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Circulating Tumor DNA as a Cancer Biomarker: Fact or Fiction?

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The notion of utilizing cell-free DNA (cfDNA)[8] in the circulation as a surrogate biomarker is not a novel concept. Mandel and Metais identified the presence of cfDNA in the blood of healthy individuals almost 60 years ago. Decades later, multiple groups were able to extend

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[9]Human genes: *KRAS*, KRAS proto-oncogene, GTPase; *BRAF*, B-Raf proto-oncogene, serine/threonine kinase; *EGFR*, epidermal growth factor receptor.

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the work of Mandel and Metais into the identification of tumor-derived cfDNA—also known as circulating tumor DNA (ctDNA)—in the blood of cancer patients. These findings suggested that a “liquid biopsy” may be a feasible clinical tool because tumors seem to release fragments of DNA into the circulatory system that are both detectable and specific to the tumor.

In the past decade, we have witnessed a surge in both new technologies and improvements on existing technologies for sequencing DNA that have made this once-laborious process cheaper and faster. In 2009, the cost of sequencing per genome was $100 000, whereas in 2014, this cost dropped to $5000 (taking into account labor, administration, management, utilities, reagents, and consumables). As a result, the use of ctDNA as a liquid biopsy has become ever more feasible.

Clinically speaking, a ctDNA-based liquid biopsy would be the optimal mode of cancer management owing to various advantages including: (*a*) Retrieval of ctDNA would be minimally invasive especially compared to a tissue biopsy; (*b*) ctDNA could provide a full representation of the tumor (as well as any clonal metastases); and (*c*) ctDNA would provide a personalized snapshot of the patient’s disease. Although the clinical use of ctDNA as a surrogate biomarker is still hampered by biological and technological hurdles, the implications of a liquid biopsy could be enormous as there would be numerous potential applications including (but not limited to): early detection, monitoring of minimal residual disease (MRD), assessment of treatment response, and triaging based on risk of recurrence.

In this Q&A article, 4 experts offer their insight into the current state of ctDNA as a clinical tool for cancer management. Specifically, they will discuss the biological and analytical challenges this technology still faces, as well as the potential benefits of this rapidly growing area of translational research.

[8]Nonstandard abbreviations: cfDNA, cell-free DNA; ctDNA, circulating tumor DNA; MRD, minimal residual disease; NGS, next generation sequencing; TAM-Seq, tagged-amplicon deep sequencing; CAPP-Seq, CAncer Personalized Profiling by deep Sequencing; FDA, US Food and Drug Administration; NSCLC, non–small cell lung cancer.

In your opinion, what is the principal mechanism behind the release of ctDNA into the circulation?

Dave S.B. Hoon: The principal mechanism can be explained by taking into consideration the multiple events associated with tumor cells. ctDNA in plasma can originate from primary, metastatic, or circulating tumor cells. It can be released from tumors by apoptosis, necrosis (programed or not), tumor cell destruction, and/or cell secretion. Likely, multiple factors affect the total ctDNA composition in the blood, which is dependent on the tumor status, burden, and histopathology.

Kenneth Kinzler: One possibility is that the DNA is released by the death of circulating tumor cells. The second possibility is that the DNA is released by the death of tumor cells in the tumor bed. There are several pieces of evidence that support the latter explanation as the primary source of ctDNA. First, the majority of cases with detectable ctDNA do not have detectable circulating tumor cells. Second, in those cases where both can be detected, the level of ctDNA is 1–2 orders of magnitude greater than that present in circulating tumor cells. Third, advanced cases of cancer are often associated with increased concentrations of cfDNA from normal cells, presumably due to death and destruction of normal cells in the tumor beds.

Klaus Pantel: In principle, ctDNA can be released from primary tumors, circulating tumor cells, micrometastasis, or overt metastases in cancer patients. The majority of ctDNA is most likely derived from apoptotic and necrotic tumor cells that release their fragmented DNA into the circulation. DNA is also released by nonmalignant host cells and this normal DNA dilutes the ctDNA in cancer patients, particularly in situations where tissue-damaging therapies such as chemotherapy or radiotherapy are administered. The fragment length might provide some information on the origin of the cfDNA. However, this issue is still under debate because different groups have reported different cutoff values in cancer patients. Part of these discrepancies might be due to the different technical approaches used to determine ctDNA and tumor types. Nevertheless, we still need to know more about the biology behind the release of ctDNA into the circulation.

