Plasma tumor DNA: on your markers, get set, go!

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Abstract: Metastatic breast cancer is incurable, yet highly treatable with endocrine, HER2 directed and chemotherapies improving survival for many patients. Successful treatment depends on the ability to monitor disease burden and response to therapies. Recently, a proof of principle study has shown that plasma tumor DNA (ptDNA) can be used as a reliable breast cancer biomarker in metastatic disease, due to its sensitivity and wide dynamic range. ptDNA more accurately reflects changes in response to therapies, and absolute levels of ptDNA demonstrate prognostic significance. Thus, ptDNA as a liquid biopsy shows great promise in the clinical management of metastatic breast cancer though further technical challenges and larger confirmatory studies are needed.

Keywords: Plasma tumor DNA (ptDNA); circulating tumor DNA (ctDNA); metastatic breast cancer; plasma biomarker

Although metastatic breast cancer in not a curable disease, it is highly treatable due to better therapies including endocrine, HER2 directed and chemotherapies. Unlike other cancer types, the ability to control disease progression and prolong life often results in women who live many years if not decades with metastatic disease. In many ways, this level of success has created new conundrums in breast oncology, notably how to best determine when and if to change therapies when so many effective options are available. Traditionally, symptoms, clinical exam and imaging have remained the mainstays for assessing response to therapies and disease burden, and yet there is emerging evidence that these methods may not be reliable or reproducible between physicians and treatment centers. As an example, the recently published BOLERO-2 trial demonstrated notable differences between local and central assessments when cataloging responses to therapies (1). In addition, tumor heterogeneity due to clonal evolution and the emergence of drug resistant clones has highlighted new challenges in reassessing metastatic disease for eligibility for targeted therapies and clinical trials (2,3). Thus, the

development of reliable biomarkers for following disease burden and response to therapies in patients with metastatic breast cancer have been a focus of much research during the past few decades.

Recently Dawson *et al.* reported an intriguing study using a novel and reliable method to follow tumor burden in patients with metastatic breast cancer (4). This group used the emerging technique of assaying circulating tumor DNA (ctDNA) to qualitatively and quantitatively measure metastatic disease in a prospective study of breast cancer patients. Importantly, the study also compared the dynamics of ctDNA in a "head to head to head" fashion with circulating tumor cells (CTCs) and a currently clinically approved breast cancer protein tumor marker CA15.3, and then correlated all changes with conventional parameters of CT scanning using standard RECIST criteria (5). The results provide an exciting glimpse into the future of monitoring tumor burden and response to therapies.

For background, it is now well-established that cells naturally secrete or shed small DNA fragments into the circulation with most of this DNA being compartmentalized

in the plasma component of whole blood. Small amounts of cell-free circulating DNA can be detected in plasma from healthy individuals (6,7), is often highly elevated in metastatic cancer patients (6,8) and correlates with disease burden (4,9,10). Interestingly, how ctDNA enters the plasma is not precisely known. While the fragment size of circulating DNA in healthy individuals suggests that it originates from apoptotic cells (7), circulating DNA is also shed from non-apoptotic cells and in this instance it is often larger and more variable in size (11-13). For clarity, we prefer and will henceforth use the term plasma tumor DNA (ptDNA) over ctDNA since there is confusion regarding ctDNA versus DNA derived from CTCs, which are distinctly different. In addition, ptDNA is more specific as it is a subset of ctDNA, since ctDNA also includes urine tumor DNA (utDNA) as well as ctDNA in other bodily fluids. Recent work, including that from our own group, supports the utility of ptDNA as a facile means for liquid biopsy (4,8,14). Our group and others have shown that cancer specific somatic mutations can be detected in ptDNA from metastatic breast cancer patients with up to a 100% concordance when blood is taken concurrently with tissue biopsy (4,8,14). In contrast, we also demonstrated that mutational discordance can occur between ptDNA and primary breast cancer tissues when there is a prolonged period (greater than 3 years) between initial diagnosis and recurrence (14). This speaks to the now proven concept of tumor heterogeneity and clonal evolution (15). PtDNA has also been used to identify treatment associated mutational changes (2,8). Thus, assessment of ptDNA can relay the mutational status of a patient's cancer with the ability for serial testing. Thus, although biopsying sites of metastatic disease can lead to clinically useful information, it is simply not feasible to biopsy every metastatic site and indeed it is often impossible to biopsy any metastatic lesion depending on the clinical circumstance. The ability to obtain a safe, non-invasive liquid biopsy adds greatly to the ability to match the right targeted therapies with the correct genetic profile from each individual patient's tumor.

Dawson and colleagues collected serial blood samples from 30 women with metastatic breast cancer over a course of 2 years, at intervals of at least 3 weeks. As mentioned, they collated and compared the results of three possible cancer biomarkers in these liquid biopsies, namely, mutations in ptDNA, CA 15-3 levels, and the number of CTCs, along with the corresponding findings from CT radiographic imaging at the respective times of blood draw. In order to assess the genetic mutational status of the patients, Dawson and colleagues attained genomic DNA (gDNA) of archived tumor tissues from 52 accrued patients and sequenced them for common *PIK3CA* and *TP53* mutations using targeted tagged-amplicon deep sequencing (TAm-Seq) (16) and/or paired-end whole genome sequencing to identify structural variants (17). From the initial 52 patients, 30 were identified as having ptDNA biomarkers that could be prospectively followed. The group then used these mutations and structural alterations as biomarkers to track tumor progression and response to treatment by querying for these changes in ptDNA both qualitatively and quantitatively to assess disease burden and response to therapies. Although there are now many methods for assessing ptDNA including BEAMing (Beads, Emulsions, Amplifications and Magnetics) (9,18), and other digital PCR platforms (19), the group used TAm-Seq and a microfluidics based digital PCR for subsequent ptDNA analysis. Using this strategy, they were able to monitor the progression of disease based on the number of quantified copies of ptDNA per milliliter (copies/mL) of blood. They demonstrated the utility and comparability of their markers by showing similar trends in multiple mutations through time and treatments in the analyzed patients. The temporal ptDNA data also showed tumor heterogeneity within the same patient, where certain mutations appeared dominant in the plasma when compared to the archived tissue. Clonal evolution was also suggested by the serial ptDNA data, because novel mutations not detected in the archived tissue were detected in the plasma, and their concentrations varied during treatments.

