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Liquid biopsy - emergence of a new era in personalized cancer care

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Abstract

The most successful treatment for cancer involves identifying druggable, biological markers for targeted therapy. In the clinical setting, surgical removal of tumors is the only procedure for identifying such targetable molecules. Shed from tumor cells, these markers are also present in circulating blood, albeit in very negligible amounts. Liquid biopsy is a procedure performed on a blood sample to look for such circulating cancer markers cells or pieces of nucleic acid from the tumor. The procedure shows promise in revolutionizing personalized cancer treatments. Here we briefly review the technique, characterization, and its utilization in clinics.

Keywords: Liquid biopsy, Circulating tumor cells, Cell free DNA, Cell free RNA, Clinical utility

Background

Today, cancer remains the leading cause of premature deaths worldwide. Treatment relies on profiling a piece of biopsied tumor tissue. However, the ease of acquiring biopsy depends on patient condition and tumor accessibility. In the case of advanced or metastatic non-small cell lung cancers (NSCLC) as many as 31% of cases do not have accessible tissue [1]. Likewise, majority of patients with pancreatic cancer progress to either locally advanced or metastatic disease in the asymptomatic phase and as many as 80% presents late with metastasis at diagnosis [2]. Progress remains hindered also by diverse landscape of tumor and technical limitations involved in sampling of biopsied tissue. Following excision, biopsy samples are fixed and sections, similar to a bread loaf, are cut and the tddop most layers are sliced again for pathological staining. But tumors are characterized by intra-tumor heterogeneity arising from clonal evolution of individual tumor cells (Fig. 1) [3]. So, the technique may fail in capturing newly evolving, genetically distinct cells that do not lie on the surface of the bread-loaf section (Fig. 2). Similarly, primary tissue from pancreatic ductal adenocarcinoma patients is usually available only by fine-needle aspiration biopsies and there is a high chance of missing aggressive clones [4]. Besides, a standard protocol in cancer management involves periodic monitoring for progression and/or recurrence of the cancer. Because a tissue biopsy can be painful and expensive, most patients shy away from a repeat biopsy. And in most cases the physician does not know where to look for metastasis. Even, commonly used imaging techniques like ultrasonography, positron emission tomography (PET), computed tomography (CT) and magnetic resonance imaging (MRI) [5] cannot detect many early-stage cancers and very small metastases [6]. While challenges in obtaining adequate tumor tissue and issues of heterogeneity continue to hamper tissue profiling, minimally invasive technologies to capture genomic contents of tumor in various bodily fluids like blood, urine, saliva, sweat and tears combined with sensitive genotyping assays, have become available. “Liquid biopsy” is the term coined to describe such diagnostic procedures performed on cancer-derived material captured in a blood sample.

Since, cancer cells that detach from solid tumors circulate in the peripheral blood, analyzing blood of patients with cancer holds the possibility for capture and molecular analysis of diverse tumor-derived materials. In normal, healthy individuals, cellular debris from apoptotic or necrotic cells is normally phagocytized by infiltrating macrophages and cleared from the circulation. However, this clearance mechanism does not proceed effectively in cells derived from tumor mass, leading to an accumulation of cellular debris including DNA, and its release into the circulation [7]. By and large, cellular components sampled

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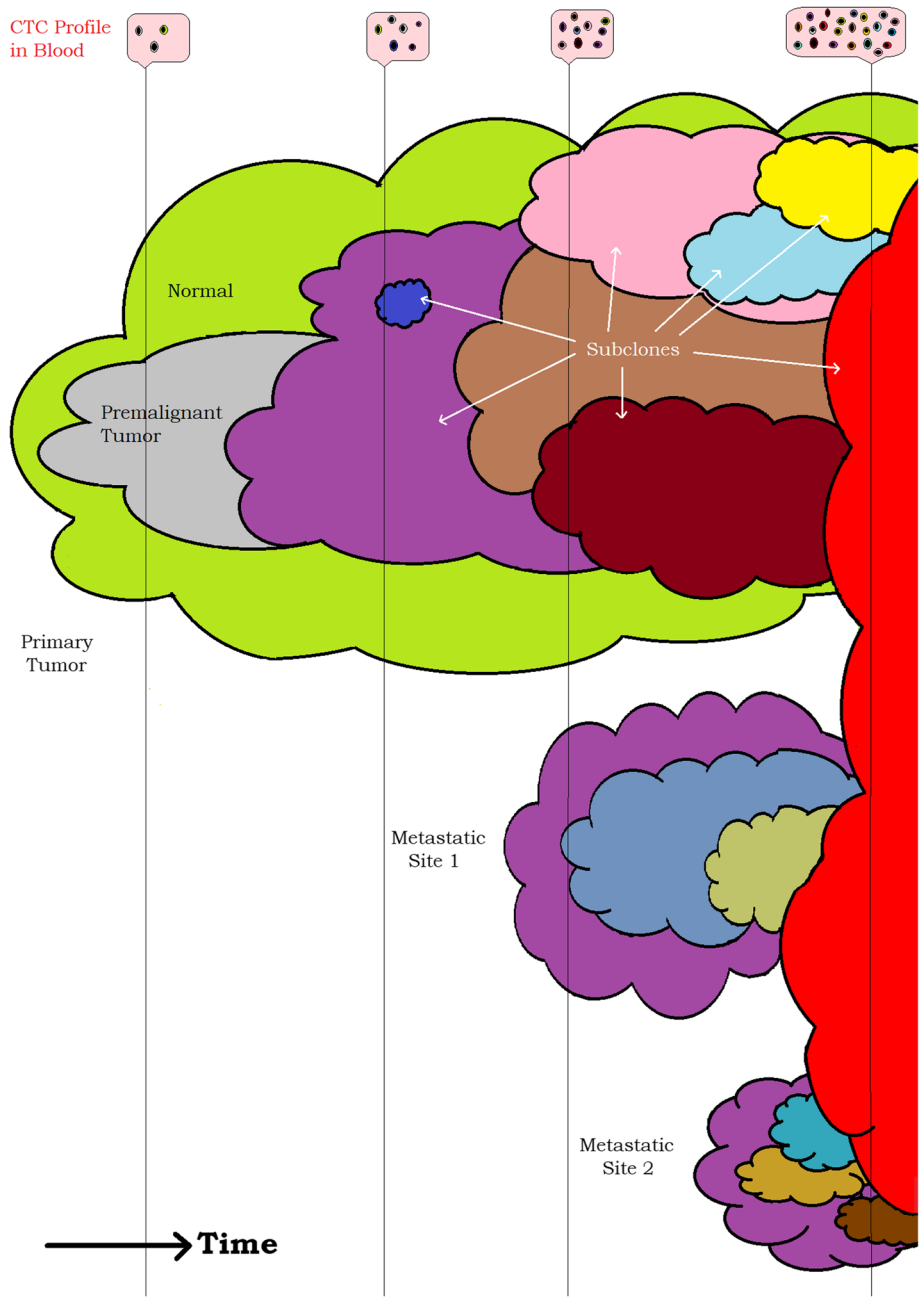

Fig. 1 Snapshot of the evolving tumor landscape. Tumor cells undergo frequent mutations resulting in emergence of distinct sub clones. This leads to ever evolving/ changing landscape of tumor cells which are difficult to monitor by traditional standard biopsy. But with recent advances in single cell detection technologies, it has become possible to capture and characterize cells shed from these distinct sub clones into the blood. They are shed either by dying tumor cells or are tumor cells that have detached from parent primary tumor and have metastatic abilities

from the blood of cancer patients is highly consistent with gene alteration patterns reported in traditional tumor tissue testing (https://www.cancer.gov/news-events/cancer-currents-blog/2016/asco-liquid-biopsy) [8, 9], which makes these circulating cancer-derived materials in the bloodstream an appealing alternative to overcoming some of the challenges described above. Because blood collection is simple and minimally invasive, this alternative method is currently being developed by many investigators, particularly with the aim of obtaining a complementary tool to tumor biopsy to predict what drugs will work for a patient and monitor how a tumor changes over time. Herein, we provide a brief overview of the various types of tumor-derived material that can be sampled using liquid biopsies. Subsequently, we discuss the available technologies for extraction of molecular information from liquid biopsy