Catherine Alix-Panabières: When cancer cells die by necrosis or apoptosis, some of the released DNA ends up passively in the bloodstream, thus the release of cfDNA into the blood by dying cells is not restricted to cancer patients; in fact, cfDNA can be detected in the blood of healthy individuals, with even higher amounts in patients with benign diseases or inflammatory diseases or when subjects are aging. *"Necrosis"* is caused by factors external to the tumor cells or cancer tissues, which result in the unregulated digestion and release of cell components. "*Apoptosis*" is defined as a controlled type of cell death that can be induced by a variety of physiologic and pharmacologic agents, followed by DNA fragmentation and cell lysis. In both cases, dying tumor cells release small pieces of their fragmented DNA in the circulation. As fragmentation of ctDNA seems to be higher following apoptosis than following necrosis, it should be possible to determine how ctDNA was released in cancer patients. Specifically, ctDNA fragmented to 160–180 bp in length corresponds to nucleosome-protected DNA observed in apoptotic cells. In addition to the release of DNA by dying tumor cells, recent studies indicate that a very small amount of DNA might be released in exosomes actively by living cells but this is still controversial. How ctDNA is released into the circulation is a key question because to adopt the ctDNA as a clinical biomarker in cancer patients, it will be crucial to elucidate in the near future how the ctDNA release is related to tumor biology by understanding: (1) from which cells it is derived, (2) which specific clones contribute to total ctDNA level that reflects their clonal burden, and (3) how the ctDNA level changes over time depending on the applied cancer therapies. The source of ctDNA is of utmost importance, as it must reflect the genetic information of the clinically relevant tumor cells. As it is mainly dying tumor cells that release DNA, depending on when ctDNA is detected in blood of cancer patients, the information we can obtain from its detection may not reflect mutations of the resistant

clones of tumor cells but those of sensitive subpopulations of tumor cells, mostly after the initiation of treatment—e.g., chemotherapy, targeted therapy.

How is ctDNA cleared from the circulation and how does that affect its stability?

Dave S.B. Hoon: Clearance of ctDNA follows similar physiological mechanisms to that of normal DNA that is released into the circulation and cleared routinely by various organs, such as tumors draining into lymph nodes, kidney, and liver. In the tumor microenvironment, lymphatic drainage is likely to clear the majority of DNA fragments that are released. The ctDNA is generally unstable except for some forms that appear to have a longer half-life for a reason that we do not fully understand. The form (i.e., exosome), size, bound molecule substance (i.e., lipid, protein), and mechanism of release collectively play a role in ctDNA clearance.

Kenneth Kinzler: The rate of clearance of cfDNA from blood has been examined using exogenous DNA, fetal DNA, and tumor DNA in human and animal models. These studies all indicate that DNA is rapidly cleared from the blood. By evaluation of a single subject whose plasma was sampled at multiple times following complete tumor resection, we estimated the half-life of ctDNA after surgery to be 114 min. Dennis Lo and colleagues followed the clearance of circulating fetal DNA in 8 women after delivery and found the mean half-life for circulating fetal DNA to be 16.3 min (range 4–30 min). The mechanism of clearance for ctDNA has not been well studied but is likely to be similar to that for cfDNA from other sources and may vary with the physiological state of the patient. A combination of nuclease degradation, renal clearance, and uptake by the liver and spleen are likely to play a role.