Levels of CA 15-3 and the number of CTCs were also measured in the blood samples. These were then compared to the detection sensitivities of ptDNA. When ptDNA was compared to CA 15-3, the former had a sensitivity of 85% whereas the latter was only 59%. When ptDNA was compared to CTCs, the former had a sensitivity of 90% whereas the latter was 67%. Thus, Dawson *et al.* showed that ptDNA is currently the most sensitive blood-based marker for metastatic breast cancer analysis. However, ptDNA did not achieve 100% detection sensitivity, meaning even though the genetic variations were detected in archived tissues, some were undetectable in the blood samples of ptDNA. This may be due to extremely low tumor burden at the time of sampling, and/or the limitation of sensitivity for the digital PCR platform and TAm-Seq methods utilized for the study. Interestingly and excitingly, newer digital PCR platforms should be able to overcome sensitivity issues while increasing speed and decreasing costs of these assays (19).

The authors then compared the performance of the three blood-based biomarkers to the findings of CT radiographic imaging in 20 of the 30 patients, since only 20 patients had measurable disease under the RECIST criteria. Unsurprisingly, the concentrations of detectable ptDNA correlated with the treatment responses as assessed by CT imaging. For instance, ptDNA concentrations decreased when a patient was diagnosed with stable disease after treatment, but the ptDNA concentrations increased as the patient recurred with progressive disease. Impressively, ptDNA was sensitive enough to reflect partial response to therapies, where concentrations decreased slightly. These changes in ptDNA concentrations showed an appreciable dynamic range that correlated with the diagnosis from CT imaging, which allowed oncologists to monitor the patients' metastatic disease progression. However, there were two patients that demonstrated discordant correlations. Similar trends were also shown for CTCs counts, but only when the number of CTCs was at least five per 7.5 mL of blood, otherwise, the CTCs data was uninformative. CA 15-3 levels only showed correlative trends with CT imaging when they were above the threshold of 50 U per mL of blood, but with a very modest dynamic range compared to ptDNA concentration changes. When CA 15-3 levels fell below the threshold, trends were inconsistent. Therefore, the authors demonstrated that ptDNA, when compared to CA 15-3 and CTCs has the widest dynamic range that corresponds to CT imaging findings, and thus may be the most accurate blood-based biomarker to monitor metastatic breast cancer progression and response to therapies.

Finally, within the same study, the authors used a Cox proportional-hazards model to plot survival curves for their patients, using ptDNA as a continuous time-dependent variable. Importantly, they showed a prognostic value for increased ptDNA levels, as they were associated with an inferior overall survival. CTCs also showed prognostic significance as in the past [20], but CA 15-3 did not. These results demonstrate that the liquid biopsy concept of identifying mutations and targets of therapy within ptDNA can be taken a step further in that quantifying levels of ptDNA not only allows for monitoring of disease in a timely fashion, but also by itself lends prognostic value.

Taken together, Dawson and colleagues have highlighted that ptDNA provides a wealth of genetic information in monitoring and tracking cancer disease progression. Beyond the fundamental detection of genetic alterations in ptDNA, serial analysis of ptDNA from cancer patients pieces together information regarding tumor burden and adds insights into acquired resistance. This opens the therapeutic and prognostic possibilities of utilizing ptDNA in the clinic to guide crucial decisions on patients as they undergo treatments. For example, therapies could be changed earlier if responses in ptDNA are not observed, which in turn avoids unnecessary costs, time, and adverse side effects of ineffective drugs. However, it should be noted that this study, while an outstanding proof of principle, will require a larger confirmatory study prior to acceptance as a routine clinical tool. In addition, other technical challenges will need to be addressed including the time and cost efficiency of identifying genomic biomarkers (somatic mutations and structural alterations), as well as the sensitivity of the employed methods for querying ptDNA. However, given the improvements in genome sequencing with decreasing costs, along with newer digital PCR technologies with greater sensitivity [19], the future of using ptDNA for routine monitoring in cancer patients is indeed bright.

In conclusion, utilizing the analysis of ptDNA in blood as a liquid biopsy to monitor disease progression is excitingly feasible. However, the analytical methods must be extremely sensitive for clinical usefulness. In addition, although in theory cancer mutations and structural alterations are 100% specific, ideally ptDNA alterations should be matched with the patient's tumor tissues to ensure that they are not the result of contamination and/or occult malignancies. However, we envision a future where ptDNA analysis will enable clinicians to monitor progress and residual disease burden for both metastatic cancer patients and early stage disease. In early stage disease, the ability to measure microscopic tumor burden could potentially change the paradigm of how systemic therapies are currently administered in the adjuvant and neoadjuvant settings. Indeed, periodic monitoring of ptDNA has the potential to revolutionize the accurate and reliable monitoring of cancer progression using the readily obtainable source of blood. Thus, the liquid biopsy will allow for a faster, less invasive and more robust assay to help oncologists best manage patients with cancer.

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