PREDICTIVE MARKERS IN THE LIQUID BIOPSY

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OUTLINE

• Introduction
• Techniques for the Laboratory Analysis of Liquid Biopsy
• Approaches for Liquid Biopsy
• Major Limitations for Introducing Liquid Biopsy into Clinical Practice

DEFINITIONS

Predictive Markers: Help guide treatment decisions and identify subsets of patients who would be “exceptional” responders to specific cancer therapies or individuals who would benefit from alternative treatment modalities.

Liquid Biopsy: A promising clinical tool for the molecular analysis of a liquid marker can easily be isolated from many body fluids (blood, saliva, urine, ascites fluid and pleural effusion), that uses technologies to examine and assess non-invasively the heterogeneous genetic landscape of the tumor (primary tumor and metastases).

• Assesses the tumor burden
• Monitors the evolution of tumor genomes
• Unravels mechanisms of resistance
• Establishes tumor heterogeneity
• Identifies predictive and prognostic markers

Currently used to define the following:
• Circulating tumor cells (CTCs)
• Circulating DNA (tumor DNA (ctDNA) and free DNA (cfDNA)
• Cell free RNA (exosomes)

Summary

Predictive Markers in the Liquid Biopsy

• Liquid biopsy and predictive marker development is a rapidly growing field in cancer which is a dynamic and heterogeneous disease
• Tumor cells never reach a steady state
• Metastatic cells are NOT merely more aggressive “clones” of the primary tumor
• Metastatic disease is an entirely different condition – which requires re-biopsy
• Patient comorbidity and safe access to the lesion make re-biopsy unfeasible
• Liquid biopsy is an effective alternative to re-biopsy

ADVANTAGES OF THE LIQUID BIOPSY

1. Easy, non-invasive access to molecular information about the tumor genome
2. Permits frequent examination of the genomic landscape of the tumor since tumors shows considerable intertumoral and intra-tumoral heterogeneity
3. Early Diagnosis using cfDNA assessment
4. Assessment of progress using tumor-associated genetic alterations
5. Early detection of disease recurrence
6. Assists in the clinical management of difficult-to-diagnose patients with advanced-stage cancer
7. A surrogate for the traditional biopsy with the purpose of identifying therapeutic targets or acquired resistance mechanisms (predictive information)
8. Can be used as a diagnostic / screening tool
9. Improves patient stratification and real-time monitoring of therapy
10. Identifies therapeutic targets and acquired resistance mechanisms (predictive information)
11. Obtains the need for tissue biopsies in metastatic patients
12. Whole genome profiling helps in the rapid diagnosis of molecular based prognostic
13. Provides wider representation of genomic data from multiple metastatic sites
14. Enables the study of tumor dynamics and mutational drift over time
15. Makes possible the characterization of new lesions
16. Estimates risk for metastatic relapse (prognostic information)
17. Understands metastasis development in cancer patients and plays a role the in future management of cancer patients

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Techniques for the quantification of tumor-associated genetic mutations (not yet standardized)

1. ARMS (amplification refractory mutation system)
2. BEAMing (beads, emulsion, amplification, magnetics)*
3. Digital PCR (droplet PCR)
4. MAP (MIDI-activated Pyrophosphorolysis)
5. MASA (mutant allele specific amplification)
6. Mass spectrometry geno-typing assay-mutant-enriched PCR
7. me-PCR (mutant enriched PCR)
8. NGS (next generation sequencing) – targeted or whole genome*
9. COLD-PCR (co-amplification at lower denaturation temperature PCR)
10. PCR-SSCP (single-strand conformation polymorphism)
11. qPCR (quantitative PCR)
12. RFLP-PCR (restriction fragment length polymorphism PCR)
13. SNV (single nucleotide variants)
14. WGS (whole-genome sequencing)

The amplification-refractory mutation system (ARMS)

• A simple method for detecting any mutation involving single base changes or small deletions.
• Based on the use of sequence-specific PCR primers that allow amplification of test DNA only when the target allele is contained within the sample.
• Following an ARMS reaction the presence or absence of a PCR product is diagnostic for the presence or absence of the target allele.

