDNA. One of the major issues of cfDNA is to decide what to analyze on it, in terms of type of cells and genetic alterations. Considering that multiple tests could be done to study gene copy number alterations, mutations, and variant fusions, the proper molecular test should be chosen, on the basis of the clinical need, starting from the treatment choice to disease monitoring.

Keywords

CTCs, cfDNA, breast cancer, STING, inflammation

Liquid biopsy biomarkers such as cell-free deoxyribonucleic acid (cfDNA) and circulating tumor cells (CTCs) exhibit great interest for personalized treatment and there is the need to have their evaluation from the same specimen to provide complementary and comprehensive information. Keup and colleagues provide preliminary results by sequencing variants in CTCs and in cfDNA “all from one tube” format, in order to use the same blood sample under the same isolation conditions of both analytes to reach an unbiased comparability and consistency¹.

CfDNA is released mainly passively and could represent dying and therapy-sensitive cells¹, even if it could not be

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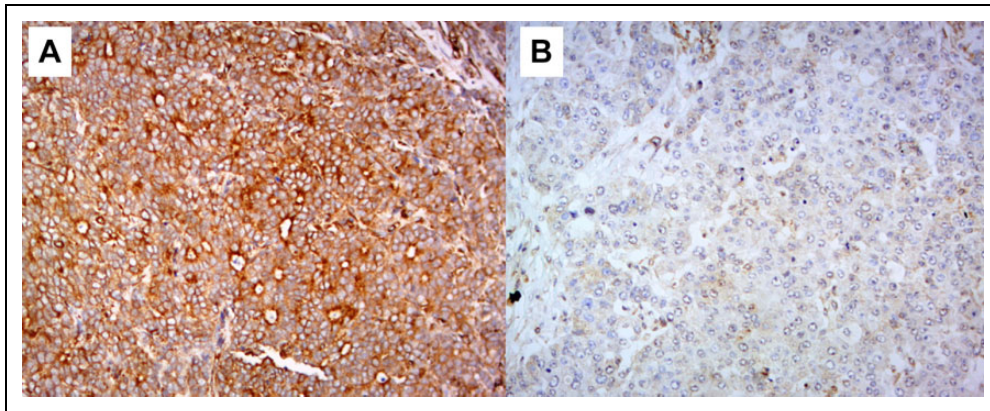


Fig. 1. STING expression assessed by immunohistochemistry in breast cancer tissues (20× magnification): (A) a case with high cfDNA content showing a strong staining intensity in the cytoplasm of tumor cells; (B) a case with low cfDNA content showing no staining in the cytoplasm of tumor cells. cfDNA: cell-free deoxyribonucleic acid; STING: stimulator of interferon gene.

closely related to a cancerous condition, but can be related to an inflammatory status. On the other hand, CTCs are rare cells actively migrating as potential seeds of metastasis and could indicate minimal residual disease¹. However, their detection remains feasible only in the presence of substantial tumor burden and/or in the metastatic setting. In fact, many studies comparing cfDNA and CTCs considered the same patient inclusion criteria, but different cohorts for either cfDNA or CTC analysis¹. In those studies analyzing both cfDNA and CTCs from the same patients, the cohort sizes were quite small and the two analytes were obtained from blood samples taken at different time points¹. We appreciated the attempt of the authors to improve technical procedures in liquid biopsy research area, but we wanted to raise several issues related to cfDNA detection, reporting our research experience.

Keup and colleagues analyzed whether cfDNA variant sequencing from CTC-depleted blood (CTC-depl. B) obtained after positive immunomagnetic isolation of CTCs (AdnaTest EMT-2/Stem Cell Select, QIAGEN, Germany) impacts the results compared to cfDNA variant sequencing from matched whole blood (WB)¹. CfDNA was isolated using matched WB and CTC-depl. B from 17 hormone receptor positive/human epidermal growth factor receptor 2 negative (HR+/HER2-) metastatic breast cancer (bc) patients¹.