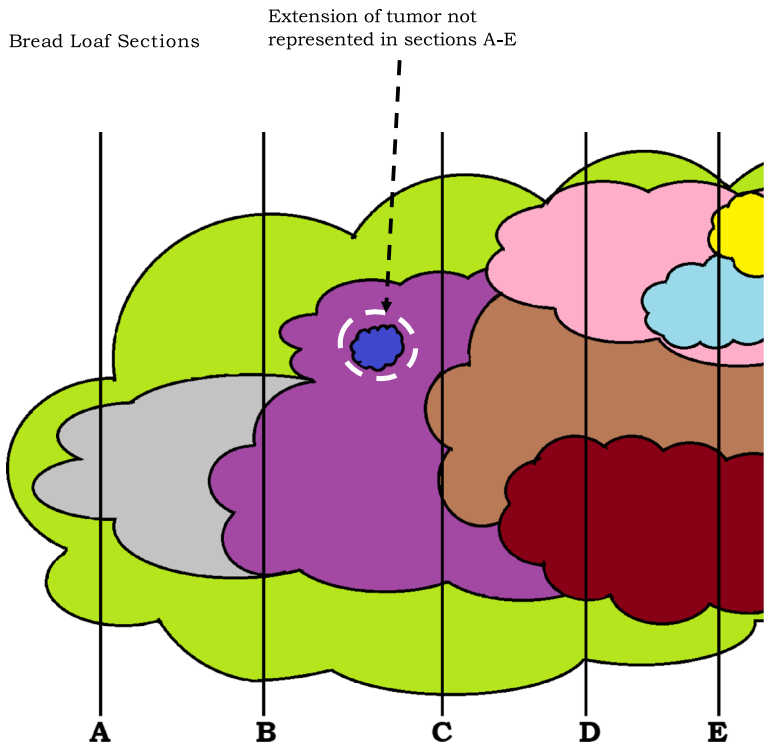

Fig. 2 Conventional sectioning of tissues using the bread loaf sectioning technique. Standard tissue biopsy evaluation of excised tissues involve bread-loaf technique wherein vertical sections of the tissues are embedded in paraffin or frozen, sectioned with microtome and stained. As illustrated here, residual tumor or tumor extensions may not be found and may recur

samples and their clinical use, focusing mostly on those associated to DNA derived from blood samples.

Approaches to liquid biopsy

Cancer at any stage can shed tumor cells as well as fragments of cancer-causing DNA into the blood system and may give good indication of mutations in the tumor at the time of sampling. The more advanced the cancer, the more likely tumor cells and cancer-causing DNA can be found in the blood. Liquid biopsy techniques detect different blood-based biomarkers including circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), also known as cell free DNA (cfDNA), circulating RNA (cfRNA), and exosomes.

Circulating Tumor Cells (CTCs)

CTCs represent intact, viable non-hematological cells with malignant features that can be isolated from blood [10]. As early as 1869, Ashworth described CTCs in cancer patients [11] and its presence in blood stream was demonstrated by Engell in 1955 [12]. However, interest in CTCs increased when high CTC counts in blood samples from patients with metastatic breast cancer correlated with poor prognosis and therefore showed prognostic potential. CTCs are released into the bloodstream during metastatic spread of the cancer through blood and are present as single cells or clusters [13]. They have been detected in various metastatic carcinomas including lung [14], breast [15, 16], prostate [17], and colorectal cancer [18] but are extremely rare in healthy subjects and patients with nonmalignant diseases [19]. Even in patients with metastatic cancer, they occur on average at a frequency of 1 in 100 million cells and are mixed with approximately 10 million leukocytes and 5 billion erythrocytes per 1 ml blood [20]. And, due to apoptosis of CTCs, which begins soon after separation from the tumor of origin, they are extremely fragile [21]. Next generation sequencing (NGS) has revealed CTCs carry mutation signatures that resemble the signatures of their primary tumors including driver and druggable mutations like *APC, KRAS, TP53, ERBB3, FBXW7* and *ERBB2* [22].

Cell free DNA (cfDNA)

Circulating cell-free DNA (cfDNA) are small DNA fragments found circulating in plasma or serum, as well as other bodily fluids. The presence of cfDNA in blood plasma was discovered in 1948 by Mandel and Metais [23]. But it was only recently shown that molecular profile of cfDNA is similar to tumor tissue DNA. A major breakthrough came with the advancement of NGS technologies for mutation detections which allowed for the

sequencing of DNA and RNA much more quickly and cheaply than the previously used Sanger sequencing. Many clinical trials are intensively investigating correlation of mutations in cfDNA to disease progression especially for hard to access cancers such as NSCLC and pancreatic cancer [24]. Patients with distant metastases have a significantly higher level of cfDNA compared to patients without metastases. Total mutation–specific cfDNA decreases during treatment but the levels increase later on in patients with recurrence [25, 26].

Plasma cfDNA usually are fragments of about ~170–500 bp, thought to originate mostly from apoptotic cells [27]. Initial studies, such as those performed by Diaz et al., suggest that when both cfDNA and CTCs are present, cfDNA fragments outnumbered CTCs by 50 to 1 [28]. In addition, a direct comparison of mutation detection on cfDNA vs CTCs showed a higher abundance of the mutation on the cfDNA from the same patient [29]. An advantage of cfDNA is that they are relatively stable and can be analyzed from bio-banked biofluids, such as frozen plasma. The most commonly used vacutainers for blood collection for isolation of cfDNA are proprietary Streck Cell-Free DNA BCT® and CellSave tubes and standard K2EDTA tubes. Studies comparing the optimal conditions for blood collection and storage temperature for cfDNA have found similar abundance and stability for up to 6 h in all tube types with no effect on the yield [30, 31].

Exosomes and circulating RNA

Tumor cells actively release several species of cfRNAs, into the blood including non-coding RNAs (e.g., microRNAs or miRNA, small nucleolar RNAs, PIWI-interacting RNAs, and long non-coding RNAs). Such species are enriched in exosomes and strongly resist RNases. High levels of exosomes are found in several bodily fluids from cancer patients [32] and because of their resistant nature, exosomes and miRNA can be isolated from biofluid samples and stored for many years in the freezer [33]. Their composition seems to reflect that of the parental cells and therefore provide a novel type of biomarker for various patient scenarios [34]. Like cfDNA, circulating miRNA seems to give a better picture of variations present in the tumor than CTC [35]. In the last few years, different alterations have been described inside the exosomes derived from NSCLC cells mirroring the processes inside tumor cells, such as *EGFR* mutation, translocations, or miRNA deregulation [36].

Recent studies have shown that plasma miRNA levels significantly correlate with larger tumor size, chemoresistance, and recurrence of tumors [37]. miRNA-144-3p and miR-210 are significantly up-regulated in plasma and tissues from clear cell renal cell carcinoma compared to healthy control plasmas or urine samples [38, 39]. A study of plasma miR-224 levels in patients with hepatocellular carcinoma found that plasma levels could accurately predict presence of small tumors which were less than 18 mm preoperatively [37]. Similarly, the overexpression of miR-21 and its detection in plasma contributed to chemo-resistance in esophageal squamous cell carcinoma [40].

