

Liquid Biopsy:

An Emerging Market for Radically Improved Cancer Management

Cancer diagnostics based on measuring biomarkers in tissue samples has already in the past decade provided revolutionary advances in diagnosis, prognosis, and therapy selection. A major drawback of the tissue-based approach centers on the need for invasive surgical procedures in sample collection, which in a great many instances preclude following the progression or regression of disease during therapy.

In recent years, an impressive number of cancer biomarker researchers have turned their attention to the analysis of markers present in biological fluids, which can be collected with minimal invasiveness and permit following the disease over time. This highly dynamic field has come to be called liquid biopsy. In the past few years a significant and growing number of startups and several major companies have taken up the challenge of commercializing and offering liquid biopsy products and services to the market

These procedures, for the most part, query blood samples for information to be gleaned from circulating tumor cells (CTCs), circulating tumor DNA (ctDNA) fragments, and extracellular vesicles (EVs). CTCs have the longest history as subjects for liquid biopsy. Indeed, one decade-old commercial product has already garnered FDA approval for in vitro diagnostic use. Circulating tumor DNA, a more recent entry on the liquid biopsy scene, is fast becoming an alternative or adjunct to CTC assays. EVs are the newest and least developed of the three liquid biopsy sample sources, and while highly promising, their ultimate value has yet to be fully established.

This report explores the background, history and basic research of liquid biopsy covering the three sample categories that dominate liquid biopsy today: circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), and extracellular vesicles (EVs, also known as exosomes). This report also details the commercial aspects, market dynamics, and trends of liquid biopsy.

Plus, transcripts from extended interviews with:

- Nicholas Dracopoli, Ph.D., Vice President Oncology Translational Research, Janssen Pharmaceuticals
- Paul Dempsey, Ph.D., Chief Scientific Officer, Cynvenio Biosystems
- Johan Skog, Ph.D., CSO and Founding Scientist, Exosome Diagnostics
- Lidia Sambucetti, PhD, Sr. Director of Cancer Biology, and Ted Ira, Director of Sales, SRI Biosciences Div.
- Harry Glorikian, Life Sciences Consultant

Executive Summary

This report, *Liquid Biopsy: An Emerging Market for Radically Improved Cancer Management*, deals with recent progress and new directions in this highly dynamic emerging modality for cancer research and in vitro diagnostics. Following a brief introduction, Chapter 2 covers historical and evolutionary aspects of liquid biopsy, along with background material to aid in understanding the material to follow. The third chapter, which deals with recent basic research activities and results, is arranged according to the three sample categories that dominate liquid biopsy today: circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), and extracellular vesicles (EVs, also known as exosomes). Chapter 4 enters the realm of commercial activity in each of the aforementioned categories. The next chapter views liquid biopsy from the perspective of market dynamics, and covers: market size estimates along with five-year projections by application segment; the competitive environment; deal activity; and results from an extensive market survey. Chapter 6 examines some developing trends in liquid biopsy, and the final chapter presents complete transcripts of interviews with six people who are knowledgeable in the field. Extracts from the interviews also appear at relevant places elsewhere in the report.

Background and history

In the past, biopsied tissue has been evaluated for malignancy by highly trained pathologists peering through microscopes and applying hard-won heuristic knowledge in pattern recognition. Some tissue micrographs yield unequivocal results, but many do not. Instead, many provide ambiguous data that can easily confound interpretation. The advent of routine immunohistochemistry improved that state of affairs somewhat by adding the ability to visualize the presence and distribution of certain cellular antigens. Molecular diagnostics improved things further, bringing DNA and oncogenes into the picture via FISH (fluorescence in situ hybridization), which gave pathologists another powerful tool to enhance their armamentarium. Next-generation sequencing systems came on the scene during the past decade, enabling routine determination of genetic variants in tumor nucleic acids, which provided information in support of matching patients with therapies.

Even with all these advances, cancer pathology still operated within limits set by the availability of biopsied tissue. In most instances, laboratorians had only a single shot at obtaining tissue from a patient. Repeat biopsies over the course of disease could have been extremely valuable, especially in monitoring the success or failure of selected therapies. Liquid biopsies, which first came on the scene in 1994, do in fact permit longitudinal time

studies of an individual patient's condition.

CTCs were first observed in the blood of a cancer patient in 1869, but it took more than a century before their entry into commerce, which happened first in 1994 at a company called Immunicon, which soon thereafter was absorbed into Veridex, a J&J subsidiary. Veridex introduced the first commercial liquid biopsy test, CellSearch, in 2000.

