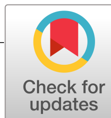

ally, breaking up weaker binaries and assembling new, tighter binaries. Globular clusters can therefore give rise to exciting and puzzling stellar populations, including millisecond pulsars, gravitational-wave events, and fast radio bursts (transient radio pulses as fast as a fraction of a millisecond, whose sources remain unclear).

Barr *et al.* point out that the eccentric orbit, fast pulsar spin, and high total mass of the PSR J0514–4002E binary system suggest that the pulsar and its companion did not start off in a stellar binary. Instead, the pulsar exchanged its initial binary companion for the present, higher-mass object in a dynamical encounter. It is even possible that the companion in question is itself the product of a previous binary system inside the globular cluster—perhaps the merger of two neutron stars, even though mergers involving neutron stars are thought to be rare inside globular clusters (*15*).

Regardless of its origin—whether a stellar remnant, the merger product of two neutron stars, or the result of another type of merger or mass transfer event—the discovery of a compact object with a mass between 2.09 and 2.71 solar masses in a globular cluster has fascinating implications. If a neutron star, it is probably the heaviest one known to date, with lessons for the uncertain physics of extremely dense nuclear matter. If a black hole, it may be the lightest known, which could affect the understanding of supernova explosions or dynamical interactions such as neutron star mergers inside globular clusters. With upcoming electromagnetic and gravitational-wave observations, the growing population of compact objects between 2 and 5 solar masses will allow further resolution of the mass distribution and reveal the details of their formation. ■

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MEDICINE

Surpassing sensitivity limits in liquid biopsy

Attenuation of cell-free DNA clearance in vivo is an alternative strategy to maximize recovery

By **Tina Moser**[1,2] and **Ellen Heitzer**[1,2]

Cell-free DNA (cfDNA) can be found in the blood and contains tumor-derived fragments [circulating tumor DNA (ctDNA)] when extracted from cancer patients. In advanced-stage tumors, ctDNA analyses to detect drug resistance mechanisms or actionable targets have already been translated into clinical practice (*1*). In recent years, ctDNA approaches have continuously been expanded to earlier disease stages. However, a persistent challenge of cancer screening and the molecular detection of minimal residual disease (MRD) has been the need to increase sensitivity, allowing for the detection of minute amounts of ctDNA with unprecedented accuracy. Most attempts to enhance sensitivity have focused on ex vivo strategies, such as sampling, analytical parts (e.g., library preparation), and bioinformatics. On page 274 of this issue, Martin-Alonso *et al.* (*2*) report confronting this challenge by transiently modulating the natural clearance mechanisms of cfDNA in vivo to increase its concentration in blood samples.

The amounts of cfDNA (and ctDNA) in the blood are determined by the interplay of its release—mainly driven by cell turnover—and its degradation and clearance, which are mediated through nuclease digestion, renal excretion, and uptake by macrophages of the mononuclear-phagocyte system (MPS) in the liver (*3*). Given the rapid clearance of cfDNA, with an estimated half-life of 30 to 120 min, a standard blood draw of 10 ml typically yields limited quantities of cfDNA. From 10 ml of blood, ~5 ml of plasma can be isolated, providing an average of 10 ng of cfDNA per milliliter, corresponding to roughly 15,000 haploid genome equivalents (GEs)—i.e., the amount of DNA in one copy of a genome (*4*). On average, ctDNA may make up as much as 10% (sometimes much higher) of the overall cfDNA pool in patients with advanced-stage cancer. However, the ctDNA fractions drop substantially to 0.1 to 1% in locally advanced disease and to <0.1% in early-stage disease or after curative-intent treatment. Therefore, when ctDNA fractions are as low as 0.1 or 0.01%, this translates to only 15 or 1.5 GEs, respectively, derived from the tumor. Such a blood sample may not contain sufficient ctDNA fragments for effective sequencing or detection.

Increasing sample volumes through plasmapheresis is one option to recover greater numbers of GEs and overcome sampling biases (*5*). However, this requires expensive instrumentation, is time-consuming, and might not be feasible for critically ill patients. To capture ctDNA more efficiently, the concept of proximal sampling, which refers to taking samples and body fluids more proximally to the tumor, has been introduced (*6*). Promising results were reported for the analysis of urinary cfDNA in bladder cancer (*7*). More recently, in vivo interventions, such as focused ultrasound (*8*) or radiation (*9*), have been proposed to increase ctDNA shedding temporarily. Yet, both proximal sampling and enhanced ctDNA release require a priori knowledge of tumor location, making the approach unamenable for screening approaches to detect cancers early.

Martin-Alonso *et al.* present a new proof-of-concept study using a preclinical model including healthy mice and mice bearing bilateral grafts of colorectal carcinoma cells to maximize the amount of ctDNA recovery by using intravenous priming agents that transiently delay cfDNA clearance in vivo (see the figure). In tumor-bearing mice, they demonstrated that liposomal nanoparticles mirroring the size of native cfDNA—that is mostly bound to histone proteins and circulates as mononucleosomes (~11 nm in size)—can attenuate phagocytic clearance by competing with uptake by the MPS. To further enhance the abundance of cfDNA and ctDNA, the authors used monoclonal antibodies (mAbs) with abrogated FcγR binding to protect cfDNA from enzymatic digestion by circulating deoxyribonucleases (DNases). Administered 1 to 2 hours before a blood draw, both agents (the liposomes and DNA binding mAbs) enabled a >10-fold increased recovery of cfDNA and ctDNA molecules. Application

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of the liposomes improved the sensitivity of their ctDNA test up to 60-fold, particularly in mice with the lowest tumor burden. Although this approach enabled an improved recovery of tumor genomes and may substantially enhance the sensitivity of ctDNA diagnostic assays, it remains to be determined how such strategies would translate into humans in terms of formulation testing and tolerability.