Not all miRNA is upregulated in cancer, however. Fan and coworkers found five serum miRNAs (miR-16-5p, miR-17b-5p, miR-19-3p, miR-20a-5p, and miR-92-3p) that were significantly downregulated while miR-15b-5p was significantly upregulated in NSCLC [41]. Treatment procedures also affect the levels of circulating miRNA. After chemotherapy, levels of miR-199b-5p, miR-301b, miR-326, miR-361-5p, miR-625 and miR-655 reduced in plasma from patients with acute myeloid leukemia though they were abundant at diagnosis [42]. Similarly, plasma miR-375 could differentiate between patients with prostate cancer and benign prostatic hyperplasia [43].

Extraction of molecular information

The first step in attaining valuable information pertaining to cancer treatment is efficient isolation and specific recovery of the circulating CTC, cfDNA and cfRNA shed by the tumor cells while leaving behind those molecules shed by normal cells. Since very few CTC, cfDNA, exosomes and cfRNA are circulating in the blood, the initial step involves enrichment or purification of these tumor released materials from other blood components in order to increase sensitivity and specificity. They have been purified on the basis of physical properties, including size, density, and electric charges. CTCs can also be positively or negatively enriched on the basis of the expression of cell surface antibodies like EpCAM. Most authors use QIAamp Circulating Nucleic Acid (Qiagen) which utilizes magnetic bead based technology to isolate plasma tumor derived DNA. A comparison of three popular kits by authors Pérez-Barrios and group found no significant differences in recovery of cfDNA extracted using kit from Qiagen or Maxwell® RSC ccfDNA Plasma Kit (Promega) but the cfDNA yield from MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche) was significantly less [44]. But others have found that the yield of cfDNA using the traditional phenol/chloroform/ethanol method in the presence of glycogen was better than that isolated in the absence of glycogen or by commercially available kit [45]. After isolation, subsequent analysis includes fluorescence in-situ hybridization (FISH), microarray, immunofluorescence, sequencing, flow cytometry and RT-PCR. Digital PCR comprising of droplet-based systems (ddPCR), microfluidic platforms for parallel PCR, NGS, BEAMing (Beads, Emulsions,

Amplification and Magnetics) and amplification-refractory mutation system (Scorpion-ARMS) assay are additional techniques that can detect rare circulating tumor DNA sequences without the need for reference standard or standard curves.

Current approaches for detection of liquid biopsy components from blood can be divided into three different categories: methods targeting specific druggable driver mutations or all possible aberrations in cfDNA, methods to isolate and identify CTCs, and methods to isolate and identify exosomes, which are discussed in detail below.

Methods targeting druggable mutation and other aberrations in cfDNA

Majority studies report screening for mutations that have been already validated in cancer tissue biopsy and compare the concordance of patient-matched plasma and tumor tissue samples. Over expression of mutated epidermal growth factor receptor (*EGFR* or *ErbB1* or *HER1*) is one such major marker seen both in tissue biopsy and cfDNA. The two most common mutations are exon 19 deletions (Del19) and L858R missense substitutions resulting in constitutive activation of the receptor without ligand binding. Presence of these mutations are strong predictors of efficacy for tyrosine kinase inhibitors (TKI) such as Gefitinib, Afatinib, and Erlotinib, or mAb such as Cetuximab. Therefore, these mutations on *EGFR* are also known as TKI- sensitive mutations [46]. A point mutation that substitutes methionine for threonine at amino acid position 790 (T790 M) produces majority of TKI acquired resistant cases for first generation *EGFR* inhibitors and is known as TKI- resistant mutation [47]. For detection of *EGFR* mutations, maximum authors use commercially available primer and probes and amplification by real time PCR. However, there have been instances of misdiagnosis of mutations in *EGFR* by routine *EGFR* mutation tests [4, 48].

Wu et al. [49], recently published a paper on evaluating the feasibility of detecting *EGFR* mutations in lung adenocarcinoma. Using the Therascreen EGFR 29 kit (Qiagen), they tested cfDNA purified from either 3.0 ml serum or 4.0 ml plasma to detect 29 somatic mutations in the *EGFR* by real time PCR. They found higher mutation detection rates in plasma than in serum (60.5% and 28.6% respectively). Patients who were cfDNA+ for *EGFR* mutations exhibited characteristics associated with more advanced disease compared with cfDNA− patients. Afatinib significantly improved progression free survival vs chemotherapy in patients with common *EGFR* mutations, the effective benefit being more pronounced in patients with Del19 vs L858R mutation-positive tumors (8.3–9.7 months vs 3.3–4.6 months; $P = 0.0009$). Karachaliou and colleagues [50] found serum *EGFR* L858R mutation correlated with overall survival, progression-free survival, and response to Erlotinib therapy in a cohort of NSCLC patients enrolled in EURTAC trial. For their screening, they used an in-house developed peptide nucleic acid mediated 5′-nuclease real-time PCR. Yu et al., developed a multiplex picoliter-droplet digital PCR for quantitative assessment of *EGFR* mutations in cfDNA derived from advanced NSCLC patients. They found fluctuations in *EGFR* mutant abundance in serial plasma cfDNA (collected over 2 months) correlating with the changes in tumor size as assessed by imaging scans [51].

Mok and colleagues [25] extracted cfDNA from blood and used allele-specific Cobas 4800 PCR assays from Roche Molecular Systems Inc. to detect *EGFR* mutations in NSCLC patients. The study cohorts included patients randomized to receive platinum-based chemotherapy plus sequential Erlotinib or placebo. The authors considered samples showing at least one activating mutation (Del19, L858R, G719x, or L861Q) to be positive for *EGFR* mutations. Patients with *EGFR* mutation–specific cfDNA and treated with Erlotinib presented a significantly better outcome than patients treated with placebo [progression-free survival-13.1 vs 6.0 months; $P < 0.0001$], while no difference emerged between those who were negative for *EGFR* mutation–specific cfDNA and treated with Erlotinib or placebo [progression-free survival-6.2 vs 6.1 months]. The ASSESS trial in Europe and Japan (NCT01785888, designed for real-world diagnostic validation of Therascreen EGFR PCR Kit (Qiagen), cobas EGFR Mutation Test (Roche), Cycleave (Takara Bio Inc., Kusatsu, Japan) and PNA-LNA PCR Clamp kits (Qiagen), for testing *EGFR* mutation found identical sensitivity when both plasma and tissue was tested using the same commercial kit [52]. Phase I expansion component of the AURA Phase I/II study (NCT01802632) conducted in USA and Japan, also, validated the use of various *EGFR* testing methodologies including cobas® EGFR Mutation Test, Sanger sequencing, Therascreen®, PNAClamp™, and Sequenom MassARRAY® [53].

TKI-sensitive and TKI-resistant mutations are not the only mutations detected in blood cfDNA. Seki et al. [54], did find TKI-sensitive (L858R and Del 19) mutations in patients with lung adenocarcinoma who received TKI therapy but developed resistance. And, only half of resistant patients harbored TKI-resistant (i.e., T790 M) mutations in cfDNA and tissues. Instead, L747P substitution mutation was found in a patient who was TKI-resistant, harbored TKI-sensitive mutant DNAs and did not have the TKI-resistant - T790 M mutation. L747 participates in a key hydrophobic core that stabilizes the inactive form of *EGFR*. Therefore, leucine to proline substitution would disfavor the formation of this hydrophobic core, thereby leading to constitutive activation of

the mutant *EGFR* [55] and could explain resistance to *EGFR* inhibitor. Chabon and colleagues [56] performed CAPP-Seq cfDNA analysis from 2 to 4 ml of plasma to identify mechanisms responsible for resistance to a third-generation *EGFR* inhibitor, rociletinib, in NSCLC patients. They found a novel L798I mutation which directly lies adjacent to the covalent binding site of rociletinib in *EGFR*, thereby preventing rociletinib binding and resulting resistance.