Klaus Pantel: The clearance of ctDNA is not fully understood. Previous reports indicate that ctDNA has a half-life of 16 min. However, a recent study from the same group used next generation sequencing (NGS) to study the kinetics of ctDNA, which revealed a biphasic clearance with half-lives of about 1 h for the rapid phase and 13 h for the second phase. It is assumed that the majority of ctDNA is cleared through the kidneys, which has raised recent interest to detect ctDNA in the urine, not only for cancers of the urogenital tract. It can be envisaged that the clearance rate of ctDNA is affected in patients with renal dysfunction. This aspect needs further investigation because it might be an important confounding factor modulating the amount of ctDNA in the circulation. In addition to renal clearance, DNA might be cleared by other mechanisms from the circulation. For example, DNA is sticky and may also adhere to host cells such as endothelial cells lining the blood vessels; it is not impossible that this DNA is released again into the blood. Several reports indicate that ctDNA can even be taken up by host cells and this uptake affects the biology of these cells. Thus, the mechanisms of ctDNA clearance and their effects on ctDNA stability still need further investigation.

Catherine Alix-Panabières: The clearance of ctDNA, “how long it takes to be cleared and how it is done,” is currently not well understood and is still under investigation. What is known is that ctDNA has indeed a limited stability in the blood of cancer patients, as

DNases also present in the blood digest it quickly. Thus, ctDNA has a short half-life of a few hours in the bloodstream, suggesting opportunities for early readouts. This genetic and fragmented material may undergo rapid changes in cancer patients and the time points of blood sampling are crucial and not easy to determine upfront when designing a clinical trial. Moreover, the release of ctDNA by dying tumor cells is most likely quite variable between cancer patients depending on the tumor type, tumor stage, response of cancer patients to therapy, tumor burden, and cell replication.

ctDNA can be investigated in various regards such as point mutations, aneuploidy, rearrangements, and methylation. What are current sequencing technologies capable of investigating?

Dave S.B. Hoon: There are multiple techniques available to investigate individual forms of genomic and epigenomic ctDNA aberrations. Each has its merits and some have been verified more efficiently than others in clinical trials. Most important is the specificity and sensitivity of the individual assay, which is highly related to the ctDNA clinical utility. Digital small molecule sequencing currently allows a very sensitive and specific approach for point mutation and amplification detection of ctDNA.

Kenneth Kinzler: Somatic point mutations, rearrangements, aneuploidy, and methylation have all been used to identify ctDNA using NGS. These approaches for distinguishing normal from tumor DNA vary in terms of their sensitivity, specificity, and practicality when applied to ctDNA. While the specific clinical application may determine which approach is optimal, we have found that using point mutations has the best combination of attributes for most of the clinical applications that we have studied.

Klaus Pantel: Highly sensitive and specific methods have been developed to detect ctDNA, such as BEAMing, Safe-SeqS, TamSeq, and digital PCR to detect single nucleotide mutations in ctDNA or whole-genome sequencing to establish copy number changes. In principle, the technologies can be divided into targeted approaches aimed to detect mutations in a set of predefined genes [e.g., KRAS proto-oncogene, GTPase (*KRAS*) in the context of EGFR (epidermal growth factor receptor) blockade by antibodies] or untargeted approaches (e.g., array-CGH (comparative genomic hybridization), whole-genome sequencing, or exome sequencing) aimed to screen the genome and discover new genomic aberrations, e.g., those that confer resistance to a specific targeted therapy (Murtarza et al., Nature 2013;497:108–112). The strengths and limitations of these technologies have been recently discussed in excellent reviews. In general, targeted approaches have a higher analytical sensitivity than untargeted approaches, despite strong efforts to improve detection limits. A European IMI (Innovative Medicines Initiative) consortium of more than 30 institutions from academia and industry [called CANCER-ID www.cancer-id.eu; scientific coordinator, Klaus Pantel; EFPIA (European Federation of Pharmaceutical Industries and Associations) coordinator, Thomas Schlange] is focusing now on liquid biopsies; the main goal is to validate the different cfDNA technologies side by side, first in ring experiments between different institutions and subsequently in multicenter clinical trials.