The liquid biopsy field has now matured in terms of the sensitivity and precision of CTC detection and isolation. It is now well established that levels of CTCs in blood increase as cancer progresses and decrease as it disappears. It's also becoming increasingly apparent that molecular analysis of biomolecules contained in CTCs provides powerful supportive information for determining, monitoring, and guiding the course of therapy. In recent years, the liquid biopsy spectrum has expanded beyond CTCs to include ctDNA and EVs. ctDNA, which typically constitutes a small subfraction of an individual's total circulating cell-free DNA, can now be readily evaluated for genetic variations using state-of-the-art target amplification and sequencing technologies. EVs, currently dominated by the type called exosomes, constitute the most recent addition to the liquid biopsy armamentarium. Once isolated, they provide ready access to subsets of biomolecules found in whole cells for subsequent assay. Much of the attention in EV liquid biopsy has centered on finding mutations in mRNA and classifying the increasingly important family of microRNAs.

Basic research

The availability of sophisticated analytical tools for analyzing the contents of cancer cells, for detecting genetic variants at extremely low allele frequencies, and for isolating extracellular vesicles, has in recent years triggered a great deal of research using liquid biopsy to yield valuable knowledge not readily accessible before liquid biopsy became available. CTCs, the first such entities on the liquid biopsy scene, have accounted for much of what's been learned to date. However, the relative ease of isolating cell-free DNA has quickened research activity in this field, and ready access to ctDNA has provided important insights that enhance and complement those derived from CTCs. EVs, though a recent arrival on the liquid biopsy scene, are rising rapidly in importance based on their perceived ability to add yet another important perspective to cancer diagnostics.

CTCs must be separated or otherwise distinguished from a much larger number of cells found in blood, and several approaches have been applied to do so. The oldest, and still most prevalent of these methods, rely on immunocapture via surface antigens, often using magnetic particles as a solid phase to achieve separation of CTCs from other blood components. Typical CTCs are positive for surface cytokeratins, negative for CD45 protein, and stain with the dye called DAPI. Early and more recent CTC technologies use the EpCAM protein for immunocapture. However, EpCAM and some other surface antigens decrease in expression when cells undergo

EMT (epithelial to mesenchymal transition) on the road to metastasis. Other CTC isolation techniques rely on cell size, morphology, behavior in electric fields, or deformability.

Microfluidic devices, which have come to play a prominent role in CTC-based liquid biopsy, can simplify assay protocols and reduce experimental times. Paucity of CTCs in blood has encouraged researchers to develop methods for obtaining extensive molecular information from single cells. Additionally, CTCs need not be isolated for enumeration. Automated imaging techniques have been applied to seeking out CTCs in the buffy coat fraction of blood. Much information can be so gleaned in situ, and CTCs so pinpointed can later be isolated for further analysis.

Another approach to liquid biopsy that's been gaining popularity in recent years foregoes the need to work with cells, focusing rather on ctDNA found in the cell-free portion of blood. The specificity of this approach relies on the presence of somatic alterations present in cancer cell genomes but not in matched normal cells. ctDNA fragments, which tend to average around 200 bases in length, cover a broad range of concentrations and can be found in blood from patients with many common cancer types at both early and late disease stages. The half-life of ctDNA in blood is said to be about two hours, which means that changes in levels and composition caused by the disease process or treatment effects will likely become detectable in relatively short order. ctDNA levels have been reported to decrease markedly after surgical removal of tumors, and to increase again as small metastatic lesions develop.

Much ctDNA research relies on digital PCR technologies, some of which can detect genetic variants at allele fractions down to 0.01% or even less. Digital PCR divides a sample into many portions or partitions, each of which is then subject to amplification. This arrangement enables more accurate quantitation than conventional means, and is often used for clonal amplification of samples meant for next-generation sequencing. Droplet digital PCR goes further by doing PCR in individual oil-water emulsion droplets, which range in size from a few femtoliters to nanoliters. Droplets can also serve for a variety of downstream experimental manipulations common in liquid biopsy. Other techniques used in ctDNA work include dielectrophoresis and CAPP-Seq (cancer personalized profiling by deep sequencing).

Extracellular vesicles have become increasingly popular in liquid biopsy for several reasons, one of which is their abundant microRNA content, a subject of growing pertinence and interest in cancer research. The field of cancer EV isolation and analysis is still maturing. Many current approaches rely on such properties as size, density, and solubility. Methods used in research settings include centrifugation, filtration, immune precipitation, and affinity purification. These modalities are, in general, not readily adapted to the diagnostic milieu and often are not terribly efficient in terms of EV yield. Several commercial products which recently became available employ filtration or size exclusion. Exosome Diagnostics (see Chapter 4), for instance, has joined with Qiagen to develop the exoRNeasy Serum Plasma Kit. The kit uses separation on a spin column filled with a proprietary

resin followed by affinity capture of EVs on a filter. Several companies also provide beads for affinity capture. For instance, Miltenyi Biotech provides magnetic beads linked to EpCAM. The same technologies used for downstream analyses of cell-free nucleic acids apply also to EV contents, although the emphasis here tends to center on RNA rather than DNA.