BEAMing (beads, emulsion, amplification, magnetics)

• Is a powerful method for detecting known genetic mutations in a blood sample, even when at very low copy numbers - capable of detecting a single mutant allele in a background of 10,000 wild-type alleles.
• Relies upon the utilization of high-fidelity DNA polymerases which minimizes error rate and ensures the detection of false positives/negatives.
• Does not significantly lower the diagnostic value of the technique.
• Probes for the detection of a mutated allele must be personalized for each patient. Requires a screening tumor biopsy in advance to capture the mutational profile which is almost always carried out in the clinic through either surgery or initial tumor biopsy.
• Has many applications including optimizing patient recruitment during clinical trials.
• In the future may also prove valuable as a diagnostic technique for making informed treatment decisions, as well as for tracking the occurrence of resistance mutations during and after treatment, so that medication can be adjusted accordingly to maximize therapy effectiveness.

Digital PCR (droplet PCR)

• A new approach to nucleic acid detection and quantification that offers an alternate method to conventional real-time quantitative PCR for absolute quantification and rare allele detection.
• Works by partitioning a sample of DNA or cDNA into many individual, parallel PCR reactions; some of these reactions contain the target molecule (positive) while others do not (negative).
• A single molecule can be amplified a million-fold or more. During amplification, TaqMan® chemistry with dye-labeled probes is used to detect sequence-specific targets.
• When no target sequence is present, no signal accumulates.
• Following PCR analysis, the fraction of negative reactions is used to generate an absolute count of the number of target molecules in the sample, without the need for standards or endogenous controls.
• The use of a nanofluidic chip provides a convenient and straightforward mechanism to run thousands of PCR reactions in parallel.

Digital PCR (continued)

• No need to rely on references or standards
• Ability to increase precision by using more PCR replicates
• High tolerance to inhibitors
• Capability to analyze complex mixtures
• Linear detection of small-fold changes

MIDI-Activated Pyrophosphorolysis (microinsertions/deletions/indels) - MAP

• MAP has an analytical selectivity of one part per billion for detection of MIDIs and an analytical sensitivity of one molecule.
• MAP provides a general tool for monitoring ultra-rare mutations in tissues & blood.
• A method of ultra-high analytical selectivity for detecting MIDIs.
• The high analytical selectivity of MAP is putatively due to serial coupling of two rare events: heteroduplex slippage and mini-pyrolyophosphorylation.
• MAP generally has an analytical selectivity of one mutant molecule per >1 billion wild type molecules and an analytical sensitivity of one mutant molecule per reaction.
• The analytical selectivity of MAP is about 100,000-fold better than that of Pyrophosphorolysis Activated Mutagenization Allele-specific amplification (PAP-A) for detecting MIDIs.
MASA (mutant allele specific amplification)

- A method for point mutation detection.
- The target gene is amplified by a biotinylated mutant specific sense primer and a Ru(bpy)(3)(2+) (TBR)-labeled universal antisense primer.
- Only the mutant allele can be selectively amplified by the mutant specific primer pair.
- Then, the MASA product is captured onto the streptavidylated magnetic beads through biotin-streptavidin linkage and detected by measuring the electrochemiluminescence ECL emission of TBR.
- This method is applied to detect a possible point mutation at codon 12 of K-ras oncogene in 30 colorectal cancer clinical samples.

Mass Spectrometry geno-typing assay-mutant-enriched PCR

- An accurate, flexible and cost-effective genotyping service.
- Saves cost across a huge range of project types.
- Is used for hundreds of different species.

me-PCR (multithreaded e-PCR)

- A refined ultrafast algorithm for identifying sequence-defined genomic elements.
- Adapted the originally described electronic PCR (e-PCR) algorithm to perform string searches more accurately and much more rapidly than previously possible.
- Runs sufficiently fast to allow even desktop machines to query quickly large genomes with very large genomic element sets.
- It multithreaded, interprets all IUPAC nucleotide symbols, allows searches with elements specified by long sequences (such as SNPs).
- Requires substantially less memory for analysis of large sequences and corrects a number of minor flaws causing misreporting of hits in exceptional cases.
- Provides increased annotation capabilities for complex genomes to non-expert laboratories.

