Minireview

Liquid biopsy and its role in an advanced clinical trial for lung cancer

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Impact statement

Liquid biopsy technology is providing a new source for cancer biomarkers, and adds new dimensions in advanced clinical trials. Utilizing a non-invasive routine blood draw, the liquid biopsy provides abilities to address perplexing issues of tumor tissue heterogeneity by identifying mutations in both primary and metastatic lesions. Regarding the assessment of response to cancer therapy, the liquid biopsy is not ready to replace medical imaging, but adds critical new information; for instance, through a temporal assessment of quantitative circulating tumor DNA (ctDNA) assay results, and importantly, the ability to monitor for signs of resistance, via emerging clones. Adjuvant therapy may soon be considered based on a quantitative cfDNA assay. As sensitivity and specificity of the technology continue to progress, cancer screening and prevention will improve and save countless lives by finding the cancer early, so that a routine surgery may be all that is required for a definitive cure.

Abstract

Liquid biopsy methodologies, for the purpose of plasma genotyping of cell-free DNA (cfDNA) of solid tumors, are a new class of novel molecular assays. Such assays are rapidly entering the clinical sphere of research-based monitoring in translational oncology, especially for thoracic malignancies. Potential applications for these blood-based cfDNA assays include: (i) initial diagnosis, (ii) response to therapy and follow-up, (iii) tumor evolution, and (iv) minimal residual disease evaluation. Precision medicine will benefit from cutting-edge molecular diagnostics, especially regarding treatment decisions in the adjuvant setting, where avoiding over-treatment and unnecessary toxicity are paramount. The use of innovative genetic analysis techniques on individual patient tumor samples is being pursued in several advanced clinical trials. Rather than using a categorical treatment plan, the next critical step of therapeutic decision making is providing the "right" cancer therapy for an individual patient, including correct dose and timeframe based on the molecular analysis of the tumor in question. Per the 21st Century Cures Act, innovative clinical trials are integral for biomarker and drug development. This will include advanced clinical trials utilizing: (i) innovative assays, (ii) molecular profiling with cutting-edge bioinformatics, and (iii) clinically relevant animal or tissue models. In this paper, a mini-review addresses state-of-the-art liquid biopsy approaches. Additionally, an on-going advanced clinical trial for lung cancer with novelty through synergizing liquid biopsies, co-clinical trials, and advanced bioinformatics is also presented.

Keywords: Bioinformatics, biomarkers, cancer, genomics, liquid biopsy, precision medicine

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Introduction and background

Cancer is a molecular disorder, characterized by the corruption of genetic information at the cellular level, with consequences resulting in changes to key proteins and molecular circuits. Loss of regulation and the onset of uncontrolled growth are the overarching hallmarks of the genomic and proteomic derangements.[1] Genomics, which includes the study of the structure, function, evolution, and mapping of genomes, continues to rapidly progress from advances in basic science and technology. Genomics is now entering the clinic to enable individualized cancer care.

Advanced genetic analysis of a patient's tumor is utilized in precision medicine to obtain an individualize therapy plan. This is contrasted historically with cancer treatment regimens assigned in a categorical manner, largely based on organ of origin. The NCI-MATCH Trial (Molecular Analysis for Therapy Choice)[2] is an example of an advanced precision medicine clinical trial, where genomic sequencing of a patient's tumor is performed. The cancer treatment regimen is derived based on the genomic findings, not the organ in which the cancer originated. The major goal for NCI-MATCH is the assessment of a complete or partial response. Minor goals include evaluation of progression-free survival for at least a six-month time point. Additional goals include assessment of time to progression and treatment side effects.[3]

Further advances in precision medicine depend on the development of novel diagnostic assays, which are needed to provide feedback (preferably quantitative) to oncologists regarding efficacy of therapy. This model exists in the field of infectious disease. For instance, with a systemic bacterial infection following antibiotic sensitivity testing, the white blood cell count (WBC) and in some cases C-reactive protein (CRP) are used as quantitative indicators for response to therapy. Similar strategies are employed in HIV medicine. HIV-1 genotyping may be performed to detect mutations in the reverse transcriptase (RT) and protease (Pr) genes: (i) before therapy is initiated, (ii) clinical indications of antiretroviral failure, (iii) suboptimal viral suppression, (iv) pregnant women with HIV-1 infection.[4] The viral load and CD4 counts are used as empirical indicators of response to complex combination therapy in HIV. The dynamics of these lab values can be followed, which allows for adjustments and optimization of therapy for patients on an individual basis; these approaches have been instrumental for definitive clinical guidance and improving outcomes.

Besides blood, other body fluids such as cerebrospinal fluid, pleural effusions, and saliva are known to contain tumor DNA shed products.[5] In this paper, a liquid biopsy is defined as the analysis of cfDNA and circulating tumor cells (CTCs) via a non-invasive routine blood draw or urine sample. For many years, the concept of the liquid biopsy has been considered the "holy grail" of medical oncology. This is due to the immediate clinical applications of liquid biopsies which include: (i) early detection of cancer, (ii) estimating overall tumor heterogeneity (primary lesion and metastatic foci), (iii) tracking temporal-based tumor dynamics, (iv) effectively mapping the deranged tumor molecular circuitry for targeted therapies, (v) assessing response to therapy early in treatment, (vi) more effective minimal residual disease monitoring strategies, and (vii) quantifying resistance to therapy and disease evolution in real time.[6] Another important feature of liquid biopsies is tremendously reducing risks associated with most standard cancer-based biopsies. For instance, an adverse event rate reported in a review of investigative biopsies at MD Anderson revealed a rate that was more than 17% for thoracic biopsies.[7] Finally, exosomes and circulating RNA, although scientifically important and exciting, are not quite ready for the clinical arena and will not be discussed in this paper.

