Liquid biopsies and the promise of what might(o) be

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Current approaches to cancer diagnosis and management are predicated on several fundamental principles including early detection, accurate and precise diagnosis and staging, and the induction and long-term maintenance of complete remission (1). Public health measures over the past decades have contributed immensely to increased patient appreciation for the value of routine screening procedures such as mammography and colonoscopy and for the need to reduce behaviors such as smoking and excessive alcohol consumption that predispose to certain cancers (2). Improved imaging techniques have also facilitated the earlier detection of cancers and their recurrence. Finally, highly targeted, more specific, and less toxic therapies have contributed significantly, albeit unevenly, to increased cancer survival rates. Key to this success is the initial biopsy of a suspected malignant tumor and its evaluation in ways that allow for the most precise diagnosis, sub-type classification, and therapeutic stratification. The necessity for biopsy has only increased since becoming a routine part of cancer management and has been aided by virtue of the procedure being made safer and less invasive. Adding to its value is the realization that most cancer types are now appreciated to be comprised of multiple subtypes with different behaviors, therapeutic responses, and long-term prognoses thus underscoring the importance of accurate evaluation (3). To this end, biopsies are increasingly subject to an array of sophisticated special stains, immuno-phenotyping and molecular analyses to improve diagnosis and inform treatment decisions.

While the direct biopsy of tumors is the gold standard of cancer diagnosis, it remains costly, inconvenient, and time-consuming. It also nearly always requires the participation of surgeons, interventional radiologists and anesthesiologists and is subject to complications such as bleeding and infection. Non-diagnostic or insufficient tissue sampling may also be an issue, particularly when relying on fine needle aspiration, which can necessitate re-biopsy (4). For these reasons, and with the exception of the leukemias, where the tissue is readily accessible and remains so following the procedure, re-biopsy is used sparingly if at all to monitor a tumor's response to therapy or to confirm recurrence and is of no value for long-term monitoring after remission has been achieved and no detectable tumor remains. Moreover, repeat biopsies typically demand repeat imaging to again localize the tumor and guide the procedure.

Not surprisingly then, the development of so-called "liquid biopsies" has garnered considerable attention (4,5). Based on the analysis of free DNA released from dead tumor cells or extracted directly from small numbers of circulating tumor cells following their dissociation from the primary tumor, liquid biopsies theoretically reduce the direct tissue biopsy and its attendant complications and inconveniences to a procedure involving nothing more than obtaining a tube of peripheral blood. More generally, the same approach can be applied to other bodily fluids or even stool although the range of tumor types evaluable with these is much more restricted. Other obvious advantages include the ease of serial sampling, the dispensing with tumor imaging prior to biopsy and, in some cases, the ability to make clinically relevant predictions and therapeutic decisions based on the amounts of tumor DNA present. One such example is the PCR-based approach

to detect the *Bcr-Abl* gene rearrangement associated with chronic myelogenous leukemia (CML). Serial sampling of peripheral blood or bone marrow allows accurate and quantitative assessments of therapeutic response and early recurrence and informs decisions regarding altering or even discontinuing therapy in those individuals who have achieved deep and durable remissions (6).

The CML analogy also underscores what is currently perhaps the major limitation of liquid biopsies, namely the need to accurately distinguish tumor-derived DNA from non-tumor-derived DNA. Circulating DNA is found in all individuals as a result of normal cell death and ongoing tissue wear and tear. This makes the detection of tumor cell-derived DNA challenging against this high background. Liquid biopsy thus demands that the identity of at least one oncogenic driver mutation be known so that its presence in the blood can be documented, quantified, and followed over time. However, with few exceptions such as the example of CML noted above, the association of a particular mutation with a particular tumor type is not assured and no "universal" cancer marker exists. Moreover, even detecting an oncogenic mutation in DNA obtained from a liquid biopsy is no guarantee that a malignant neoplasm actually exists; it may simply be originating from a pre-neoplastic lesion destined not to evolve into an actual malignancy for years, if ever. At least currently, the best way to identify a true driver mutation originating from an actual malignant cell is through tissue biopsy. This undercuts one of the originally intended purposes of its liquid counterpart, namely its use as a screening tool. Thus, prior documentation from a solid biopsy of at least one bona fide oncogenic driver is necessary to allow for the choosing of a bespoke liquid biopsy probe (typically a set of PCR primers) that can unequivocally identify DNA of tumor origin.