Catherine Alix-Panabières: ctDNA detection among total cfDNA in plasma requires the use of molecular methods and is based on the genetic or epigenetic differences between normal and tumor-derived DNA. The targets for ctDNA detection are (1) mutations with known or predicted functional relevance (oncogene-activating mutations and tumor suppressor–inactivating mutations), (2) mutations of no or unknown functional relevance (somatic chromosomal rearrangements, noncoding DNA mutations and DNA variants), and (3) epigenetic modifications (methylation status of key sequences and histone modifications). Some of the current technologies capable of investigating ctDNA include: (*i*) technology combining pyrophosphorolysis-activated polymerization and allele-specific amplification during PCR; (*ii*) a set of digital genomic methods that improve identification of genetic alterations in ctDNA; (*iii*) a PCR-based method that allows single-molecule PCR reactions to be performed on magnetic beads in water-in-oil emulsions, the BEAMing approach; (*iv*) Other digital PCR technologies that include droplet digital PCR and microfluidic systems; (*v*) PCR amplification followed by NGS. Plasma cfDNA analyzed by NGS can determine (*a*) the presence of a given mutation and estimate its allelic frequency within a sample and (*b*) the whole-genome characterization of the entire repertoire of mutations in a cancer. Finally, to get an idea about the high sensitivity of improved technologies for ctDNA detection, it has been reported that digital PCR and BEAMing can detect somatic point mutations with sensitivities ranging from 1% to 0.001%. In addition to investigating point mutations and copy number changes, the evaluation of the methylation status of tumor suppressor and metastasis suppressor genes in ctDNA can be evaluated by real-time methylation-specific PCR assays. All these technologies have been reported with various in vitro sensitivities using cancer cell lines; however, clinical results and their respective evaluation are still in progress in clinical trials.

Recently, we have seen an emergence of new sequencing technologies such as tagged-amplicon deep sequencing (TAM-Seq) and CAncer Personalized Profiling by deep Sequencing (CAPP-Seq). What are the advantages of these newer technologies compared to the “older” ones?

Dave S.B. Hoon: The newer techniques offer more direct targeting but are not necessarily better than newer approaches of NGS. NGS in digital small molecule analysis is a more highly sensitive and specific approach than conventional NGS. Traditional parallel NGS is costly and limited. Techniques of detection are rapidly evolving and improving. Newer approaches can only be deemed efficient when clinical utility is robustly demonstrated.

Kenneth Kinzler: Many methods can detect ctDNA in advanced cases where the fraction of ctDNA can represent more than 5% of the total DNA. However, maximizing the fraction of cases with detectable ctDNA, especially in the case of early disease, requires methods that can detect a few mutant molecules when they represent <0.1% of the total DNA. Fortunately, digital approaches (e.g., digital PCR, BEAMing, and SafeSeqS) allow detection and accurate quantification even at these low levels.

Klaus Pantel: The main advantage of these newer technologies is their higher sensitivity. “Older” technologies have been used in proof-of-principle studies analyzing the blood of

patients with advanced cancer where the ctDNA amount is very high and can exceed 50% of the total amount of cfDNA. However, these technologies are not able to detect smaller amounts of ctDNA in the “sea” of normal circulating cfDNA, which is a clear limitation in particular if we extend our analyses to patients with MRD or early detection of cancer. For these situations, an increasing number of much more sensitive technologies have been developed that can detect ctDNA at remarkably low concentrations such as 0.00025% for the CAPP-Seq technology. However, now the total amount of cfDNA and the available numbers of genome equivalents might become an important limitation. Thus, to reach the remarkable sensitivities of these new assays, the size of the plasma sample needs to be increased considerably to obtain a sufficient amount of ctDNA for analysis. In this regard, ctDNA analyses in early stage cancer patients face the same problems as circulating tumor cell analyses. Thus, the promise that ctDNA analysis will detect cancer from a drop of blood is misleading.

Catherine Alix-Panabières: Standard PCR–based assays have a relatively limited sensitivity and cannot detect mutations that represent <5% to 10% of the total pool of alleles. Indeed, they produce many artifacts, particularly if the ctDNA amounts are rather low, whereas new technologies are able to detect very low amounts of ctDNA. Identification of somatic genetic and epigenetic aberrations now has been facilitated by the advent of highly specific and sensitive techniques (e.g., range from 1% to 0.001% for digital PCR and BEAMing). This involves getting different clinical information: with newer, more sensitive technologies, we can imagine the utility in the diagnosis of cancer, the detection of early-stage cancer, and the detection of MRD, whereas with older technologies, only advanced and metastatic-stage diseases could be detected and evaluated.