Temporal based molecular diagnostics for following the clinical progress of a human cancer by a routine blood draw has not yet been established. The true realization of precision medicine requires advanced non-invasive assays, which will provide quantitative feedback that can monitor or predict a patient's response. This will allow for rational therapeutic assessments with appropriate adjustments as is routinely done in the field of infectious disease. These innovative genetic analysis techniques will advance understanding enabling a more precise personalized treatment. Providing the "right" cancer therapy, including correct dose and timeframe based on the molecular analysis of the tumor in question, is the next critical step for effective individualized therapy.

The next technologic leap will be the frequent and serial application of genomic technologies to non-invasive biological fluid analysis (e.g. plasma, urine, saliva) at the bedside or home. Soon it will be possible to analyze the state of DNA shed products in the context of molecular pathways and networks, before, during, and after therapeutic actions. Such advances, and advances in proteomics and potentially metabolomics of biofluids, are on the horizon and will represent the initiation of true precision medicine. Further understanding the role that molecular pathways and networks play in cancer create new clinical opportunities, since detecting and disabling aberrant pathways and networks should provide more targeted and durable therapeutic responses.[8]

Improved methods for frequent assessment of therapeutic efficacy and resistance are needed. Currently, cancer patients are assessed using tissue biopsies and radiology scans, neither of which are without risk,[9] especially thoracic oncology biopsies. In contrast, techniques utilizing routine blood draws are safer having virtually no risk. The challenge with liquid biopsy is not patient safety but rather the complex analytical methodologies and analysis. In order to better assess therapeutic efficacy, minimize toxicity, and effectively profile the clonal and sub-clonal dynamics of the cancer process, liquid biopsy approaches as they stand today need further refinement. These efforts are being pursued by the FDA Sequencing Quality Control Phase II (SEQC2) project[10] the and the Blood Profiling Atlas in Cancer (BloodPAC)[11] consortium.

The precision medicine field is moving beyond solid tumor genomics to the analysis of body fluids obtained in a non-invasive fashion, and analyzed for DNA shed products, and aberrant circulating cells. DNA is an information archive. Shed products from cellular turnover, via necrosis, secretion, or apoptotic events, provide molecular clues that can be exploited for cancer prevention and early diagnosis, where cure may be achieved by a routine surgery alone. There have been many recent reviews concerning liquid biopsy.[12–16] In this paper, a salient overview of the current state of liquid biopsy-based approaches will be discussed along with aspects pertaining to incorporating such assays into an evolving advanced precision medicine clinical trial for lung cancer. Unique to our evolving lung cancer study has been the development of a scientific program in conjunction with an advanced clinical trial, with a focus on synergy between: (i) liquid biopsy methodology,

(ii) co-clinical trials, and (iii) advanced bioinformatics. This will be illustrated in the manuscript.

Liquid biopsy – Background

Biology of cfDNA and CTCs

Tracing the history of cell-free DNA (cfDNA), it was initially identified in the blood of healthy subjects by Mandel[17] and Metatis in 1948. There was not much interest in this finding until ~30 years later when it was reported that increased amounts of cfDNA were found in cancer patients.[18] A decade later, the presence of neoplastic characteristics in the circulation was reported.[19] Studies relating burden of disease and cfDNA first appeared in 2001, where it was reported that as the tumor size increases, the cellular replacement processes and the quantity of cellular debris also increase.[20] The finding that cfDNA exists at stable levels, which may grow significantly due to cell-injury was reported in, 2008.[21]

The release of cfDNA occurs through a combination of apoptosis, necrosis, and secretion. This is illustrated in Figure 1. The genetic alterations detectable include: point mutations, methylation patterns, chromosomal rearrangements, structural rearrangements, and copy number variations. Examples of cells contributing to cfDNA include cells turning over due to: (i) normal processes (e.g. lining of gut), (ii) inflammatory events or other immune mediated processes, and (iii) neoplastic phenomena. ctDNA is a tumor shed product. Normally phagocytes clear cellular debris. However, this does not happen competently in solid tumors, since cellular debris accumulates and is released into the blood.[6]

The detection of ctDNA in plasma depends upon an evolving concept referred to as cfDNA "shed."[12] It is the difference in rates between the releases of DNA by tumor cells vs. the renal clearance. Mitotic rate, necrosis, degree of vascularization, and tumor size are key variables. If the cancer is metastatic, bone or liver involvement are significant factors along with the overall burden of disease, all favoring ctDNA detection.[21] On average, the amount of cfDNA found in a normal person is ~5 to 10 ng/mL. In cancer patients, depending on the type of cancer and burden of disease, the cfDNA concentration is in the range of ~1 to 50 times normal.[22,23]