It is here that querying mitochondrial DNA (mtDNA) offers potential advantages over nuclear DNA. Because malignancies almost always express high levels of reactive oxygen species that disproportionately originate in mitochondria, mtDNA often incurs the brunt of oxidative damage (7,8). This is reinforced by the lack of efficient mtDNA repair mechanisms. Together, these factors conspire to allow the mtDNA to accumulate mutations more quickly and to preserve them more efficiently than those in nuclear DNA (7,8). The greater abundance of mtDNA also makes its sampling and analysis far easier, particularly when "Deep Sequencing" approaches are employed (9,10). Perhaps most importantly, even though some mtDNA mutations are actual oncogenic drivers (11),

most are not, thus probably appearing only after full cellular transformation has been achieved and the mutagenic oxidative environment already established. Simply detecting mtDNA mutations reliably and at significantly higher levels over background might therefore suffice to signal with high probability that a tumor is lurking somewhere and that confirmatory studies, including actual biopsy, are needed.

In the current issue of BMC Medical Genomics, Campo *et al.* (12) have now made two major observations concerning the mtDNA from patients with hepatocellular carcinoma (HCC). First, they compared mtDNA sequences from primary tumors, matched neighboring non-malignant liver tissue, and peripheral blood from the 85 patients from whom all three sets of sequences were available through The Cancer Genome Atlas. They found mtDNA sequence variability or "entropy" to be significantly higher in livers than in tumors or peripheral blood samples. They also found that tumor and peripheral blood mtDNA sequences differed by an average of 0.92 mutations across the entire mitochondrial genome. Second, the sequence differences between tumor and blood mtDNAs were usually, but not invariably, non-recurrent and had a greater likelihood of clustering within the non-coding ca. 400 bp hypervariable segment of the D-loop region (HVS1) that is highly susceptible to mutation even in normal tissue (13). 169 differences were found to distinguish HCC mtDNA from peripheral blood DNA.

Given that cancers in general and epithelial cancers like HCC in particular sustain hundreds-thousands of mutations during the course of their evolution (14), how does one explain the finding that both the non-malignant liver and peripheral blood mtDNA from these HCC patients in fact displayed less mtDNA sequence heterogeneity? Mitochondria are present at hundreds-thousands of copies per cell and often show sequence variation (heteroplasmy), the degree of which can differ even among normal tissues in the same individual (15,16). These changes can be subject to both positive and negative selection such that under certain conditions, individual mutations may achieve dominance, thus reducing the overall heterogeneity or "entropy" of the mtDNA pool (13,17,18). The lower entropy of mtDNA in HCCs seen by Campo *et al.* (12) is consistent with the long-held notion that cancer cell mtDNA is subject to higher mutational stress than it is in normal tissues but that some mutations may undergo positive selection, presumably because they confer a survival advantage within the continuously evolving tumor landscape (11,19). As these mutations come to dominate the mtDNA via natural selection, overall entropy is reduced. This mtDNA then

escapes the tumor, mixes with and dilutes the mtDNA originating from more heteroplasmic normal tissues, and reduces its entropy as well. The tumors' contribution to this is seen in the differences that distinguish the mtDNA sequences of peripheral blood and liver.

The study by Campo et al. (12) shows that it is possible to distinguish mtDNA derived from peripheral blood of patients with HCC from that of a healthy population by measuring the degree of nucleotide heterogeneity across the entire 16,569 bp mitochondrial genome without relying on previously identified recurrent mutations. They accomplish this through a machine learning pipeline where Iterative Relief was used to select relevant features from mtDNA entropy profiles, which were then used to train a Random Forest classifier. They achieved nearly 100% accuracy in distinguishing individuals with HCC from those without based solely on the mtDNA profiles of blood. A somewhat less robust but still impressive 92% accuracy was achieved with a cross-validation cohort.