What is the greatest technical challenge that we are still facing with regards to genotyping ctDNA?

Dave S.B. Hoon: The major technical problem or Achilles’ heel is the isolation of ctDNA from small amounts of serum/plasma. This barrier of isolation will influence downstream analysis. Do we accurately get all the ctDNA? Assay sensitivity remains a major problem. The most important technical challenge is to identify what level of ctDNA is of clinical utility. This will likely vary amongst cancer types, depending on the clinical status of the patient and what question is being addressed by the specific assay biomarker ctDNA.

Kenneth Kinzler: Advancing technology has made detection of ctDNA feasible and future advances are likely to make such assays even more robust and cost-effective. However, these assays, like current ones, are likely to be limited by practical and biological rather than technical issues. On the practical side, we are limited by the number of cell free molecules present in a plasma sample. For example, 1 mL of plasma typically contains about 3000 copies of any given gene, limiting our ability to detect ctDNA to 1 in 15 000 copies given a 5-mL plasma sample. On the biological side, only a small fraction of benign tumors and some tumor types (e.g., glioblastomas) release detectable concentrations of ctDNA, even when tested with very sensitive assays under near optimal conditions. Some of the limitations of using ctDNA as a biomarker may be circumvented by larger sample volumes

and alternate protocols or sites for blood collection. Finally, tumor DNA has been shown to be a promising cancer biomarker in other readily available clinical samples such as stool, urine, sputum, saliva, and Pap smear samples.

Klaus Pantel: The greatest technical challenge is the identification of very low amounts of ctDNA in blood samples containing variable amounts of cfDNA and the choice of the right panel of cancer-specific genomic aberrations. If the genomic aberrations are defined by the choice of available targeted therapy, this task seems to be doable with the existing technologies. However, if ctDNA analysis is used for screening of MRD in cancer patients or even for early detection of primary disease in "healthy individuals," the genomic aberrations in an individual patient are unknown. In patients with MRD, the primary tumor (or at least a biopsy) might be available for sequencing. Nevertheless, the quality of these formalin-fixed paraffin-embedded samples is highly variable. For early detection, there is no tumor material available and the panel of possible genomic aberrations is substantial for most solid tumors. Moreover, it is well known that cancer-associated mutations occur with increasing age even in individuals that never develop cancer during their lifetime. For example, a recent study has shown that up to 15% of healthy individuals over the age of 65 years harbor leukemia somatic mutations but leukemia was only diagnosed in a minority of these patients. Thus, the detection of cancer-associated mutations on cfDNA might not indicate that the individual tested already has cancer or will develop cancer in her/his lifetime but it might precipitate extensive diagnostic procedures such as CT and MRI scans to search for a putative unknown tumor lesion.

Catherine Alix-Panabières: First, preanalytic steps need to be standardized before detecting ctDNA in an optimized manner, e.g., blood volume, plasma or serum, blood centrifugation, specific reagent designed to isolate high quality of DNA, DNA preparation through extraction strategy, and storage conditions to define the most reproducible and robust methodology; then a complete evaluation of the analytical performance (e.g., robustness, reproducibility, sensitivity, specificity, internal and external quality controls) as well as the analysis time from the blood sample collection to the result back to the cancer patient is needed. All these technical points need to be considered but, more importantly, we need to evaluate the clinical relevance of ctDNA at different time points to answer different key questions regarding applications such as stratification of patients for therapy, evaluation of treatment efficacy, early diagnosis of metastatic relapse, or defining treatment resistance. Moreover, for early diagnosis of cancer, we have to keep in mind that (1) specific mutations must be known to detect ctDNA [e.g., *KRAS* or B-Raf proto-oncogene, serine/threonine kinase (*BRAF*) mutation in colon cancer] and (2) many people have benign tumors (e.g., skin tumors) which carry mutations that can overlap with cancer-associated mutations and may cause false-positive findings if ctDNA is used for cancer screening.

How long do you think it will be before we see the introduction of ctDNA into clinical practice?

