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Rationale for the LiquidBiopsy® Triple Play

Cynvenio Biosystems | April 2015

The treatment of cancer is dependent on effective tools for evidence-based clinical decision making. The advent of next generation sequencing (NGS) has focused much attention on an entire new class of biomarkers that are the result of mutation analysis and biologically specific. These genetic mutations drive virtually all forms of cancer. Traditionally, this analysis has been performed on biopsy material. However, many tumors are difficult to sample, accessible only using fine needle aspirates or are located either in unknown sites or in sites that are challenging and risky to access. Moreover, recovery of biopsy material is only one part of the problem. Once a treatment is begun, evidence of resistance or recurrence is necessary to avoid unnecessary or ineffective treatments. Radiographic approaches are often insensitive to changes in tumor response and the recovery of additional biopsy material assumes we can effectively identify the most important sample of the disease for analysis. The LiquidBiopsy® can solve all these problems. It is a supplementary biopsy mechanism that samples different parts of the disease from traditional surgical biopsy, has none of the associated side effects or risks being just a blood draw, can be repeated as needed for effective monitoring, and samples the part of the tumor that is associated with metastatic events.

A LiquidBiopsy® approach to tumor monitoring is effective because there are two sources of tumor DNA that can be examined in a blood sample: ctcDNA (circulating tumor cell DNA) derives from circulating tumor cells (CTC) that either sloughed off or are metastasizing in the blood. DNA can also be recovered as small randomly fragmented pieces of DNA from plasma (cell free DNA or cfDNA). These fragments are waste products of either cell death or turnover and derive from both normal and tumor cells. This DNA does not provide specific information about cancer. The small population of nucleic acids that are informative are from ctDNA (circulating tumor DNA). The different biological source of these two templates has implications. cfDNA is abundant and easy to recover. However, the fraction that is tumor derived is mostly a tiny fraction of the total amount of cfDNA and cannot be enriched. As CTC can be enriched, CTC of different characteristics can be recovered. Enriched CTC can provide DNA sequence informed about a specific biological compartment in the cancer life cycle. However, until the advent of the LiquidBiopsy® platform, this population of cells could not be recovered in a way that allowed direct NGS analysis. Fundamentally, both templates are very rare templates. As such, a system that allows analysis of both templates will increase sensitivity. Furthermore, the two analytes provide different types of information depending on the clinical need.

In supporting both biomarkers, the LiquidBiopsy® system uses exacting scientific methods that affect the analysis. Recognizing the rarity of any tumor DNA in blood and its heterogenous nature, whether ctDNA or ctcDNA, the approach requires case control sequencing. This means, for any analysis, the LiquidBiopsy® workflow also analyzes DNA from normal healthy cells in the patient. This allows the noise caused by any sequencing test as well as changes that are not specific to the tumor to be distinguished. Second, while ctDNA and ctcDNA can be recovered from one tube of blood, ctcDNA is not restricted to epithelial populations that have proven to be insensitive and applicable in only certain diseases. Third, the use of case control supports expression analysis of mRNA signatures or discovery of rearrangements that require cell based analysis. Examples include targetable alterations such as RET fusions in lung cancer or Androgen Receptor splice variants in

prostate cancer. Biological changes like changes in ER expression in breast cancer or protein targets for immunotherapeutic targets like PD-L1 can also be monitored on cells.

Unlike ctcDNA, ctDNA cannot be selected. Furthermore, for reasons that are not clear, ctDNA is not produced at the same levels by all cancers. The LiquidBiopsy® test requires 1% representation. This 1% threshold allows rapid detection of mutations without having previous knowledge of the mutations that are present. There is a particular challenge of finding mutations at low frequency where there is an increased risk of false positives. This risk is due to unfavorable signal to noise ratios and the catabolite nature of ctDNA. Risk is mitigated and assigned by the threshold and to a clinically relevant quantity of template (representation significantly below 1% often equates to much less than 100 molecules, a frequency that is interesting in a research setting but has not been shown to be clinically relevant). By supporting the analysis of both templates from one tube of blood, the LiquidBiopsy® supports the greatest breadth of applications, the greatest chance of generating information for any particular sample, and an orthogonal sample - the best solution to the problem of false positives.