Although fragmented, cfDNA is stable in the circulation. This contrasts with free RNA molecules, which apart from micro-RNA, do not appear to survive.[16] cfDNA is normally quite fragmented with an average length of ~170 bp. This size corresponds to the length of DNA wrapped around a single nucleosome. cfDNA at lengths corresponding to two or three nucleosomes (i.e. ~340 and 510 bp) may appear at lower quantities.[24,25] The route of elimination of cfDNA is largely by the kidneys.[26] The half-life of ctDNA has been reported to be of a relatively short duration, < 90 min.[21]

Liquid biopsy – CTCs

Large clinical studies for breast and prostate cancer have employed the use of CTCs as markers of response to therapy and indicator of prognosis.[27,28] CellSearch® is an FDA approved assay, which uses whole blood for the evaluation of CTCs of epithelial origin. It has been found that the presence of CTCs in blood is associated with a decrease in overall survival in patients treated for metastatic breast, colorectal, or prostate cancer.[29] Although the capture of CTCs is difficult, they do allow for the analysis of their contents, namely, DNA, RNA, and proteins. Advanced CTC platform technologies, such as high-definition single cell analysis (HD-SCA) that is being developed at the Kuhn Lab at USC, allow for such extensive molecular profiling, namely DNA, RNA, and protein analyses. Through a collaboration, HD-SCA is being used in the Arkansas Bioinformatics Consortium (AR-BIC) advanced lung cancer clinical trial. AR-BIC is an alliance consisting of the research universities in Arkansas and the National

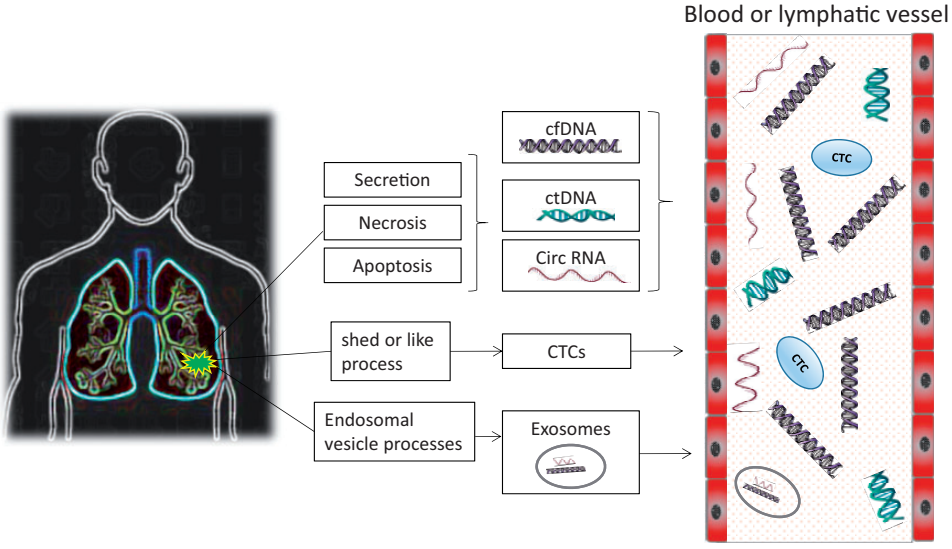

Figure 1. Cell-free DNA (cfDNA) origins. cfDNA is released by normal cells and cells involved with pathologic processes (e.g. inflammation, neoplasia). Circulating tumor DNA (ctDNA) is a subset of cfDNA released by tumor cells, and may contain a variety of genomic alterations (e.g. point mutations, chromosomal rearrangements, copy number variations). The vast majority of ctDNA is felt not to be derived from circulating tumor cells (CTCs). Although scientifically interesting, circulating RNA and exosomes are not quite ready for the clinic. (A color version of this figure is available in the online journal.)

Center for Toxicological Research (NCTR), a center of the Food and Drug Administration (FDA). CTC findings will be integrated with NGS results providing a more complete molecular study of the lung cancer biology.

There are growing classes of CTCs that are helping to explain and explore cancer biology. This is a very active and dynamic area of cancer-related biotechnology. Importantly, there are no universal markers for these cells. One of the first assays to detect, measure, and characterize carcinoma cells (epithelial origin) in the blood was developed by Racila *et al.*[30] in 1998. Technical advances have now made it possible to detect and characterize single CTCs in a patient's blood. Epithelial cell adhesion molecule (EpCAM) detection is the basis of many CTC methods. However, Non-EpCAM approaches for CTC capture are an active area of investigation.[31] By classifying CTCs by more advanced molecular criteria, e.g. epithelial to mesenchymal transition (EMT) markers, helps to identify more aggressive subpopulations and may have clinical utility.[32] CTCs provide an opportunity to capture and profile individual aspects of a patient's malignancy and are proving to be a vital cornerstone of precision medicine.