Dave S.B. Hoon: We are currently entering a time whereby ctDNA assays are in CLIA-accredited laboratories. The ctDNA assays are being reimbursed by healthcare payers and

also by individual patients. Hopefully, in a few years we may see US Food and Drug Administration (FDA) approval of particular ctDNA assays for specific cancers.

Kenneth Kinzler: While it is speculative to predict when any new technology will be introduced into clinical practice, there is a growing number of studies that indicate that ctDNA can outperform currently used clinical cancer biomarkers in specific clinical situations. Accordingly, I would hope and expect that ctDNA measurements will be used to aid clinical decisions in an increasing range of situations over the next 5–10 years.

Klaus Pantel: The blood-based stratification of targeted therapies is probably the ticket for the introduction of ctDNA analysis into clinical practice. Recently, the first ctDNA test for epidermal growth factor receptor (*EGFR*) mutations in non–small cell lung cancer (NSCLC) has been approved by the FDA for the situation when no tumor tissue can be biopsied. More tests for mutations in genes encoding therapeutic targets and/or the corresponding resistance genes will follow in the near future. NSCLC is an interesting tumor entity for this application because various mutations directing specific targeted therapies in small cohorts of responsive patients have been recently identified and biopsies are not easily obtained in a considerable number of patients. However, it needs to be emphasized that neither DNA analyses of ctDNA nor tumor tissue will guarantee that the patient will have a durable and life-prolonging response to the respective therapeutic agents. The clinical utility of ctDNA analysis (like any other diagnostic test) needs to be proven in randomized clinical intervention studies in which therapy decisions are based on ctDNA analysis, i.e., can the ctDNA analysis direct the treatment in an individual cancer patient and does this change lead to an improved survival? It may take many years before these studies are completed and accepted by the medical community. The recent results of the multicenter, open-label, proof-of-concept, randomized, controlled phase 2 SHIVA trial were rather disappointing; the use of molecularly targeted agents outside their indications did not improve progression-free survival compared with treatment at physician’s choice in heavily pretreated patients with cancer. Monitoring ctDNA for mutations conferring resistance to targeted therapies might be also hampered by the fact that we still know little about the dynamic biology of ctDNA release. ctDNA represents mainly the genome of dying tumor cells but viable tumor cells drive cancer progression and cause therapy resistance. Thus, the selection of the appropriate time points for ctDNA screening will be crucial to detect those ctDNA species that are derived from the resistant tumor cell clones. In this context, the parallel determination of viable circulating tumor cells as early indicators of failure to therapy superior to standard tumor markers used in the clinic might be helpful.

Catherine Alix-Panabières: The development of molecular tests for treatment decision-making, coupled with technological developments, has become fashionable these last years and we still hope that real-time liquid biopsy will lead to noninvasive and sensitive ways of detecting and monitoring cancer in patients. However, the introduction of ctDNA into clinical practice may take several years as we need to define what will be the best technology for ctDNA preparation (preanalytic steps) and detection, what will be the end points, and whether ctDNA analysis will be more informative and independent of current standards such as tissue biopsies. As indicated earlier, we need to evaluate the clinical relevance of ctDNA and, primarily, its clinical utility, the key question for personalized

medicine. In addition, for primary screening, very large cohorts of cancer patients and matched control individuals need to be analyzed prospectively, including patients with benign diseases as a control group. Considering other screening approaches [e.g., PSA (prostate-specific antigen) for prostate cancer or mammography for breast cancer], this effort requires thousands of patients and controls and observation periods of 10 years or more.

Note added by Moderators: After this Q&A was completed, the sequencing giant Illumina announced the formation of a new company, Grail, with a goal to develop blood tests that would cost less than $1000 and have the ability to detect many types of cancer at asymptomatic stages. The tests will be based on “liquid biopsies” (blood tests) and sequencing of isolated ctDNA. The company already has raised $100 million and it includes high-profile investors such as Bill Gates (Microsoft) and Jeff Bezos (Amazon). Illumina’s CEO projects that these tests will reach consumers by 2019 and they will be available in doctor’s offices.