Next generation sequencing (NGS)

- "Massively parallel" or "deep NGS sequencing" is a DNA sequencing technology which has revolutionized genomic research.
- An entire human genome can be sequenced within a single day. The previous Sanger sequencing technology, used to decipher the human genome, required over a decade.
- NGS can be used to sequence entire genomes or constrained to specific areas of interest, including all 22,000 coding genes (a whole exome) or small numbers of individual genes.
- Each of the three billion bases in the human genome is sequenced multiple times, providing high depth to deliver accurate data and an insight into unexpected DNA variation.
- All NGS platforms perform sequencing of millions of small fragments of DNA in parallel. Bioinformatics analyses are used to piece together these fragments by mapping the individual reads to the human reference genome.

LIMITATIONS OF NGS

1. Although in genome research NGS has mostly superseded conventional Sanger sequencing, it has not yet transitioned into use in routine clinical practice.
2. Reagent costs are still high (despite improvements in technology).
3. Requires high-quality DNA (which are not found in cfDNA).
4. Extensive workflow (2-100 Peta Bytes (Pb)) and data analysis (requiring a bioinformatician).
5. Extensive data storage requirements.
6. Difficulty in distinguishing the intrinsic background noise of deep sequencing from true tumor-associated tumor alterations which requires professional management of the data collected.

COLD-PCR (coamplification at lower denaturation temperature-PCR)

- Is a novel form of PCR that selectively amplifies low-abundance DNA variants from mixtures of wild-type and mutant-containing (or variant-containing) sequences.
- This amplification is produced irrespective of the mutation type or position on the amplicon.
- It uses a critical denaturation temperature.
- The use of a lower denaturation temperature in COLD-PCR results in selective denaturation of amplicons with mutation-containing molecules within wild-type mutant heteroduplexes or with a lower melting temperature.
- COLD-PCR can be used in lieu of conventional PCR in several molecular applications, thus enriching the mutant fraction and improving the sensitivity of downstream mutation detection by up to 100-fold.
### PCR-SSCP (polymerase chain reaction-single-strand conformation polymorphism)
- One of the simplest and perhaps one of the most sensitive methods for detection of mutations based on PCR technology.
- It is a simple and efficient means to detect any small alteration in PCR-amplified product. It is based on the assumption that subtle nucleic acid change affects the migration of single-stranded DNA fragments.
- Results in visible mobility shifts across a nondenaturing polyacrylamide gel (PAGE).
- Polyclarimide gel is used for analysis of DNA with specialized buffer systems and without area.
- The PCR-SSCP procedure includes following steps: PCR amplification of the gene locus, resolution in nondenaturing PAGE, visualization using silver staining.
- PCR conditions are optimised for PCR-SSCP by testing a number of variables such as concentration of DNA, Taq polymerase, dNTPs (deoxynucleotide triphosphate), MgCl2, and temperature profile.
- The PCR amplification protocol for all the SSCP primers used is same except the annealing temperature, which varies between primers.

### Restriction Fragment Length Polymorphism (RFLP)
- RFLP probes are labeled DNA sequences that hybridize with one or more fragments of the digested DNA sample after they were separated by gel electrophoresis, revealing a unique blotting pattern characteristic to a specific genotype at a specific locus.
- Are co-dominant (both alleles in heterozygous sample will be detected) and highly locus-specific.
- Are typically short, single- or low-copy genomic DNA or cDNA clones.
- Are frequently used in genome mapping and in variation analysis (genotyping, forensics, paternity tests, hereditary disease diagnostics, etc.).
- Detect differences in homologous DNA sequences that can be by the presence of fragments of different lengths after digestion of the DNA samples in question with specific restriction endonucleases.
- Are molecular markers (are specific to a single clone/restriction enzyme combination).

### SNV (single nucleotide variants)
- Detected from the results of NGS studies.
- SNVs requires determining the individual’s genotype at each locus, the phrase “SNP (single nucleotide polymorphism) genotyping” has been used to refer to this process.
- NGS based methods for SNV detection are designed to detect germline variations in the individual’s genome. Somatic SNVs can be detected within an individual using multiple tissue samples.
- Due to the increasing abundance of NGS data, these techniques are becoming increasingly popular for performing SNP genotyping to identify rare SNPs within a population.