Non responders to anti-*EGFR* therapy is also seen in individuals with somatic mutations in the *KRAS* proto-oncogene. Majority cases present various single point mutations at codon 12. Biorad has a commercial kit comprising of ddPCR amplification using the QX200™ Droplet Digital™ PCR System and the PrimePCRTM *KRAS* Mutant Probe assays to detect G12D, G12R and G12 V mutations in *KRAS*. These are the most frequent *KRAS* mutations found in sporadic pancreatic ductal adenocarcinoma primary tumors. The specificity for detection of G12D and G12R is 100%. However, there are reports of non-specific amplification of G12D mutant DNA with the G12 V assay. The system is quite sensitive for the detection of mutant DNA as about 0.5 ng corresponding to 37 copies can be detected by this technique. Pancreatic cancer patients that tested positive for any of the *KRAS* mutation in plasma cfDNA, using the Biorad system, had a significantly shorter overall survival than patients who tested negative for a mutation (60 days vs 772 days respectively) [4].

Nygaard and group [26] used in-house designed primers and qPCR to detect six mutations of codon 12 and one in codon 13 of the *KRAS* gene in patients with newly diagnosed, histopathologically confirmed stage III-IV NSCLC and at follow up following chemotherapy alone or in combination with bevacizumab. Only 10% (7/69) of patients were identified with a plasma *KRAS* mutation before start of treatment which persisted during the treatment course. But in two patients there was no mutation at the start of treatment, but mutation was detected at progression. In another study, analysis of metastases from colorectal cancer patients who developed resistance to cetuximab or panitumumab showed the emergence of *KRAS* amplification in one sample and acquisition of secondary *KRAS* mutations in 60% (6/10) of the cases [57]. Comparable results were found in a prospective study of patients with metastatic colorectal cancer during treatment with third line anti-EGFR therapy, cetuximab and irinotecan. Plasma *KRAS* status, unlike that in tumor tissue, was a strong predictive and prognostic factor for response to these third line anti-EGFR therapy [58, 59].

Others have tried a technique referred to as enriched polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP) to distinguish between wild type and mutant allele. In this technique a mismatched primer is used for PCR, which introduces restriction site and can be used to differentiate the wild type allele from mutant allele. The technique has been tested in differentiating early colorectal lesions based on the presence of mutant *KRAS* status in cfDNA and was found to have significant predictive capability [60–62].

Generally, *KRAS* mutations occur in a mutually exclusive manner along with *BRAF* mutations. Together mutations on these genes lead to the constitutive activation of *EGFR* signaling through the oncogenic *Ras/Raf/Mek/Erk* pathway. The most common mutation in *BRAF* is V600E substitution and individuals with the *BRAF* mutation benefit from targeted inhibition of BRAF protein with BRAF inhibitors [63]. Janku and group analyzed *BRAF* mutations in cfDNA using the Idylla BRAF Mutation Test (Biocartis) - a real-time PCR-based system. *BRAF* V600E mutation was detected in 29% plasma cfDNA samples from patients with diverse advanced cancers. There was 88% agreement with mutations in tissues and sensitivity of 73% and specificity of 98%. A higher percentage of mutant *BRAF* (V600E) corresponded with shorter overall survival (10.7 months vs 4.4 months respectively) and shorter time to treatment failure in patients receiving BRAF/MEK inhibitors. Idylla assay showed 100% concordance with those of ddPCR QX200 (Bio-Rad) and BEAMing (SysmexInostics) [64]. A significant decrease in plasma *BRAF* V600E concentrations was found in patients with melanoma being treated with BRAF inhibitors, dabrafenib or vemurafenib and at the moment of best response but at progression, there was a significant increase in the concentration of plasma *BRAF* V600E [65]. Vemurafenib and dabrafenib are selective inhibitors of activated *BRAF* V600E, and tumor tissues positive for *BRAF* V600E by Cobas 4800 BRAF V600 Mutation Test (Roche Molecular Systems, Inc.) (https://www.cancer.gov/about-cancer/treatment/drugs/fda-vemurafenib), are approved by the US FDA and the European Medicines Agency (EMA) for the treatment of unresectable or metastatic melanoma.

Clinical need for *KRAS* mutation testing is largely related to the use of anti-EGFR antibody therapy. Therascreen ARMS assay (Qiagen), Competitive Allele-Specific TaqMan PCR (castPCR, Life Technologies), and Invader Plus assay with peptide nucleic acid clamping (InvClamp assay (Hologic, Inc. Marlborough, MA, USA) are widely used in both clinical and trial settings to determine equivalence for *KRAS* mutation. castPCR plate includes primer and probes for additional *KRAS* mutations and *BRAF* V600E, which are not included in Therascreen or Invader Plus [66]. There is US FDA approval of the Therascreen KRAS RGQ PCR Kit (Qiagen) and the Cobas® KRAS Mutation Test (Roche Molecular Systems, Inc.) for detecting druggable mutations in

formalin-fixed paraffin-embedded (FFPE) tissues of specific cancers. Routine *KRAS/BRAF* screening is performed before initiating anti-EGFR therapy in patients with colorectal cancers to predict non-responsiveness to anti-EGFR therapy and to prevent drug-induced toxicity thereby avoiding heavy expenses related to the treatment [67]. Tabernero et al., investigated the use of cfDNA and plasma protein biomarkers to predict the clinical utility of regorafenib and assess prognosis in patients with metastatic colorectal cancer enrolled in CORRECT trial. They used BEAMing technology to identify *KRAS*, *PIK3CA*, and *BRAF* mutations in DNA obtained from plasma and FFPE tissue specimens. Patient-matched fresh plasma and FFPE tumor samples showed concordant mutation status in 76% of patients for *KRAS*, 88% patients for *PIK3CA*, and 97% of 236 patients for *BRAF*. Correlative analyses showed a trend for regorafenib clinical benefit across patient subgroups defined by *KRAS* and *PIK3CA* mutational status. Most of the discordance in *KRAS* status were due to the detection of a mutation in plasma but not in the patient-matched FFPE tumor sample [68]. As represented in Figs. 1 and 2, technical limitations prevent complete identification of all cells within a tumor mass and could be one of the reasons for discordance in *KRAS* status between ctDNA and FFPE analysis.

Since both FFPE and cfDNA samples are fragmented and found in limited quantities, technologies for analyzing FFPE DNA has been exploited for use with cfDNA [69]. When tested using identical platforms, some studies report good agreement in the mutation status between cfDNA and FFPE [44, 70]. Higgins et al., found 100% concordance of *PIK3CA* mutation status by BEAMing between FFPE samples and corresponding cfDNA from blood [71]. Similarly, Janku and colleagues found concordance in 91% cases for *BRAF* mutations, 99% cases for *EGFR* mutations, 83% cases for *KRAS* mutations and 91% cases for *PIK3CA* mutations in FFPE and cfDNA in 157 patients with advanced cancers [72]. Using Scorpion ARMS method, Duan and colleagues found an overall concordance of *EGFR* mutation status between plasma and tissue samples to be 80% [73]. But, others have found poor concordance between FFPE and cfDNA from blood. Adamo and colleagues did not detect any mutations in the cfDNA though tumors tissues had a *KRAS* G12D mutation [74]. Similarly, Grasselli and colleagues also saw very high heterogeneity and poor correlation in the mutational load between tissue-plasma results [75]. These differences could potentially explained by low tumor burden [75] or ctDNA shedding or low total number of tumor cells in the primary tumor [76] or due to differences in protocol for isolating DNA from FFPE and plasma [44]. Also, formalin fixation introduces DNA denaturation, and introduction of non-reproducible sequence alterations in DNA [77]. Unlike cfDNA, there are also issues in identifying amplicons displaying high GC content in FFPE DNA [78].