Interestingly, it has been reported from the Vogelstein lab that the vast majority of ctDNA is not derived directly from CTCs, and have a half-life shorter than CTCs.[23,33] In their study, ctDNA was often present in patients without detectable CTCs, suggesting that they are distinct sources of biomarkers. It was further stated that ctDNA is not preferable to CTCs for the monitoring or detection of cancer. Both are exhibiting significant scientific and clinical utility.[23]

Liquid biopsy – Clinical status and challenges

Accurate molecular assays are essential for the successful targeted treatment of several non-small cell lung cancer (NSCLC) adenocarcinoma subtypes (e.g. *EGFR, ALK, ROS1*). In the latest NCCN Guidelines (Version 8.2017), evaluation of newly diagnosed patients for these subtypes is a category one recommendation. Importantly, these NSCLC subtypes are effectively treated by non-chemotherapy regimens. In these cases, effective therapy is delivered via a class of drugs known as targeted small molecules, which have a very mild toxicity profile compared to standard chemotherapy. Plasma genotyping can be used in certain clinical scenarios to identify targets in *EGFR* NSCLC subtypes. Plasma genotyping is becoming more widely utilized, as evidenced by recent FDA approval of the initial blood-based companion diagnostic assays.[34,35]

Nearly 80% of cancer patients do not have genetic mutation results available during the initial consult with an oncologist, and ~25% begin cancer treatment before receiving results. Given this scenario, molecular diagnostic companies are offering rapid services. Whole blood is shipped overnight for the identification of ctDNA mutations using commercially available droplet digital PCR (ddPCR). NSCLC *EGFR* and *KRAS* mutations are reported with a turnaround time of three days.[36] It is important to note that while numerous assay approaches have been evaluated in clinical studies, for clinical utility, ctDNA assays must be directly compared to the current reference standard of a tumor biopsy.

Basic science and clinical medicine are being transformed by next generation sequencing (NGS) and the capability to detect small frequency variants in heterogeneous tumors.[37] The detection of mutations below 3% incidence is challenging with typical NGS practices. Background noise is generated by polymerases during NGS library building.[38] In clinical situations involving liquid biopsies for biomarker applications in cancer, background noise is particularly problematic. These situations often involve the detection of very low frequency mutations. For instance, following lung cancer surgery for curative intent (i.e. a small localized tumor), post-operative ctDNA mutations will be of an extremely low frequency due to removal of the tumor (i.e. source of tumor DNA shed) and the continued clearance of ctDNA by the kidneys. In these cases, post-operative blood analysis potentially requires detection of mutations of 0.1% or fewer, which is challenging. Technology advances have been rapid. Continued improvements in liquid biopsy sensitivity and specificity will dramatically change how adjuvant therapy decisions are made and how cancer screening will be performed.

PCR-induced NGS errors are particularly challenging with liquid biopsies given the degree of low-incidence mutations. There is noteworthy progress from academic and commercial entities to overcome this dilemma. A combination of sequencing, bioinformatics, and individual molecular tagging of DNA templates is typically employed. With molecular tagging, unique molecular sequences (barcodes) are incorporated with each DNA molecule during the library preparation stage. This allows sequencing reads originating from the same starting strand of DNA to be identified following PCR amplification. A single consensus sequence can then be derived by comparing all sequencing reads from that same DNA strand. This approach is very effective in correcting for errors introduced by PCR or during the sequencing process.[14] This is illustrated in Figure 2(a). A high-level consensus analysis pipeline for molecularly tagged reads is shown in Figure 2(b).

A NGS technology called duplex sequencing has been established by the Loeb lab. It is capable of detecting a single mutation among more than 1×10^7 wild-type nucleotides.[39] The enhanced quality and accuracy of these methodologies will enable clinical utility for ctDNA assays, permitting the study of scenarios with very low frequency mutations. These approaches will be used by our study as they become available.

Specificity (true negative rate) of results also creates additional challenges, especially for future screening type ctDNA assays. Recent large population genetic analyses concerning human aging have discovered that mutations in blood cells may induce a clonal expansion and occur frequently.[40] This process has been named clonal hematopoiesis of indeterminate potential (CHIP), and importantly does not involve dysplastic processes or cytopenias.[41] CHIP is similar to monoclonal gammopathy of undetermined significance (MGUS) and monoclonal B-cell lymphocytosis (MBL), which are predecessor conditions for multiple myeloma and chronic lymphocytic leukemia,

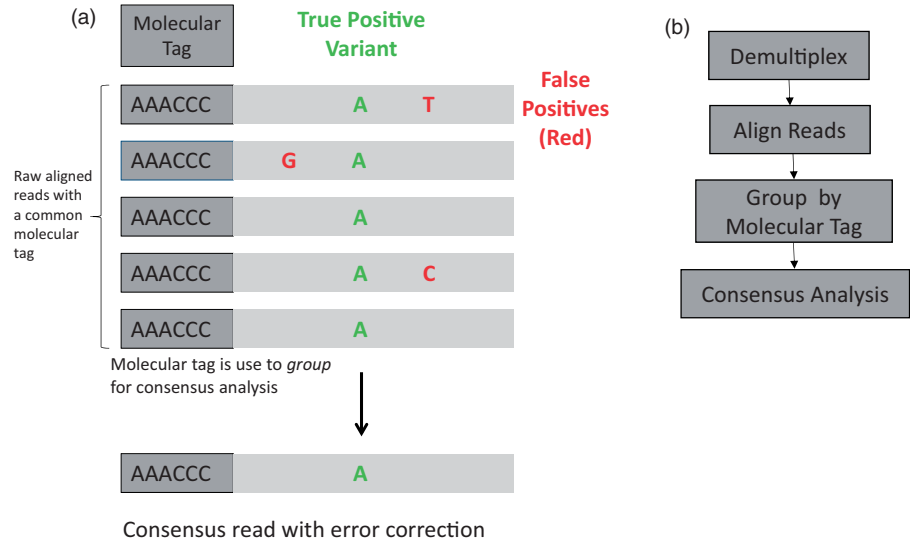

Figure 2. Consensus analysis of reads by molecular tagging. (a) Reads that align to the same region of the genome and contain the same molecular tag are grouped, and analyzed for true positive variants and false positives. Error correction is applied by eliminating false positives. Only true positive variants will appear in all of the raw aligned reads with the common molecular tag. (b) High-level consensus analysis pipeline. (A color version of this figure is available in the online journal.)