Template	Number of Mutations	Gene	Mutation Frequency
ctDNA	1	*TP53*	*<1%*
		FGFR1	*<1%*
		CTNNB1	*<1%*
		PIK3CA	22.0%
ctcDNA	4	PIK3CA	1.5%
		CTNB1	2.0%
		FGFR1	1.1%
		TP53	11.0%

Table 1: LiquidBiopsy detected alterations in the ctDNA and ctcDNA compartment. Italics indicate mutations detectable but below threshold.

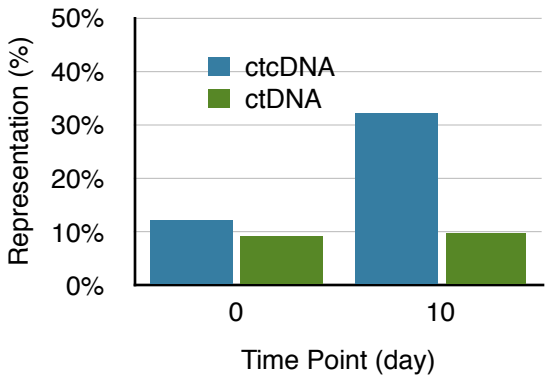

Figure 1: A LiquidBiopsy® test was performed on a patient with recurrent breast cancer at the beginning (Day 0) and after the start (Day 10) of a new line of therapy. A single activating mutation in PIK3CA was observed in both samples at day 0. After 10 days of treatment, the mutation was had incremented almost 300% in the ctcDNA template but was effectively unchanged in the ctDNA.

The complementary nature of ctDNA and ctcDNA are best seen in case examples. Figure 1 shows the impact of a change in treatment in a patient with recurrent metastatic breast cancer. The patient presented with a driver mutation in PIK3CA that was detectable in both the ctcDNA and ctDNA fractions. A decision to start the patient on ER inhibition was followed with a repeat test to monitor the response. After 10 days, a second test clearly showed a 3-fold increment in the number of cells with the mutation while the ctDNA was unchanged. These data emphasize the biomarkers are produced by different mechanisms of the disease and so reflect different aspects of the disease process resulting in complementary utility. In a second example, a patient with metastatic esophageal cancer was evaluated by LiquidBiopsy® to provide alternative treatment options. The patient presented with four alterations in ctcDNA, three of them with targeted therapies

or relevant (PIK3CA, CTNB1, FGFR1). The driver mutation in PIK3CA was observed in both ctDNA and ctcDNA. However, the other three mutations could only be seen by reducing the threshold of the ctDNA test. So while the ctDNA confirmed the observations in the ctcDNA compartment, the limiting representation of ctDNA template would not have supported their observation without previously knowing they were there.

LiquidBiopsy® uniquely supports NGS based analysis of both ctDNA and ctcDNA. As such it is the only approach that acknowledges shared and emerging utility of ctDNA and ctcDNA. By supporting both approaches, the LiquidBiopsy® also predicts the emergence of additional readouts that can be derived from non-DNA based biomarkers. The comparative value of the different templates is summarized in table 2.

The pharmaceutical community will continue to develop drugs that are more and more effective at specifically inhibiting disease specific processes. However, from the patients perspective, detecting, controlling and monitoring the disease process demands any information that can be specifically attributed to a tumor and the cancer process. The LiquidBiopsy® supports the full spectrum of such readouts.

Role	Biopsy	CTC and ctcDNA	ctDNA
Historical role	Gold Standard	Prognosis	NA
Applications	Pathology and IHC	Pathology and IF	NA
Nucleic Acids	DNA + RNA	DNA + RNA	DNA Only
Disadvantages	Invasive & Costly, infrequently sampled	Enrichment necessary	Very high signal:noise
Advantages	Validated template	Accessible sample, good concordance with biopsy, multiple readouts, biologically relevant, tunable	Accessible sample, good concordance with biopsy, detected in multiple stages

Table 2: Complementary aspects of cell based and nucleic acids based readouts from a blood sample.