In addition to the druggable mutations, there are studies showing the presence of mutations or modifications in other genes, though the clinical significance of these novel alterations needs to be determined. Most of these changes have been identified by comparing the plasma samples of specific cancers with that of healthy volunteers. Some examples include the alterations in estrogen receptor 1 and erb-b2 receptor tyrosine kinase 2 (*ERBB2*) of breast cancer patients which were not seen in tissues, but have been detected in cfDNA [79–81]. Uehiro and colleagues [82] compared the methylation signatures of nearly 140 candidate genes in plasma samples to detect early breast cancer in patients. Using the Illumina Infinium Human Methylation 450 BeadChip Assay platform, they identified methylation in 12 genes that were present both in peripheral blood mononuclear cells and primary tumor tissues from breast cancer patients, but not in peripheral blood mononuclear cells from healthy volunteers. They developed an algorithm by incorporating their results and were able to predict early breast cancer with fairly high sensitivity (86%) and specificity (82%), making the model comparable to mammography screening. This is exciting because more women are being diagnosed with higher stage cancer at a younger age and the accuracy of mammography is not reliable due to high breast density in young women.

Unlike, PCR-based methods which detect only known mutations using specific primers and probes, NGS look at entire genes which allow to identify rare and novel mutations driving cancer in a single run [83]. But due to low concentration of cfDNA, complexity of analysis and interpretation, and economics involved in NGS, targeted amplicon-based NGS platforms are also being developed [84]. In targeted NGS panels, areas of genes that are most often mutated in cancer are targeted and enriched using multiplex-PCR based library preparation [85]. Illumina has released TruSight Tumor 15 which assesses 15 genes that are frequently mutated in solid tumors. Though, it has been optimized to work with highly fragmented and degraded FFPE DNA, recent studies have shown that it can be used to analyze mutations in cfDNA (https://www.illumina.com/). The Ion Ampliseq Colon and Lung cancer panel on the Ion Torrent Personal Genome Machine selectively amplifies 90 amplicons that encompass 1825 mutational hotspots of 22 genes related to colon and lung cancer [86–88]. It has been clinically validated in a retrospective study of 39 NSCLC samples and 51 colorectal cancer samples [84]. The ctDx™ Resolution Bio ctDx Lung assay (Resonance Bioscience) is another commercially targeted NGS system for detecting driver mutations targeted by

specific FDA-approved therapy or therapies now in clinical trials [89] (http://www.resolutionbio.com/assays/nsclc.html). The sensitivity and accuracy of these targeted NGS platform are 100% for detecting variants of driver genes at an allelic frequency > 4% [79, 84, 90]. Malapelle and colleagues [91] has designed and tested a "SiRe" panel to identify 568 clinically relevant mutations in six genes (*EGFR, KRAS, NRAS, BRAF, cKIT* and *PDGFRα*) involved in NSCLC, gastro-intestinal stromal tumor, metastatic colorectal carcinoma and melanoma. In the prospective studies, SiRe panel was able to detect the emergence of the *EGFR* T790 M mutation in 42.9% of patients at tumor progression after TKI treatment. Thermo Fisher has also recently launched a commercial NGS-based assay, Oncomine Comprehensive Assay v3, to detect relevant alterations in several critical receptor kinases, DNA repair pathway and cell cycle pathway genes in both blood and FFPE tissue samples [92].

Guardant360 (G360, Guardant Health; www.guardanthealth.com) platform has been designed to identify tumor-related genomic alterations via complete exon sequencing in 73 different genes in cfDNA from blood [93, 94]. FoundationACT Liquid Biopsy Assay (F1, Foundation Medicine, Inc., http://www.FoundationMedicine.com) sequences the exons of 315 cancer-associated genes and introns from 28 genes involved in rearrangements. Both the F1 and G360 tests have high specificities (>99%) but somewhat lower sensitivities [95, 96]. Using G360, Schwaederle and colleagues were able to identify actionable aberration, matched to an FDA-approved drug, in *TP53*, *EGFR*, *MET*, *PIK3CA*, and *NOTCH1* in cfDNA of patients diagnosed with either lung, breast or glioblastoma. They also compared the concordance in genomic alterations between FFPE tissues and cfDNA and found that 35% (22 of the 63 patients) had ≥1 alteration in common between the tissue and cfDNA. In contrast, of 222 healthy volunteers, only one had an aberration in *TP53* [97].

However, full exploitation of NGS technology remains limited due to reduced efficiency by which regions of interest can be captured/enriched from cfDNA and the higher error rate of sequencing relative to the accuracy of ddPCR. Additionally, multiplexed PCR-based amplicon library preparation is needed for NGS which can introduce sequencing bias resulting in uneven read coverage and increase in the numbers of duplicate fragments present in the library [98]. ddPCR, also, seems to be better at detecting rare genetic variants, and is less susceptible to inhibitors when compared to real-time quantitative PCR [99].

Since tumor DNA is released into the blood stream during cell turnover, presence of high levels of cfDNA has also been associated with shorter median overall survival of patients. Current procedures for quantitative cfDNA analysis include simple spectrophotometry [100] and qPCR of housekeeping genes. Quantitation of both single genes like *CYC* [59], *hTERT* [61], β2 microglobulin [26, 101], or multiple genes [82] in plasma samples has been used to quantitate cfDNA. Commercially available DNA DipStick TM Kit (Thermo Fisher Scientific) is, another, simple kit for qualitative assessment of cfDNA levels. Using the dip stick, Sozzi et al. [102], showed a reduction in cfDNA levels in the relapse-free NSCLC patients, and increasing levels in patients who subsequently presented with recurrence.

Methods to isolate and identify CTC

CTCs have been purified from biological fluids and in vitro cell cultures using a variety of strategies and techniques. But CTC detection rates are generally low despite the advances in cell capture technologies [103]. However, even the simple readout of presence or absence of CTC correlates with micro-metastases or early tumor cell dissemination which are events key to developing metastatic disease. Studies done by different groups like Olsson et al. [104], and Coumans and Terstappen [105] suggest that increased CTC detection precede clinical detection of metastasis in patients with an average lead time of at least a year or less, whereas patients with long-term disease-free survival had undetectable CTC postoperatively. In a study of 231 patients with metastatic castration-resistant prostate cancer, de Bono and colleagues [106] reported that 57% of patients had ≥5 CTCs per 7.5 ml of blood. Others too have found similar CTC counts in blood [107].

A number of methods have been evaluated for separation of CTCs. As CTCs differ in size from other blood components, size-based filtration systems have been frequently used by numerous authors to enrich them [108]. Generally, the filtration system consists of a filtration tube containing the membrane, a manifold vacuum plate with valve settings, a vacuum manifold and a vacuum pump [109–111]. Following filtration, CTCs can be stained by standard immuhistochemistry techniques to identify specific proteins or nucleic acid can be extracted and analyzed by ddPCR or NGS to identify mutations [107, 112–114]. Oh and coworkers used a high-density microporous chip filter to enrich CTCs and evaluate its clinical utility in colorectal cancer. CTCs were stained with a 4-color protocol involving DAPI for nucleated cells, CD45 monoclonal antibody (mAb) as a leukocyte marker, and epithelial cell adhesion molecule (EpCAM) mAb, and cytokeratin (CK) mAb as an epithelial cell marker. In a study of 50 patients, CTC levels were at least four times higher in patients with stage IV cancer compared to patients with lower stages of cancer and healthy control subjects. Progression-free survival was lower in CTC+ patients compared with CTC- patients.