respectively. MGUS and MBL are usually benign and do not progress to a malignant state. Importantly, since mutations are commonly detected in healthy older adults (e.g. CHIP), the discovery of cancer-related mutations via cfDNA might not indicate the subject already has cancer, or will even develop cancer during their lifetime. Such results may just cause significant anxiety and possibly additional diagnostic procedures with radiation side effects (e.g. body CT scan).[42] Of note, radiation from a CT scan may be associated with a very small increase in the possibility of developing cancer later in a person's life, and repeat studies over time will compound this risk.[9]

Liquid biopsy – Preanalytical considerations and contrived samples

There are a multitude of variables and methodologies to be considered. It is difficult and impractical, at this time, for mid-size or small centers to conduct detailed and exhaustive preanalytical studies. However, blood collection tubes are critical and initial comparative studies were performed at UAMS. Initial studies to compare Streck cfDNA BCT blood collection tubes with standard EDTA were conducted at UAMS. The EDTA approach requires the sample to be put immediately on ice and to complete processing within ~60 min. This is compared to the Streck protocol, where the sample is required to remain at room temperature, and may theoretically be processed for up to 14 days. Comparing the two approaches, other than cost, a significant difference was not seen. Collaborative endeavors with the Kuhn Lab at USC made Streck the clear and practical choice.

Membership in SEQC2 and the BloodPAC are providing thoughtful guidance through discussion forums and web accessible information resources. The BloodPAC consortium includes members from academia, government as well as private and industrial entities. The goal is to speed-up the clinical utility of liquid biopsies. This includes investigation of preanalytical variables. SEQC2 is an international consortium, composed of members from academia, industry, and government and led by the FDA. Goals include the development of best practices, protocols, and quality metrics for NGS-based diagnostic assays, which support regulatory science and precision medicine. There is a dedicated subgroup concerning liquid biopsy methodology. In essence, the liquid biopsy is an application of deep sequencing. Experiments involving contrived samples are in progress to address reproducibility and level of detection for a variety of liquid biopsy technologies and assay approaches.

An advanced clinical trial for lung cancer utilizing liquid biopsies

Background and design

The prior sections of this paper have provided a scientific background and foundation for the application of liquid biopsy assays to a clinical trial. Per the 21st Century Cures Act, innovative clinical trials are integral for biomarker and drug development.[43] A number of lung cancer clinical trials utilizing liquid biopsies have been reported.[44] The novelty and uniqueness of this study are synergy between: (i) liquid biopsy methodology, (ii) co-clinical trials, and (iii) advanced bioinformatics. To this end, a lung cancer scientific program has been developed in conjunction with an advanced clinical trial, and this will now be shown. Figure 3 illustrates the overall design and information flow of the clinical trial. Figure 3(a) profiles the UAMS Clinical Trials Office, which coordinates all aspects of the approved Institutional Review Board (IRB) protocol, patient enrollment, de-identification activities for patient protection, and monitoring. The remaining sections of this paper provide background and details of an advanced