### WGS (whole genome sequencing)
- Also known as full genome sequencing, complete genome sequencing, or entire genome sequencing.
- A laboratory process that determines the complete DNA sequence of an organism’s genome at a single time.
- Used for fetal, neonatal, perinatal, pediatric, adult and rare genetic diseases (56 genes).
- Enables sequencing all of an organism’s chromosomal DNA as well as DNA contained in the mitochondria and, for plants, in the chloroplast.
- Can provide raw data on all six billion nucleotides in an individual’s DNA.
- Does not provide an analysis of what that information means or how it might be utilized in various clinical applications, such as in medicine to help prevent disease.
- Generates a lot of data (for example, there are approximately six billion base pairs in each human diploid genome). Its output is stored electronically.
- Requires a large amount of computing power and storage capacity.

### Real Time quantitative PCR (qPCR)
- Is very similar to traditional PCR.
- The amount of PCR product is measured after each round of amplification and not at the end point as with traditional PCR.
- Amplification products are measured as they are produced using a fluorescent label.
- During amplification, a fluorescent dye binds, either directly or indirectly via a labeled hybridizing probe, to the accumulating DNA molecules.
- Fluorescence values are recorded during each cycle of the amplification process.
- The fluorescence signal is directly proportional to DNA concentration over a broad range.
- The linear correlation between PCR product and fluorescence intensity is used to calculate the amount of template present at the beginning of the reaction.
- The point at which fluorescence is first detected as statistically significant above the baseline or background, is called the threshold cycle or Ct Value.
- The Ct Value is the most important parameter for quantitative PCR.
- This threshold must be established to quantify the amount of DNA in the samples.

### WGS (continued)
- Whole Exome Sequencing (hypothesis free test)
- Whole Genome Sequencing (hypothesis free test)
- Targeted Exome Sequencing for the following disorders (hypothesis driven)
  - Brain and neurodevelopmental disorders
  - Bone diseases
  - Cancer syndromes
  - Connective tissue disorders
  - Epilepsy
  - Metabolic diseases
  - Mitochondrial diseases
  - Neuromuscular diseases
  - Renal diseases
- Clinical costs which includes costs for time spent by the Genomic Odyssey Board are included (only $ 3000 is currently reimbursed)
- Extensive workflow (2-100 Petabytes - PBs) and data analysis (requiring a bio-informatician)
**Tam-Seq (tagged-amplicon deep sequencing)**

- A method for screening low-frequency mutations.
- Identifies cancer mutations present in circulating DNA at allele frequencies as low as 2%, with sensitivity and specificity of >97%.
- Tam-Seq can noninvasively identify the origin of metastatic relapse in a patient with multiple primary tumors.
- Can be used to monitor tumor dynamics, and track concomitant mutations in plasma of metastatic cancer patient over several months.
- Is a low-cost, high-throughput method could facilitate analysis of circulating DNA as a noninvasive "liquid biopsy" for personalized cancer genomics.

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**PROCEDURES/TECHNIQUES EMPLOYED TO COLLECT AND STORE SAMPLES (blood, plasma, serum or DNA)**

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Tumor-Specific Aberration</th>
<th>Source</th>
<th>Technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorectal cancer</td>
<td>APC, KRAS, PIK3CA, TP53</td>
<td>Plasma, Serum</td>
<td>BEAMing, PCR, SSCP, ME-PCR</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>PIK3CA, TP53 (structural variation)</td>
<td>Plasma, Serum</td>
<td>TAm-Seq, digital PCR, Fluorescence-PCR</td>
</tr>
<tr>
<td>Ovarian cancer</td>
<td>PIK3CA, PTEN, EGFR, BRAF, KRAS</td>
<td>Plasma, Serum</td>
<td>TAm-Seq, digital PCR, Fluorescence-PCR</td>
</tr>
<tr>
<td>Hepatocellular cancer</td>
<td>SNV</td>
<td>Plasma WGS</td>
<td></td>
</tr>
<tr>
<td>Pancreatic cancer</td>
<td>KRAS</td>
<td>Plasma</td>
<td>NGS, PCR</td>
</tr>
<tr>
<td>Oral squamous cell cancer</td>
<td>Microsatellite test</td>
<td>Serum</td>
<td>PCR</td>
</tr>
<tr>
<td>Colorectal and breast cancer</td>
<td>Chromosomal alterations</td>
<td>Plasma and Serum</td>
<td>Nested real-time PCR</td>
</tr>
</tbody>
</table>