In patients with stage I to III cancer, recurrence occurred only in CTC+ patients [115].

More recently, CTC isolation techniques have depended on antibodies against EpCAM, a protein that sticks out of the outer surface of CTCs, but not in healthy blood cells. The CellSearch CTC test, the only US FDA-approved CTC isolation platform consists of EpCAM antibodies attached to magnetic beads [116] so the cells can be pulled out of solution with a magnetic field. Presence of CellSearch CTCs have predicted the worst outcome in various cancers [117, 118]. Longitudinal analyses have, also, identified a link between the size of CTC-clusters and patient overall survival. Compared to the patients without any CTC, those with 2-cell CTC-clusters and ≥3-cell CTC-clusters had a hazard ratio of 7.96 and 14.50 respectively [119, 120]. Isolation of CTC with CellSearch CTC kit coupled with NGS on Roche 454 GS junior platform has proved successful in *EGFR* mutation analysis in the Phase II Erlotinib TRIGGER) study [121]. But studies on *KRAS* expression in CTC isolated by CellSearch did not find any statistically significant difference in clinical outcome between *EGFR* mutation-positive and *EGFR* mutation-negative patients tested for cetuximab efficacy in advanced colorectal cancer [122]. Similarly, CTC numbers did not correlate with clinical characteristics or patient outcomes in newly-diagnosed and recurrent ovarian cancer patients [123].

A major drawback of EpCAM based platform is the high variation in the gene expression between tumor subtypes and its downregulation during epithelial-to-mesenchymal transition (EMT) of cancer cells [124]. Therefore, EpCAM based platforms have provided only modest sensitivity in detecting CTCs [125]. Unlike CellSearch system, AdnaTest EMT-2 (Qiagen) test is an immuno-magnetic bead enrichment step targeting the surface proteins EpCAM, HER2 and EGFR. Use of three proteins to capture CTC seems to be better than EpCAM alone. In the patient cohort of Hanssen and colleagues, CellSearch system identified CTCs only in patients with lymph node metastases or larger primary tumors (≥T3). In contrast, multiplex RT-PCR for *PIK3CA*, *AKT2*, *TWIST*, and *ALDH1* following AdnaTest EMT-2 capture, identified CTCs with the same frequency in both lymph node positive and negative patients [126]. Alonso-Alconada and colleagues have designed and patented PrediCTC for the assessment of CTC in metastatic colorectal cancer patients. The assay evaluates the expression of *LOXL3*, *ZEB2*, *GAPDH*, *VIL1*, *TIMP1*, *CLU* and *TLN1* of captured CTCs [127].

Perhaps due to limitations in their detections technologies, *KRAS* mutant cfDNA was detected in patients with both resectable and advanced pancreatic disease whereas CTC was detected only in patients who had metastatic disease [4]. Therefore many authors have used modified kits to increase the specificity of these commercial kits. Antonarakis and co-workers [128] and Steinestel and group [129] found that including primers to detect specific splice variants or point mutations of androgen receptor in the AdnaTest gave more reliable information on cells resistant to drugs that target androgen receptor signaling. Others have taken advantage of the fact that CellSearch has one free channel position for addition of an extra antibody. Lindsay and colleagues added FITC-labelled anti-vimentin antibody or anti-Ki67 antibody to the free channel in the CellSearch system to fish out CTCs that were positive for vimentin or Ki67. Though only 32% and 45% of the CTC were positive for vimentin or Ki67 respectively, the presence of either vimentin + or Ki67+ CTC correlated with significantly reduced overall survival [130]. Recently Chikaishi and group developed a novel microfluidic platform, a 'CTC-chip' comprised of light-curable resins that has a unique advantage to bind any antibody. Using CTC-chip coated with an anti-podoplanin antibody, they successfully captured human mesothelioma cells (ACC-MESO-4) with no EpCAM expression, but with podoplanin expression [125].

NGS analysis has revealed mutational heterogeneity in actionable genes between individual CTCs [131]. In order to circumvent the issue of heterogeneous expression of EpCAM on tumor cells, Bulfoni and group adopted a CTC enrichment strategy based on red blood cell lysis followed by the immunomagnetic depletion of leukocytes from blood samples (i.e., a negative selection) and subsequently stained the recovered cells with a cocktail of antibodies recognizing epithelial and mesenchymal markers and sorted them by multiparametric fluorescence. They found presence of CTCs co-expressing epithelial and mesenchymal markers (EM CTC) were significantly associated with poorer progression free survival and overall survival [132]. Zhang et al. [133], used anti-CD45 antibodies to deplete CD45 positive cells and enrich CTCs in blood collected from pancreatic cancer patients followed by immune-staining of CK and CD45, DAPI and fluorescence in situ hybridization with the centromere of chromosome 8 (CEP8) probe to identify CTCs in 31 cases of pancreatic cancers, and 30 healthy individuals. With a cutoff value set at 2 CTC cells/3.75 mL blood, the sensitivity and specificity in the diagnosis of pancreatic cancer was 68.18% and 94.87%, respectively. During a one and a half year follow-up, CTC positive pancreatic cancer patients showed metastasis and worse survival rate.

Expression pattern of a 10-gene liver-specific transcript panel to amplify RNA in CTC, was used by Kalinich and group, to differentiate hepatocellular carcinoma (HCC) from other nonmalignant liver conditions. They used a CTC-iChip microfluidic device which depletes hematopoietic cells from blood by size-based exclusion of

red blood cells, platelets, and plasma, followed by magnetic deflection of white blood cells tagged with magnetic bead-conjugated CD45, CD16, and CD66b antibodies for enrichment of CTCs. The transcript expression pattern was able to correctly distinguish HCC with 88% specificity and 50% sensitivity from other malignancies. Positive CTC scores declined in treated patients receiving therapy [134].

Due to challenges in obtaining enough CTCs from blood, culture of CTCs in conventional media [135] or into mice [136, 137], is also being attempted in hopes of obtaining sufficient amounts for molecular analysis. Cultured CTCs maintain a similar genomic profile compared with primary tumor tissues and maintain their ability to grow long-term in vitro and show tissue specific metastasis properties. EpCAM-negative breast cancer CTCs containing stem-cell properties (CD44 +/CD24−) have been isolated by multiparametric flow cytometry from blood of breast cancer patients. These cells possessed high competence to generate breast cancer brain metastasis in xenografts [138, 139]. Incidentally, breast cancer is the second most common cancer to metastasize to the brain and the prognosis of patient diagnosed with brain metastasis remains poor [140].

Methods to isolate and identify exosomes

The stability of miRNA in blood have encouraged several investigators to identify and develop clinically relevant miRNA signatures as liquid biopsy markers. Ultracentrifugation is the most common method for separation of exosomes and miRNAs from blood. Allenson and coworkers, who compared exosome-derived DNA to cfDNA in liquid biopsies of patients with pancreatic ductal adenocarcinoma, found higher percentage of detectable *KRAS* mutations in exosome DNA than previously reported for cfDNA. They isolated exosomes using serial ultracentrifugation and characterized them with electron microscopy, flow cytometry and particle analysis [141]. Concerns of contamination of protein/RNA/membrane aggregates arising from similarities in sedimentation properties during high-speed ultracentrifugation, have prompted some protocols to use sucrose density gradients or adjusting the centrifugation duration in a "swinging bucket" or "fixed-angle" rotor for efficient separation of the exosomes from the protein- or lipid-aggregates [142, 143]. Helwa et al. [144], did a comparative study using differential ultracentrifugation and three commercial reagents (miRCURYTM exosome isolation kit (miRCURY) (Exiqon, Woburn, MA), ExoQuickTM Serum Exosome Precipitation Solution (ExoQuick) (System Biosciences, Mountain view, CA), and Total Exosome Isolation Reagent for serum (TEIR) (Life Technologies, Carlsbad, CA) for isolation of exosomes using different volumes of pooled and individual human serum. They found that commercial kits produced a significantly higher yield (80–300 fold) of exosomes from serum as compared to ultracentrifugation, irrespective of the starting serum volume.