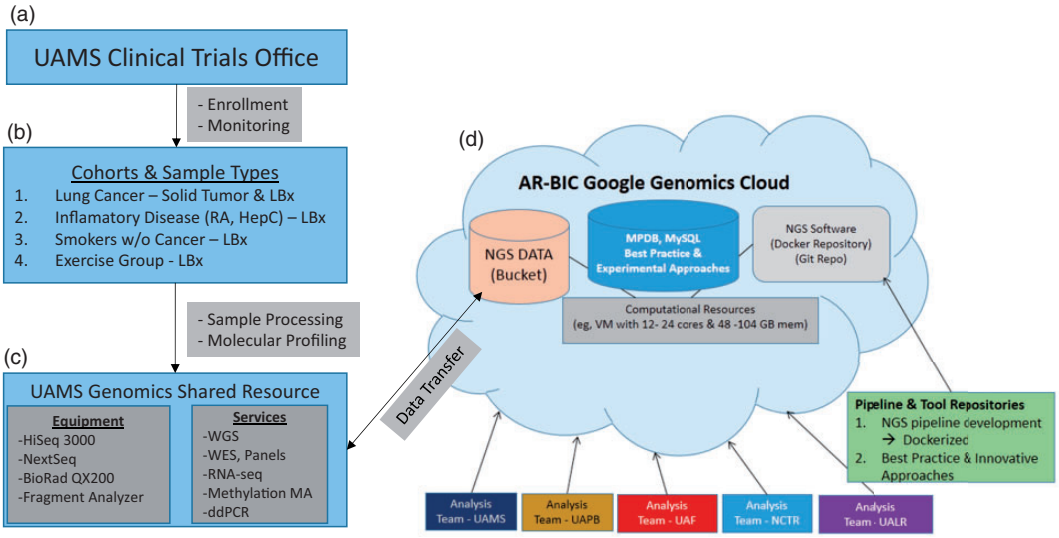

Figure 3. Clinical trial design and information flow. (a) The UAMS Clinical Trials Office coordinates all aspects of the approved Institutional Review Board (IRB) protocol, as well as patient enrollment, de-identification of all patient information and samples, and performs protocol monitoring. (b) The cohorts and sample types included in the clinical trial are: (i) lung cancer patients, all stages, and histologies, (ii) inflammatory disease patients (Rheumatoid Arthritis and Hepatitis C), (iii) heavy smokers (≥ 30 pack years) without cancer per low dose CT (LDCT) testing and, (iv) a normal volunteer exercise group. Regarding liquid biopsy (LBx), both plasma and urine are collected from all cohorts for the study of cfDNA in both health and different disease states. (c) UAMS genomics shared resource. (d) The Google Cloud is utilized to enhance collaborative endeavors across the member organizations of the Arkansas Bioinformatics Consortium (AR-BIC), which is an alliance consisting of the research universities in Arkansas and the National Center for Toxicological Research (NCTR), a center of the Food and Drug Administration (FDA). (A color version of this figure is available in the online journal.)

clinical trial for lung cancer along with the salient translational science and collaborative role of AR-BIC.

The leading cause of cancer related deaths in the United States is lung cancer.[45] This is true for both men and women. Lung cancer is difficult to detect; many times there are no outward symptoms or clinical indications until the disease is advanced. Problematic strategies for early detection along with the fact that it is commonly resistant to standard therapeutic modalities (i.e. chemotherapy, radiation) make it particularly lethal. Since 2003, there has been a significant increase in knowledge and disease subtyping for NSCLC. Advanced genetic analyses have been useful in identifying subtypes of this cancer enabling improved molecular understanding resulting in more effective therapeutic options having significantly reduced toxicity profiles.[46]

Unfortunately, for ~20 years, the occurrence of NSCLC in Arkansas has been about 25% higher than that reported nationally.[45] UAMS is committed to improving lung cancer outcomes as part of its mission to the State of Arkansas. An important aspect concerning consideration for NCI cancer center designation is addressing specific needs of the regional population. In this regard, lung cancer is a logical choice and UAMS is formulating and administering programs on both a public health and scientific basis. As part of the scientific endeavors, AR-BIC is working to improve outcomes for lung cancer patients.

As illustrated in Figure 3(b), the clinical trial involves four cohorts: (i) lung cancer patients, (ii) inflammatory disease patients (Rheumatoid Arthritis and Hepatitis C), (iii) heavy smokers (≥ 30 pack years) without cancer per low dose CT (LDCT) testing and, (iv) a normal volunteer exercise group. A major aim of the trial is to better understand cfDNA in health and disease. In this regard, liquid biopsies are being collected in a variety of patient disease states and normal volunteer cohorts. For all cohorts, each time a liquid biopsy is performed, a routine blood draw protocol is utilized, and three tubes of blood are collected in Streck cfDNA BCT blood collection tubes, with the order of tube filling noted. For blood sample handling, the Streck protocol is followed. Germline DNA via buffy coat and plasma are isolated and processed usually between 24 and 48 h. Currently, all plasma samples are being banked. Following the completion of the SEQC2 liquid biopsy contrived sample experiments, plasma samples will be processed. Given recent NSCLC reported findings that mutations in both urine and plasma can be detected with high sensitivity, urine for cfDNA is also being collected for all cohorts for liquid biopsy analysis.[47]

For lung cancer patients undergoing surgery for curative intent, liquid biopsies are obtained: (i) pre-operative, (ii) post-operative (~day 2 or 3), and then at each follow-up appointment. Lung cancer patients with advanced disease undergoing systemic therapy have monthly liquid biopsies. A single liquid biopsy is obtained from the inflammatory disease and heavy smoker cohorts. For the exercise group, a liquid biopsy is obtained before and then after ~30 to 45 min of cardio-level exercise. In addition to cfDNA analysis, CTCs are also being profiled. To avoid contamination from skin cells, either the second or third Streck tube collected during the liquid biopsy procedure is overnight shipped to collaborators at USC for an advanced CTC analysis.

Advanced clinical trial – Molecular profiling and bioinformatics

Extensive molecular profiling of the solid tumor and matched normal is performed for the lung cancer patients from either surgically resected tumor or a research biopsy (metastatic disease). This is illustrated in Figure 3(b) to (d).