The authors analyzed the biomarker status for a small case series at a single time point, and the patient characteristics were well described. They concluded that cfDNA variants from matched WB and CTC-depl. B showed no relevant differences and that parallel isolation of cfDNA and CTCs from only 10 ml of blood in all from one tube format was feasible¹. This is a feasibility study as the authors analyzed only one single sample from a small case series of patients at an advanced line of treatment. The small amount of necessary blood is a point of strength, even if no conclusions about clinical associations with disease progression and/or response to therapy may be derived from this work. In the real clinical

practice for disease monitoring and/or predict the response to therapy, the analysis of the biomarkers should be done at multiple time points. Moreover, the basal value of the biomarker should be done before starting any line of treatment, to be able to see any biomarker modification for disease monitoring². The biomarker evaluation should be performed whenever a new line of treatment is performed and at the disease re-evaluation. We agree with the fact that the author performed a depletion of CTC to avoid blood cell contamination for cfDNA analysis to detect variants¹, because it is important to distinguish germline mutations from somatic mutations, although cfDNA alterations may not reflect the whole tumor biology. In fact, some authors reported that concordance between alterations in the tumor tissue and in circulating cfDNA could greatly vary². Chae and colleagues performed a study on 28 patients with advanced solid tumors to analyze the concordance between genomic alterations assessed by next-generation sequencing in tumor tissue and in circulating cfDNA². The overall concordance defined as the presence or absence of the identical genomic alterations in a single gene on both molecular platforms was 91.9%–93.9%. Over 50% of mutations detected in either technique were not detected using the other, indicating a potential complementary role of each assay. Across five genes tumor protein 53 (TP53), epidermal growth factor receptor (EGFR), kirsten rat sarcoma virus gene (KRAS), adenomatous polyposis coli gene (APC), and cyclin-dependent kinase inhibitor 2A (CDKN2A), sensitivity and specificity were 59.1% and 94.8%, respectively². Potential explanations for the lack of concordance include differences in assay platform, spatial and temporal factors, tumor heterogeneity, interval treatment, subclones, and potential germline DNA contamination².

In addition, one of the major issues related to cfDNA is that it is not closely related to cancer and it is released in case of cell death, occurring in several conditions, such as systemic inflammation³. It is well known that cfDNA is a trigger of inflammation, given that it is also composed of

mitochondrial and cytoplasmic DNA that constitute highly pro-inflammatory molecular garbage⁴. Malignant transformation usually accompanies formation of cytosolic chromatin fragments and micronuclei, increasing the probability of DNA leakage in cancer cells⁵. Besides micronuclei, small DNA fragments derived from DNA damage could be released into cytoplasm during the whole interphase. We previously demonstrated no prognostic value of cfDNA and its integrity of four genes involved in bc breast carcinoma amplified sequence 1 (BCAS1), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit Alpha (PI3KCA), c-Myc gene and human epidermal growth factor receptor 2 (HER2) on 79 serum samples collected before surgery for women at first diagnosis of bc with different subtypes⁶. Given that a high content of cfDNA could reflect inflammatory processes, despite it being difficult to know the origin of cfDNA (e.g., epithelial cells, inflammatory cells, or both), we decided to investigate the role of STING in bc tissue in relation to cfDNA. Cytosolic DNA stimulates active cyclic guanosine monophosphate-adenosine monophosphate (GMP-AMP) synthase-stimulator of interferon gene (cGAS-STING) pathway, an important regulator of cancer cell growth, senescence, and immune surveillance, a versatile pathway that needs further investigation, especially considering that its function changes along with cancer type and disease stage.

In the primary tissue of a bc patient with high cfDNA serum content, we observed a strong expression of STING biomarker, while a patient with low content of cfDNA showed no STING expression in the tumor tissue (Fig. 1). STING biomarker analyzed by immunohistochemistry on tumor tissue could reflect a circulating inflammatory status and needs to be further investigated, not only on CTCs but also on cfDNA. We are aware that these results were derived from the analysis on only two early bc patients, but we are planning to enlarge the case series in order to validate these data. However, one of the main problems with cfDNA is to decide what to analyze of it, in terms of type of cells and genetic alterations. Therefore, multiple tests could be done to study gene copy number alterations, mutations, and variant fusions. The proper molecular test should be chosen on the basis of the clinical need, starting from the treatment choice to disease monitoring.

Authors' Note

Roberta Maltoni and Michela Palleschi are co-first authors.

Authors' Contributions

Conceptualization was by RM, MP, and SB; investigation by MP, SR, MMT, AR, and MP; original draft preparation by RM, SB, SR, and MP; supervision by RM and SB. All authors finally approved

the version to be published and agree to be accountable for all aspects of the work.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.


Ethics and Patient Consent


The present study was approved by the local Ethics Committee of Area Vasta Romagna. All procedures performed in the study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

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