Liquid biopsy has been shown to provide valuable information on cancer subtyping, prognosis, matching patients with therapies, detecting recurrence, and predicting the development of drug resistance before it starts to manifest. Notably, liquid biopsy also appears to have great potential for predicting and characterizing metastasis, the actual cause of death in a large majority of terminal cases. A vision for the future entails using liquid biopsy results in support of attacking nascent metastases before they become unmanageable.

Commercial aspects of liquid biopsy

Janssen Diagnostics' CellSearch CTC enumeration system dates back more than a decade, and the company has enjoyed, essentially, a monopoly in the liquid biopsy market, however small it's been. Recent years have seen rapid growth in the number of companies entering the market, many of which are backed with substantial venture capital funding. The commercial activities section of the report presents brief profiles of these newer entrants, with emphasis on products in development and on the market, again categorized according to the three sample types.

Companies covered that emphasize CTCs include AdnaGen, Angle, ApoCell, Biocept, Biofluidica, Clearbridge Biomedics, Cynvenio, Cytolumina, CytoTrack, Diagnologix, Epic Sciences, Fluxion Biosciences, iCellate, Janssen Diagnostics, Qiagen, Rarecells SAS, Silicon Biosystems, and SRI International. Biocept and Cynvenio exemplify companies that augment their CTC assays with ctDNA analysis.

Companies described herein that specialize in ctDNA approaches to liquid biopsy include Agena Bioscience, Boreal Genomics, Chronix Biomedical, Genomic Health, Guardant Health, Inivata, Molecular MD, Myriad Genetics, Natera, Personal Genome Diagnostics, Sysmex Inostics, and Trovagene. Companies in the extracellular vesicle segment include Exosome Diagnostics, Exosome Sciences, and HansaBiomed OU.

Market dynamics

Although liquid biopsy shows great potential to extend the capabilities of tissue biopsy and possibly even replace it, the modality is still in its early days. Considerable inputs of effort and investment will be required before the field can mature and achieve widespread, routine use in oncology clinical practice. Still, the substantial and growing numbers of companies involved and the substantial levels of investment made suggest ultimate success in establishing liquid biopsy as a valuable modality in cancer research and diagnostics.

Many current signals suggest oncoming rapid growth in the liquid biopsy market. Estimated 2014 revenues for liquid biopsy products and services total \$120 million. Projected compound annual growth during the subsequent five-year period is expected to exceed 30%. A breakdown of projected revenues into R&D and patient testing segments indicates much faster growth in the latter than the former. Although it's difficult at this early stage to predict which companies will become leaders in the liquid biopsy market, we have identified a subgroup of companies with apparent advantages. These include AdnaGen, Cynvenio, Epic Sciences, Genomic Health, Guardant Health, Janssen Diagnostics, Myriad Genetics, and Qiagen.

This market dynamics section of the report also describes 16 recent deals involving liquid biopsy. The most prevalent of these involve collaborations between two companies, each of which has complementary technology to contribute. The next most prevalent are companion diagnostic deals, in which a drug developer collaborates with a liquid biopsy company in development of a test to determine patients suitable for such treatment. Less prevalent deal categories include biomarker identification matching liquid biopsy technology and biomarker content companies, and those in which a large company sponsors technology development in a smaller one.

The market dynamics chapter also contains results from a survey of more than 100 individuals active in the liquid biopsy field. Almost two-thirds are active in CTC work, but nearly as many also work with ctDNA, and almost a third are involved with EVs. Among respondents active in CTC work, immunocapture still dominates the technology scene, although microfluidics and imaging also garnered substantial numbers of responses. The ctDNA segment technology responses were dominated by digital PCR (61%), while the droplet digital PCR variant merited 39% of respondents' votes. In the EV category, proteins were the most common constituent to be analyzed, followed closely by microRNA.

An overwhelming majority of respondents felt that liquid biopsy will come to play an important role in cancer management in the next two years, while very few disagreed. When asked to rate popularity of sample sources during the next five years, respondents rated ctDNA highest, followed by a combination of CTCs and ctDNA. Other selections had lower response ratings.

Trends

Chapter 6 of the report examines some emerging trends that may affect the future development, acceptance, and market growth of liquid biopsy. Of particular interest are trends in government regulation that could limit growth in liquid biopsy, and indeed in the overall molecular diagnostics market. We also discuss issues surrounding the relative merits of the three categories of liquid biopsy samples, and ways in which they may be competitive or complementary. Finally, we examine important trends relevant to

targeted drug resistance and metastasis.