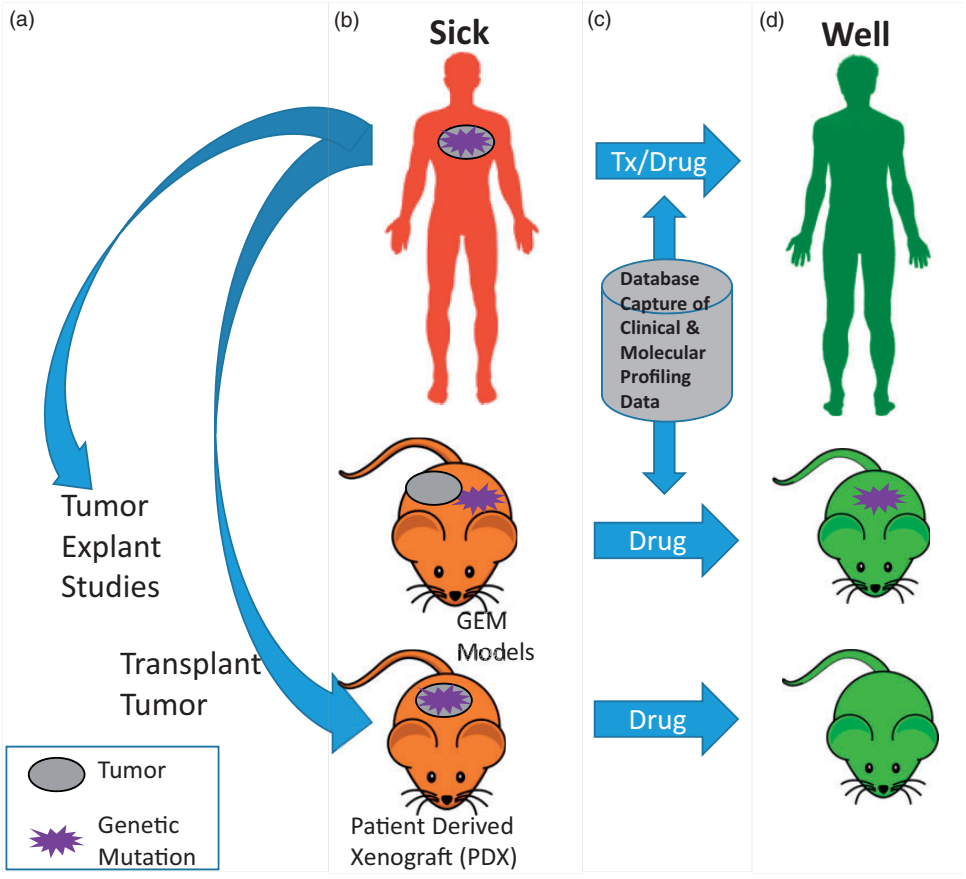

Figure 4. Towards patient-based prognostic assays. (a) Tumor material is acquired from patients with early stage disease during surgical resection for cure or a research biopsy is obtained from patients with more advanced disease. Tumor tissue is used for both patient-derived xenograft (PDX) and tumor explant studies. (b) Tumor tissue is transplanted into NOD scid gamma (NSG) mice to initiate the PDX. If molecular profiling reveals a hotspot mutation (e.g. EGFR L858R) in the patient's tumor, a genetically engineered mouse (GEM) will be purchased from a commercial vendor or developed in-house at UAMS. (c) A custom database captures all clinical and molecular profiling experimental results from patients and their corresponding mouse models. (d) Temporal analyses are performed on patients and mouse models via liquid biopsies, e.g. for investigation of therapeutic response or resistance. (A color version of this figure is available in the online journal.)

Specifically, (i) low-pass whole-genome sequencing (WGS) at ~15× for copy number analysis (CNA), (ii) a TCGA-based gene panel that utilizes molecular tagging at very high coverage (e.g. 2000–3000×), (iii) RNA-seq for total RNA on the tumor for 100M reads, (iv) methylation analysis of tumor DNA via the Illumina Infinium microarray platform. As molecular profiling data are generated, QA/QC checks are performed, and then the raw data (e.g. FASTQ files) are uploaded to the AR-BIC Google Genomics Cloud, for further processing by the consortium as shown in Figure 3(d).

The Google Cloud is used because: (i) NGS data are large and complex, (ii) collaborative activities with NGS data are difficult, and (iii) IT infrastructure and associated technical support for collaborative activities are included with the commercial cloud service. This helps to liberate investigators, allowing more focus on cancer science versus essential computational infrastructure. This may be especially relevant in the early phases of new projects. Projects that cross organizations may find economic and time benefits from a commercial provider.

An additional consideration in the computational research area is the fact that big data from NGS are outpacing information technology. In the very innovative and fast-paced science and technology of high-throughput molecular profiling, what is suitable for IT operations currently may become inadequate in one or two years. Administrative rules made sharing of data between organizations problematic, and collaborative activities challenging outside of a cloud environment. Although patient data are de-identified, it still resides in a commercial cloud, so further steps were taken. The University of Arkansas System entered into a Business Associates Agreement (BAA) with Google to further ensure HIPPA compliance. Concerning data sharing, the Genomic Data Commons has been created by the National Cancer Institute to foster a collective vision for the sharing of cancer genomic data.[48] The AR-BIC Google Genomics Cloud follows this viewpoint.

Bioinformatic pipelines were developed for all molecular profiling modalities following best practices put forth by the Broad Institute. All pipelines utilize Docker container methodology to simplify configuration management and enhance reproducibility and collaborative activities, as shown in Figure 3(c) and (d). Each molecular profiling experiment (e.g. RNA-seq for patient X) is run through a best practice pipeline and the output (e.g. Cufflinks data) is then loaded into the Molecular Profiling database (MPDB),

which resides in the AR-BIC Cloud. The MPDB is actually split into best practice and experimental databases. This allows the capture and discrimination of molecular profiling data from either best practice methodology or a new custom or experimental pipeline approach. Thus, the bioinformatic infrastructure is prepared for "*A vs. B*" type pipeline or analysis comparisons, which aids in the evaluation of new and innovative algorithmic methods.