Both *KRAS* G12D and *TP53* R273H mutations have been detected in exosomal DNA from patients with pancreas-associated pathologies, including pancreatic ductal adenocarcinoma, chronic pancreatitis and intraductal papillary mucinous neoplasm compared to healthy human subjects [145]. A combined isolation and analysis of exosomal RNA and cfDNA (together referred to as "exoNA") seems to improve blood-based liquid biopsy for *EGFR* mutation detection in NSCLC patients. For exoNA, the sensitivity was 98% for detection of activating *EGFR* mutations and 90% for *EGFR* T790 M. The corresponding sensitivities for ctDNA by BEAMing were 82% for activating mutations and 84% for T790 M. In a subgroup of patients with intrathoracic metastatic disease, the sensitivity increased from 26% to 74% for activating mutations ($p = 0.003$) and from 19% to 31% for T790 M ($p = 0.5$) when using exoNA for detection [146].

Similar to CTCs, exosomes carry surface markers from the cell of origin, which is exploited for enrichment strategies [147]. Rapid advancement of another novel method known as nanoscale fluorescence activated cell sorting call, or nanoFACS, has further advanced the methods of exosome isolation and sorting and allowed for the study of discrete, free, individual exosomes from bodily fluids [148]. Others have isolated exosomes using size exclusion chromatography [43]. Exosomes and other extracellular vesicles derived proteins are also a source of biomarkers that complement other approaches for tumor assessment. Vykoukal and colleagues isolated them from plasma by ultracentrifugation flotation through a multi-step density-gradient overlay and analysed then by mass-spectrometry. They found SRGN, TPM3, THBS1 and HUWE1 proteins could be used to distinguished lung adenocarcinoma cases from controls [149].

miRNeasy mini and microKits (Qiagen) seems to the most popular kits for isolation of exosome-incorporated miRNAs and cell-free miRNAs [43, 150].

Limitations

A major bottleneck preventing the routine clinical use of liquid biopsy are the variations of cells and genetic material in the blood of patients [151], with tumor specific mutations ranging from undetectable in some patients to over hundred thousand copies of the mutation per ml of plasma in others with advanced disease [152]. Additionally, the accuracy of these tests is grossly influenced by the quality and quantity of the DNA extracted from the tissues. Thus, mutation can be missed in cases of contamination, widespread necrosis, or when only small quantities of DNA are available [153]. Perhaps for the

same reason, there is a lack of consistent and robust results of circulating genomic materials, with many apparently contradictory reports in the literature [154]. Lee and group [107] found one of their patients had all COSMIC mutations though the patient was negative for EpCAM-positive CTCs. In another instance, the patient tested negative for all COSMIC mutations despite having the highest number of EpCAM-positive cells. COSMIC is an Ion AmpliSeq™ Cancer Hotspot Panel v2, a next-generation sequencing assay from Thermo Fisher Scientific, Inc. that can detect 2800 Catalogue of Somatic Mutations in Cancer (COSMIC) across 50 genes [107]. Similarly, some studies have found very low mutation rate in cfDNA compared to tissues. *KRAS* mutations in plasma was 3% compared with 45% rate observed in the matched tissues from colorectal adenocarcinoma and colorectal high-grade intraepithelial neoplasia [61].

The ability of tumor cells to rapidly undergo EMT is an added confounding factor in obtaining reproducible results at various time points. During EMT, epithelial cells acquire mesenchymal, fibroblast-like properties and show reduced intercellular adhesion and increased motility [155]. Under physiological conditions, it increases the ability of the tumor cells to survive in blood circulation and establish micro-metastases in peripheral tissues. The process has been implicated in acquiring resistance to anti-cancer therapy and metastasis seen in resistant tumors [156].

Another limitation challenging the promise of liquid biopsy is the presence of mutations at higher frequency in cfDNA in benign or premalignant conditions compared to their malignant higher stage counterparts. Many of these genes traditionally fall under the category of hallmark drivers of cancers. Benign and premalignant conditions of various cancers express higher frequencies of alterations in *BRAF, RAS, EGFR, HER2, FGFR3, PIK3CA, TP53, CDKN2A*, and *NF1/2* genes as compared to malignant tissues. Similarly, human *HER2* is more commonly overexpressed in ductal carcinoma in situ (~27%–56%) when compared with invasive breast cancer [157]. Though Uehiro and colleagues were able to predict early cancers with high sensitivity and specificity using their prediction model, they did not find any significant trend toward a higher detection index in patients with advanced stage breast cancer [82].

Blood processing delays, storage, temperature, agitation of the sample and shipment are also a major source of variability between samples [158]. Because of the potential for cell lysis during blood coagulation during serum collection, plasma is often preferred over serum [159]. In addition, the choice of anti-coagulant used in plasma collection can influence downstream detection technologies, such as qRT-PCR [160]. Half-life of these circulating species in blood is also a matter of concern. For example, the half-life of cfDNA in circulation ranges from few minutes to several hours [161]. An authors' choice of using either plasma or serum and their volumes seems to be guided by availability of blood samples. Similarly not all authors have mentioned if they quantified the amount of extracted cfDNA. Some authors quantified the purified cfDNA by using a spectrometer [54] while others normalized the concentration based on internal controls such as the mean of multiple [49, 82] or single genes [26, 59, 61, 101]. Knowing the concentration of cfDNA is important as Zhang and coworkers [162] found a decline in sensitivity from 82.6% to 46.7% with decreasing cfDNA inputs ($p = 0.028$). Similarly, pre-analytical factors like sample collection tube type, incubation time, centrifugation steps, plasma input volume and DNA extraction kits had a major impact on the cfDNA recovery [163]. Helwa and group [144] found a linear relationship between input serum volume and isolated exosomes. Thus, low copy number of mutant alleles and low half-life of cfDNA, together with exclusion of finer experimental details, prevent reproducibility of analysis. Most studies that have investigated the use of circulating DNA to identify tumor genotype have included a small number of patients, further restricting their relevance and ability to investigate potential genotype–clinical outcome correlations [68].

Laboratory to bedside success stories

In spite of the limitations there are several advantages of liquid biopsy over tissue biopsy and has proved useful in monitoring of the metastatic burden of cancer. In cases when tissue biopsy is unfeasible or risky, a liquid biopsy gives the much needed information with a simple, minimally, invasive test. If not enough tissue is obtained from an initial biopsy for establishing biomarker status, liquid biopsy allows the patient to avoid repeat surgical biopsies. When cancer recurs after treatment, a liquid biopsy can re-establish biomarker status and see whether clinically significant changes have taken place in the new tumor. This information is important to plan the treatment options for the patient in whom the cancer has reoccurred. When cancer spreads to an area that is not easily reachable, a biopsy at the site of origin may not give correct information. A liquid biopsy on the other hand could give a more complete understanding of metastasis. Thus, the patient can get optimal care with a simple blood analysis. The advantages of liquid biopsy over traditional tissue biopsy is listed in Table 1.