In addition to the mutationally-susceptible D-loop/HVS1 region, other "hot spots" in mtDNA-encoded genes that affect mitochondrial function in several cancer types have been reported by others (13,19). These include synonymous and non-synonymous point mutations and indels in the genes encoding the ND3, ND4 and ND5 subunits of Complex I (Complex I (NADH dehydrogenase), the COX1-3 subunits of Complex IV (cytochrome c oxidase) and the ATP6 subunit of Complex V (ATP synthase) (13). Because obtaining robust coverage of the entire mitochondrial genome can present difficulties even when Ultra Deep shotgun-based approaches are employed, an important observation of Campo et al. (12) was that 83% accuracy in distinguishing mtDNA from HCC and normal could still be achieved when mutations only within HVS1 were considered. Improved accuracy might be achievable by focusing on these additional hot spots

The Campo et al. (12) report raises a number of interesting questions. Among the most obvious is whether their work can be extended to tumors other than HCC, particularly those where evidence already suggests that this may be possible (13,17,19). Another is whether the most mutagenically susceptible mtDNA regions seen in HCC are also targeted in other cancers and whether the mutations remain stable over time in any particular individual. If they are in fact shared with other cancers then the two most likely explanations for why are that either these regions of mtDNA are selectively susceptible to oxidative attack or that the mutations confer an as yet unappreciated benefit

to the malignant cell. The reduction in HCC mtDNA entropy observed by Campo et al. (12) certainly suggests the latter possibility, but which mutations are responsible for this selection and why it occurs merit further scrutiny. Moreover, the two possibilities are not mutually exclusive. A third question is: if an initial solid tissue biopsy of the primary tumor establishes the presence of both nuclear DNA mutations and a malignancy-associated mtDNA entropy profile, then which of these should be used to monitor the patient's subsequent clinical course if employing liquid biopsy to do so? It seems logical to assume that the greater abundance of circulating mtDNA makes it the preferred candidate but this will still require head to head intra-patient testing. Finally, the idea that the malignancy-associated mtDNA entropy profile shift is a late event in the evolution of a transformed cell needs to be verified in the clinical setting if it is intended to serve as a robust and unequivocal marker of transformation. A reasonable way to test this notion might be with colorectal neoplasms where the molecular events underlying the stepwise progression from benign polyps to frankly malignant disease have been well-established (20) and where the mtDNA entropy profiles of tissues sample at each stage could be easily evaluated. If malignancy-associated mtDNA entropy profile change is indeed a late occurrence, then its mere presence might be sufficient to make a tentative diagnosis of cancer and provide yet another potential advantage over that offered by the analysis of nuclear DNA. In this scenario, prior solid biopsy would be unnecessary and mtDNA mutational entropy analysis could be employed as a useful screening tool.

These considerations return us to the original point, namely, that the best possible liquid biopsy would be one that identified indisputable cancer-associated molecular abnormalities, either nuclear or mitochondrial, from a peripheral blood sample. This could be applied as a routine screen, with more time-consuming, expensive and invasive tests being reserved to confirm the results of blood testing. It is here that mtDNA entropy profiling might be most advantageous. The inability of Campo et al. (12) to distinguish tumor stages using mtDNA profiling is actually encouraging as it implies that the lower limit of detectability has not been reached. Just how small a tumor must be before it eludes detection with mtDNA sampling from a liquid biopsy and how this compares to the sensitivity of standard imaging techniques are also questions that merit further investigation.

Given the limits of current technologies, it seems that the more likely routine application of liquid biopsies, at

least for the foreseeable future, will be for serial monitoring after a tumor is first diagnosed by classical biopsy and its driver mutations clearly delineated. Using this molecular analysis as a guide, the liquid biopsy evaluation will be designed to detect the most important molecular lesion(s) that unequivocally reflects the makeup of the tumor, thus providing one or more sensitive and specific markers. Whether these lesions are nuclear, mitochondrial or some combination of the two will only be determined by future work. In the meantime, the development of new approaches such as the one described by Campo *et al.* (12) will be watched with great interest.

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Footnote

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