Advanced clinical trial—Towards individual patient-based prognostic assays

Critically needed are effective models to test candidate therapies for patients. To avoid the insufficiencies of cancer cell lines, patient-derived xenograft (PDX) models are being utilized in our study, as illustrated in Figure 4(a) and (b**)**. These permit the patient's tumor to develop in a milieu simulating an *in vivo* setting enabling more precise tumor-drug studies, which are imperative for co-clinical trials. Notably, co-clinical trial methodology was recently used in prostate cancer and revealed a new mechanism of castrate resistance.[49] The co-clinical trial methodology involves integrative studies utilizing mouse model (xenograft and genetically engineered mice) treatment data. These are derived from or related to patient lung cancer clinical samples (Figure 4(c)). Molecular profiling data from a patient and corresponding mice will be analyzed in a joint fashion. This is an approach to focus on therapeutic efficacy (Figure 4(d)). The co-clinical trial platform is an innovative approach to improve the design, speed, and outcomes of rational and personalized cancer treatments.

An advantage of mouse-based co-clinical trials is that the PDX in both control and experimental groups can be derived from the same patient and share the same histopathology and tumor genetics. This may be particularly significant when exploring the individual activity of a new or novel combination therapy. Importantly, from the same patient, PDX models can be generated from therapy-naïve and post-treatment tumor material. This provides a background for deeper understanding of therapeutic resistance following an initial partial or complete response.[50] In our clinical trial, liquid biopsies have been successfully performed on PDX models over time, using a 100 μL blood draw, with cancer mutation detection (e.g. *KRAS* c.183A > T, p.Q61H; *PIK3CA* c.1633G > A, p.E545K[51]) by ddPCR. A disadvantage of the PDX model is that initially a large quantity of tumor tissue is needed. The PDX process is also known to be time and resource consuming. True clinical utility for individual patients may be challenging and at times impractical due to the length of time to establish an acceptable mouse model as well as the associated high failure rates.[50]

Preclinical models that can better recapitulate a patient's cancer are needed. Central to this need is the ability to predict response to standard-of-care or novel therapies. Three-dimensional *ex-vivo* cultures of fresh tumors (explant) are being used as a substitute to mouse models in order to address the high failure rates with establishing a viable PDX. Regarding explants, evidence of tumor growth correlating with that obtained *in vivo* as well as retaining a viable tumor microenvironment are reported. Approximately 70% of primary lung cancer specimens processed in a recent study were responsive to explant lab techniques and displayed viable tissue for up to three days.[52] In the trial, an explant procedure will be used along with advanced and integrative genetic analysis.

Summary and concluding remarks

For many years, the concept of the liquid biopsy was considered a *dream* for medical oncologists. Now the vision is to transform cancer medicine, making it safer and rationally driven based on quantitative molecular assays. Liquid biopsies tremendously reduce risks associated with most standard cancer-based biopsies. The immediate clinical applications of liquid biopsies are many. These include: (i) early detection of cancer, (ii) determination of the total tumor heterogeneity (primary lesion plus metastatic foci), (iii) tracking temporal-based tumor dynamics, (iv) mapping the deranged tumor molecular circuitry for targeted therapies, (v) assessing response to therapy early in a treatment course, (vi) more effective minimal residual disease strategies, and (vii) effectively quantifying resistance to therapy and disease evolution in real time. The analyses of ctDNA and CTCs via a non-invasive routine blood draw are beginning to revolutionize many aspects of cancer treatment, screening, and prevention.

The novelty and uniqueness of this study are synergy between: (i) liquid biopsy methodology, (ii) co-clinical trials, and (iii) advanced bioinformatics. Thus far, a clinical trial protocol has been established and accepted by the UAMS IRB. Patient registration is proceeding and on-track. Mouse models are in progress and tumor material is also being cryo-preserved for explant studies. Liquid biopsy approaches are ongoing, and importantly include involvement in the SEQC2 program and BloodPAC consortium. An assortment of state-of-the-art bioinformatics methodologies have been established to meet needs of the clinical trial, improve analysis, and encourage collaborative activities regarding the intricate genetic analysis data.

Advanced assays are needed for success with precision medicine. These advanced molecular diagnostics will be used by physicians to track the evolution of a patient's cancer over time. Per the 21st Century Cures Act, innovative clinical trials are integral for biomarker and drug development. There is an urgent need for advanced clinical trials employing: (i) innovative assays utilizing liquid biopsy methods, (ii) molecular profiling with advanced bioinformatics, and (iii) mouse models and tumor explants, in order to improve outcomes for cancer patients. By means of a collaborative approach through AR-BIC such a clinical trial is now in progress in Arkansas.

DISCLAIMER

The information in these materials is not a formal dissemination of information by FDA and does not represent agency position or policy.

Authors' contributions: MS, KA, DJ, LH were involved with the clinical trial study design. EP, MY, AP, DJ, JL, KW were involved with the bioinformatic study design and software implementation. IJS, DY were involved with the animal study design and implementation. JX, DJ are involved with liquid biopsy study designs. SJ performed pathologic tissue analysis. MS, ML, IJS, SJ are involved with the collection and processing of human samples. DJ wrote the manuscript.

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.

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