The FDA recently issued a draft guidance indicating its intention to restrict use of the LDT (laboratory developed test) category based mainly on degree of risk to patients. Since LDTs have played such an important role in commercializing cancer biomarker technologies, many have concerns that such regulation could slow the adoption of important liquid biopsy assays. While limited obstacles to market development are likely to result, there are, arguably, good reasons to remain optimistic that impacts will be minimal. Still, a vital factor for rapid market growth requires that liquid biopsy undergoes further standardization so that tests representing different technologies and manufacturers provide consistent results.

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Chronix Biomedical

Genomic Health

Guardant Health

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Roche and iCellate

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Paul Dempsey, Ph.D., Chief Scientific Officer, Cynvenio Biosystems

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Exhibit 4.1 CTC-based liquid biopsy companies		
Company	Technology	Comments
Fluxion Biosciences	Microfluidic-immunocapture	Multiple downstream analyses enabled
iCellate (formerly Liquid Biopsy AB)	Microfluidic-based on biomechanical aspects	Instrument prototypes in field testing
Janssen Diagnostics	CellSearch immunomagnetic capture	Only FDA-approved CTC enumeration system, 150K tests run between 2010 and mid-2014
Janssen Diagnostics	CTC-iChip, microfluidic immunocapture	In co-development with Mehmet Toner group at Mass. General Hospital
Qiagen	Therascreen EGFR RGZ Plasma PCR kit	Granted CE-IVD mark 1/15 for CDx of IRESSA in advanced NSCLC patients
Rarecells SAS	Size-based filtration capture	Broad spectrum of downstream assays possible
Silicon Biosystems	Microfluidic trapping, fluorescence imaging	Dielectrophoretic trapping principle, isolation and recovery enabled
SRI International	Laser scanning followed by automated imaging	Laser step for rapid location of CTCs followed by automated fluorescence imaging of labeled biomarkers

Circulating tumor DNA (ctDNA)

The number of companies focused on ctDNA for liquid biopsy has grown in recent years, until it's now almost even with those specializing in CTCs. Even if the information garnered from ctDNA is not as comprehensive as that from CTCs, it does appear to possess certain attributes that make it a good candidate for replacing or augmenting CTCs. And, of course, cell-free DNA is much more easily isolated than CTCs, even if the cancer-derived portion represents only a small fraction of the total.

Agena Bioscience

Agena Bioscience originated in May 2014 when San Diego venture capital firm Telegraph Hill Partners purchased Sequenom's Bioscience business, provider of the mass spectrometry-based genetic analysis system known as MassARRAY, which is claimed to offer genetic analyses with high-degree multiplexing, high volume, and precise quantitation. UltraSEEK, a prominently featured application of the system, is a high-sensitivity somatic detection technology for identifying rare mutations occurring at less than 1% abundance in as little as 40 nanograms of ctDNA. The system interrogates 26 driver mutations in 12 key oncogenes.

The UltraSEEK protocol involves: multiplex PCR; mutant-specific chain extension of amplicons; capture of mutant alleles on magnetic

and commercialization agreement in the area of EGFR mutations. Sysmex Inostics and Merck KGaA are collaborating for development and commercialization of a RAS mutation detection kit. Finally, Myriad Genetics has multiple CDx deals for its BRCAanalysis assay, notably one with AstraZeneca that has gained FDA approval.

Biomarker identification characterizes two deals: Theradiag and cancer institute IRCM are collaborating in identifying circulating microRNA biomarkers in rectal cancer; and Fluxion is sponsoring Stanford University researchers in using their CTC capture system for identification of cancer subtypes.

There are two examples of large companies sponsoring technology development: Janssen sponsored and collaborated with Mass General in development of a next-generation microfluidic CTC-iChip, which Janssen anticipates marketing; and Roche is similarly sponsoring research at iCellate (formerly Liquid Biopsy AB) for development of new

CTC detection technology.

One example represents an outright acquisition, in which Agena Bioscience absorbed Sequenom's Bioscience unit with its mass spectrometric mutation detection technology.

Market Survey

In December 2014, we conducted an online survey of more than 100 individuals whose work involves one or another aspect of liquid biopsy. Of 117 people who responded to the first question, nearly 20% work for organizations that provide liquid biopsy services (Exhibit 5.3), while just more than 10% have positions with companies that provide liquid biopsy products. Another 8.5% said they worked in both products and services. Nearly 60% of respondents are employed by a university or other nonprofit organization.

Exhibit 5.3 Type of organization where respondents work (N = 117)

Organization type	Number of responses	Percent of responses
Liquid biopsy services company	23	19.7%
Liquid biopsy products company	12	10.3%
Liquid biopsy products and services company	10	8.5%
University or non-profit	68	58.1%
Other	4	3.4%
Totals	117	100.0%