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**SELECTION OF TUMOR MARKERS AND TECHNOLOGIES IN LIQUID BIOPSY**

- Multiple mutational panels versus personalized panels based on the sequence of the cancer of an individual.
- Technology dependent.
- BEAMing used for selected genes (or single probes for rare variants designed on a personalized basis).
- NGS used for screening large panels of genes.
- Cost of personalization on a patient-by-patient basis is very high.
- Deep-sequencing of panels of somatic mutations in the clinic is not routinely performed.
- Screening panels of somatic mutations that are commonly mutated in cancer will enable the identification of infrequent mutations (such as KIT mutations in breast cancer that occur at a frequency of approximately 1%).

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Circulating Tumor Cells

CTCs Selection Methods
1. Size-based membrane filters - considerable overlay between CTCs and leukocytes
2. CellSearch system (Veridex). FDA approved for breast, prostate and colorectal cancers.
   - Semi-automated system that enriches for cells expressing epithelial-cell adhesion molecules (EpCAs) but lacking the leukocyte-specific molecule (CD45), cells are further immunostained with fluorescent-labeled anti-keratin antibodies.
   - With this system baseline and follow-up CTC levels are reported to be strong predictors for progression free and overall survival.
3. CTC-chip - a non-transparent three-dimensional array of microposts
4. micro-Hall detector - A microfluidic device for enhanced CTC capture which detects the magnetic movement of cells
5. These new CTC microfluid devices have not been validated to a level comparable as the CellSearch system.

ANALYSIS of CTCs
1. Protein-based analysis: (EPISPOT assay) - distinguishes between apoptotic and viable CTCs
2. Quantitative immunofluorescence protein analysis to simultaneously visualize differently labeled targets within the CTCs
3. Quantifiable, dual-colorimetric RNA-ISH assay that uses pooled epithelial and mesenchymal transcripts.
4. Interphase fluorescence in situ hybridization that assesses cytogenetic composition
5. High resolution single cell DA sequencing

CANCER MONITORING WITH CTCs
1. Disease Monitoring: even in the presence of peripheral blood CTCs are prognostic biomarkers and can be measured in patients with cancer.
2. By monitoring CTCs the acquisition of recurrent drug resistance can be uncovered
3. Mutation analysis for a specific resistance maker and genome-wide analysis strategies are expected to capture all possible mechanisms of resistance
4. CTCs obtained several months after diagnosis of the primary tumor and metastasis has shown a high level of amplification following treatment
5. CTC biology has the potential to discover processes that are instrumental for metastasis.

Free circulating nucleic acids
DNA, mRNA, miRNA

Circulating Tumor DNA (ctDNA)
1. ctDNA originates from both apoptotic cells (in healthy individuals) and necrotic cells (in cancer patients)
2. ctDNA analysis is non-invasive and convenient because it can be performed repeatedly
3. DNA from apoptotic cells is released in 185-200 bp fragments, DNA released form malignant cells is considerably longer
4. The whole exome sequencing of cDNA through Next Generation Sequencing (NGS) has been shown to be relevant information about the molecular status of the tumor.
**Circulating DNA (ctDNA and cfDNA)**  
*(Summary of Dr. Lin’s talk in this symposium)*

- Cancer is a genetic disease  
- Tissue is the gold standard but now plasma (liquid biopsy) are being used  
- NGS is the most widely used technique for circulating DNA  
- A very small amount of DNA can be detected in the blood (1 in 10,000)  
- The limit of detection of circulating DNA is very low (0.05 – 0.009, which is almost at signal noise levels - at the level of polymerases)  
- ctDNA and cfDNA is used for detection, monitoring, resistance, tumor heterogeneity and mutations  
- Work flow depends on the following:  
  - Disease specific  
  - Disease amplicons  
  - Patient specific targets