A recent large study comparing the effectiveness of cfDNA analysis to tissue biopsy in NSCLC shows the clinical value of the liquid biopsy approach. The authors used the Therascreen EGFR RGQ PCR kit to detect L858R, Del19, and T790 M in plasma samples enrolled in phase IV, open-label, single-arm clinical trial of

Table 1 Advantages of liquid biopsy over standard tissue biopsy

	Liquid biopsy	Tissue biopsy
Clinical sample	Blood	Effected tissue
Risk	Minimal risk/pain	Risk depends on location of tumor
Ease of collecting sample	Quick	Depends on location of tumor. Some tumors are hard to reach
Ease of monitoring patients	Simple blood test	Difficult to do repeat surgeries; also surgeons may not know where to look for metastatic tumor
Invasive	Minimally invasive	Invasive
Time for patient recovery	Quick; does not require hospitalization	Time Intensive; requires hospitalization of patients

Caucasian patients with NSCLC on first-line Gefitinib. The median progression-free survival (months) was 9.7 for mutation-positive tumor and 10.2 for mutation-positive tumor and plasma. The high concordance (94.3%), test specificity (65.7%), and test sensitivity (99.8%) between matched tumor and plasma, further established that *EGFR* mutation status in tumor tissue can be accurately assessed using cfDNA [164]. This positive result led to the approval of the use of cfDNA analysis for *EGFR* mutation analysis for IRESSA® in Europe (in patients where a tumor sample was not evaluable), making it the first EGFR tyrosine kinase inhibitor for which cfDNA testing is included in the label. Similarly, Cobas® EGFR Mutation Test v2 (US-IVD) is a recent US FDA approved platform intended to aid physicians in identifying NSCLC patients with *EGFR*- Del 19 for treatment with TARCEVA® (Erlotinib) or patients with *EGFR* T790 M substitutions for TAGRISSO™ (Osimertinib) therapy. The US FDA has given approval for detection of these mutations using Cobas® either in DNA isolated from FFPE tumor tissue or cfDNA from plasma derived from EDTA anti-coagulated peripheral whole blood in patients from whom a tumor biopsy cannot be obtained (http://www.accessdata.fda.gov/cdrh_docs/pdf15/P150047a.pdf).

Biocept, Inc. is a San Diego, California-based molecular oncology diagnostics company that specializes in CTC and biomarker analysis. Using proprietary liquid biopsy based technology, therapeutic markers for breast cancer were identified from blood though tissue biopsy was found to be negative for such markers (http://biocept.com/patients/success-stories/). Such identification of targetable molecules allow for early initiation of appropriate therapies against a tumor which has accumulated fewer oncogenic events.

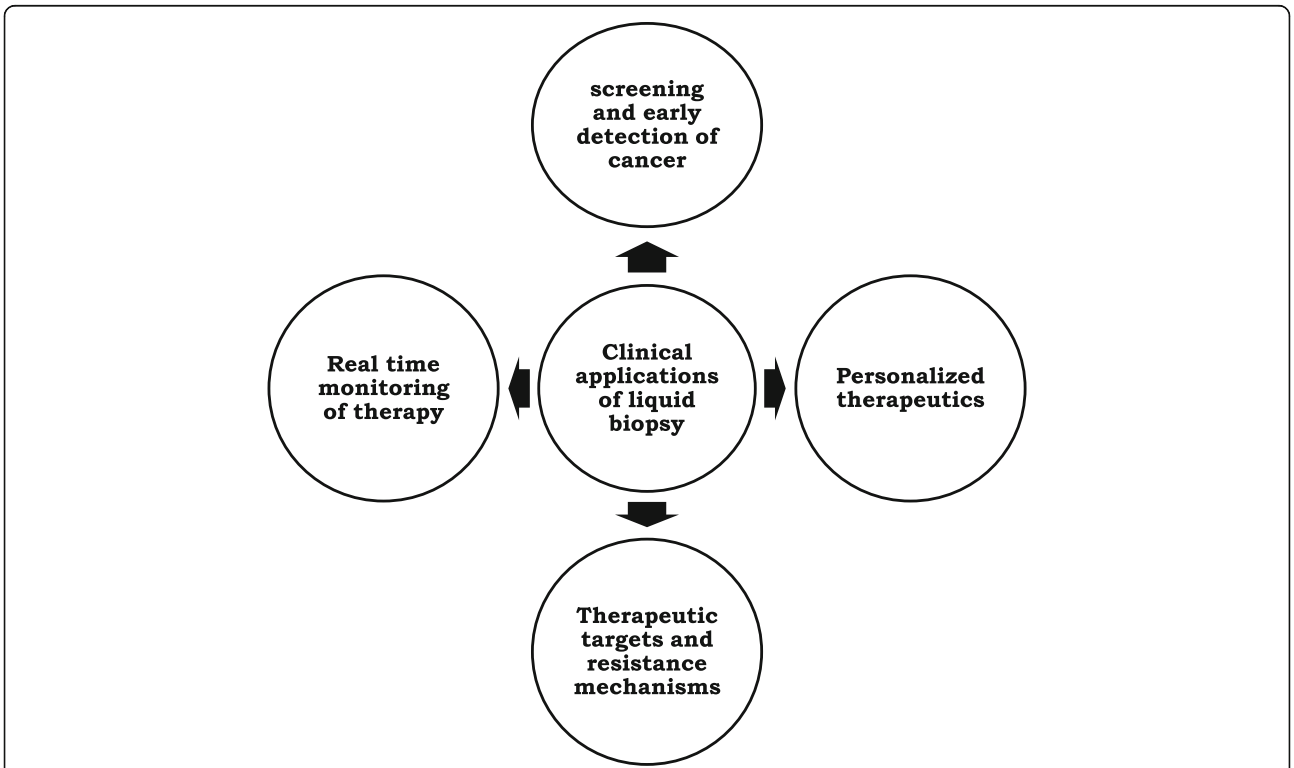

Fig. 3 Clinical application of liquid biopsy for personalized medicine. Capture of CTCs, ctDNA and exosomes as a "liquid biopsy" has several promising advantages over standard biopsy pertinent to clinical settings

In another published case report, a 70 year old woman with no relevant past medical history diagnosed with stage IV NSCLC, (adenocarcinoma histotype) was found to harbor an Del19 *EGFR* activating mutation in her exosome DNA. The patient was treated with Gefitinib and was found to respond to the treatment with stabilization of disease and an improvement in quality of her life within 10 months [165].

Conclusion

Cancer is a heterogeneous disease that is continuously evolving during its progression making clinical management difficult. To date, tissue biopsy remains the only option to identify targetable markers present in the tissue, but the procedure has inherent deficiencies which prevent identification of all such markers in a biopsied tissue. Although technically challenging, an advantage of liquid biopsies over other traditional tissue based methodologies is the enablement of longitudinal monitoring which could help clinical oncologists gain a broader molecular understanding of the disease (Fig. 3). Based on detection of specific alterations in the dying tissues contributing to the cells and DNA circulating in blood, it might be possible to diagnose disease even before the onset of clinical symptoms or progression to later, more advanced stages, where the disease burden becomes high, and is typically hard to manage or untreatable. Thus, liquid biopsy has tremendous potential as a non-invasive blood-based diagnostic test for personalized care of cancer patients.

Future perspective

There is a critical need for identifying specific signatures present in the ever evolving cancer cells for improved analytical and diagnostic sensitivity. Studies conducted within the past decade have shown that circulating tumor derived cells, DNA, or RNA in the blood harbor genetic alterations that correspond to primary tumors and metastatic sites. They hold the promise of providing a comprehensive real-time picture of the complete tumor burden in an individual patient. Despite current advances, it is not known if they are representative of all relevant primary and metastatic cell clones. Similarly, not much is known about the biological processes that shed these biomarkers into blood. Their clinical utility is restricted by the available technologies for collection, storage and isolation from blood. However, in the next few years technological refinements should allow us to further study these processes in depth and bring about a fundamental change in cancer management.

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Availability of data and materials

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Authors' contributions

This is a review and all the authors contributed to writing the review. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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