Currently, the peak of sensitivity and specificity in ctDNA diagnostics is achieved using a bespoke approach, meaning that known mutations from the tumor tissue are tracked in plasma (*10*). However, when screening for a limited number of mutations, the number of available GEs dictates the analytical sensitivity. One effective strategy to capture rare ctDNA fragments is to sequence relevant target regions to exhaustion (up to 15,000 times). To this end, an increase of signal-to-noise ratios to reliably distinguish true tumor-associated mutations is necessary. A seminal breakthrough in reducing the impact of sequencing errors and increasing confidence in mutation detection was the use of molecular barcodes combined with sophisticated bioinformatics analyses that enable error suppression (*11*). For instance, in duplex sequencing, the two strands of DNA are tagged and sequenced independently, resulting in a 1000-fold error reduction compared with standard sequencing, but at a considerable expense (*12*). To reduce the required number of sequencing reads per target, the MAESTRO technique was developed, which uses short probes to enrich for patient-specific mutant alleles and uncovers the same mutant duplexes using up to 100-fold fewer reads (*13*). Enrichment of specific cfDNA fragment subpopulations during library preparation and the selective recovery of specific fragment sizes in silico might also boost ctDNA detection and could complement or provide an alternative to deep sequencing cfDNA (*14*).

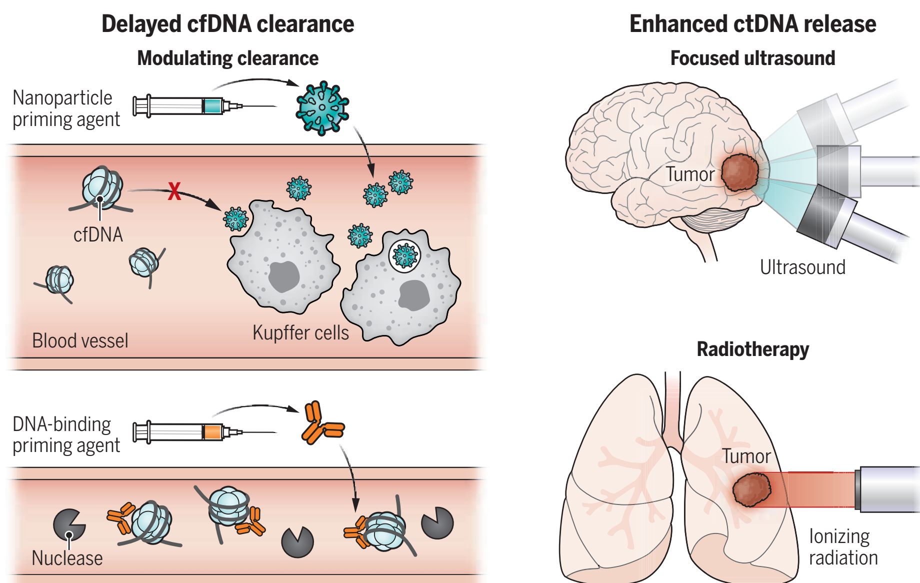
Another strategy to overcome the limited availability of GEs is to broaden the scope of mutation tracking for MRD. Instead of focusing on a single or a few mutations at high depth, this approach involves screening for a much more extensive array of mutations—potentially in the hundreds or thousands—which may greatly increase the odds of detecting ctDNA, even with limited amounts of GEs (*15*). Some groups showed that if multiple markers are simultaneously evaluated from the same plasma sample, the overall lower limit of detection of ctDNA is inversely correlated with the number of markers.

For the widespread use of ctDNA to detect early-stage cancer, more generalizable, tissue-agnostic ctDNA assays are required. These assays should offer broader patient coverage, faster turnaround times, and potential cost-effectiveness because they are not limited by tissue type. In this context, the integration of biological cfDNA features extending beyond mutations, such as fragment profiles, nucleosome features, and methylation patterns, harbor complementary information and may thereby improve the sensitivity boundaries (*3*). Regardless of which strategies (or a combination thereof) will prevail, increasing the sensitivity of ctDNA analyses remains pivotal for improving the clinical utility of liquid biopsies in cancer diagnostics and monitoring. Looking ahead, integrating multianalyte data—including RNA; exosomes; and circulating tumor cells, proteins, and metabolites—and combining different molecular features—e.g., genomics and epigenetics—holds promise for further improving sensitivity and specificity in liquid biopsy.

In addition to the requirement of amplifying sensitivity, other challenges persist, including the need for standardization of liquid biopsy protocols, addressing interlaboratory variability, and navigating ethical and regulatory considerations (e.g., handling false negative and false positive results and cost coverage). Additionally, evaluation of the cost-effectiveness of high-sensitivity technologies warrants attention to enable broad accessibility in clinical settings. Ongoing research and collaboration among academia, industry, and regulatory bodies are essential in refining existing methods, overcoming these challenges, and unlocking the full potential of liquid biopsy to revolutionize cancer diagnostics and treatment. The journey to redefine liquid biopsy continues, and with each breakthrough, a future where early cancer detection is a reality comes closer. ■

Increasing liquid biopsy sensitivity in vivo

In vivo approaches to increase the amount of cell-free DNA (cfDNA) that can be extracted from the blood in a liquid biopsy can increase the sensitivity of detection. For example, cfDNA clearance can be delayed when mice are treated with nanoparticles to compete with native cfDNA (usually in the form of mononucleosomes) for uptake by phagocytes. DNA binding monoclonal antibodies can also protect cfDNA in mice from degradation by nucleases. Other strategies include increasing circulating tumor DNA (ctDNA) release from tumors through ultrasound or ionizing radiation.



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