**Tracer X – a new technology for the early detection of circulating DNA in cancer using liquid biopsy**

1. Has been developed recently for early stage lung cancer  
2. The sequencing is done at multiple times  
3. The tumor heterogeneity is watched over time  
4. Detection levels are as follows:  
   1. 3.75  
   2. 5.01  
   3. 1.71  
   4. 0.57  
   5. 0.38  
   6. 23.00

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**Circulating Tumor DNA (ctDNA)**  
*Detection methodology in serum and plasma*

1. Avoid rupture of blood cell membranes to avoid contamination with DNA from blood cells  
2. Select the most appropriate methods to gain a sufficient amount of quality DNA  
3. Detection of ctDNA through real-time PCR or digital PCR and Next Generation Sequencing (NGS)

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**CIRCULATING CELL-FREE DNA (cfDNA)**  
*(slide adapted from: Dr. Thorsten Voss, Senior Scientist R & D PreAnalytix)*

1. Mostly strongly fragmented (<500bp)  
2. Also could include long fragments (≥ 500bp) from necrotic processes as well  
3. Very low concentrations in plasma, serum, urine and other body fluids (<100ng/ml)

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**miRNAs**

- Have been studied for their regulatory roles in many aspects of biology, including cancer and other diseases.  
- Circulating cell-free miRNA (cfmiRNA) may be useful as non-invasive biomarkers of cancer.  
- cfmiRNAs have been found in various malignancies  
- Have their diagnostic values  
- cfmiRNAs have several clinical applications.

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**Cell-free microRNAs as cancer biomarkers**

Detecting the circulating microRNAs in blood helps in cancer detection, with the cfRNA being studied for its regulatory roles in many aspects of biology, including cancer and other diseases. In recent years, it has been demonstrated that circRNA can be used as a new tool for detecting various biomarkers for cancer.

*Adapted from: Thomas Jefferson University.*
Cell-free RNA (exosomes)

- Exosome analysis in cancer is a new field.
- Exosomes are considered to be “operating/active secretions” of cancer cells.
- These microvesicles more than simple exophytic budding of a cell membrane.
- Are considered to be representative of the functional status of tumor cells.

EXOSOMES
Total RNA, DNA, Protein

Summary: Predictive Markers in the Liquid Biopsy

- Continue to improve the detection limits of cancer.
- Decrease the amount of nucleic acid template.
- Expand the number of genes available for analysis.
- Overall estimation of tumor characteristic with a snapshot of circulating nucleic acids will support treatment decisions.
- Major limitation of future treatment decisions based on liquid biopsy is the rise of resistance mechanisms that change the genotype-directed therapy in solid tumors.
- Cell-free DNA not only monitors treatment response but also detects early resistance mechanisms (Ouazit et al. 2014).
- This sets the basis for the development of cell-free DNA as a new and non-invasive biomarker that can be used routinely as a predictive marker for targeted therapies.
- Liquid biopsy, by providing new insights, is paving the way to changing the approach to patient management, guide treatment decisions and improve clinical outcomes.
- Liquid biopsy can be used as a screening or a diagnostic test for cancer.
- Formal validation will overcome the hurdle between theoretical robustness, laboratory data and translational experience.

Considerations and Major Limitations for Introducing Liquid Biopsy into Clinical Practice

1. Lack of standardization of techniques for liquid biopsies in the clinic: ranging from the material from which DNA is extracted (plasma versus cancer) to techniques for the quantification of tumor-associated genetic mutations (digital PCR, NGS, BEAMing).
2. Validation in prospective clinical studies.
3. The value of Circulating Tumor Cells (CTCs) - which is presently mainly correlated only with prognosis.
4. There is no information available regarding the molecular characterization of CTCs.
5. Except for the Veridex system, there is no other FDA-approved validated technique for either cell-free DNA or cell-free RNA (exosomes) analysis.
6. Cell-free DNA (cfDNA) evaluation procedures and procedures for blood collection and downstream analysis are not yet standardized, optimized, and approved.
7. Comparison with standard assessment for treatment response (RECIST criteria) will be required.
8. Tumor/liquid biopsy discrepancies will have to be resolved.
9. Numerous passive and active mutations that accumulate during the lifetime of a tumor make selecting multiple biomarkers difficult.