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Liquid Biopsies in the Screening of Oncogenic Mutations in NSCLC and its Application in Targeted Therapy

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Abstract

Non-small cell lung cancer (NSCLC) still dominates cancer-related deaths in America. Despite this, new discoveries and advancements in technology are helping with the detection and treatment of NSCLC. The discovery of circulating tumor DNA in blood and other biofluids is essential for the creation of a DNA biomarker. Limitations in technology and sequencing have stunted assay development, but with recent advancements in the next-generation sequencing, droplet digital PCR, and EFIRM, the detection of mutations in biofluids has become possible with reasonable sensitivity and specificity. These methods have been applied to the detection of mutations in NSCLC by measuring the levels of circulating tumor DNA. *ALK* fusion genes along with mutations in *EGFR* and *KRAS* have been shown to correlate to tumor size and metastasis. These methods allow for noninvasive, affordable, and efficient diagnoses of oncogenic mutations that overcome the issues of traditional biopsies. These issues include tumor heterogeneity and early detection of cancers with asymptomatic early stages. Early detection and treatment remain the best way to ensure survival. This review aims to describe these new technologies along with their application in mutation detection in NSCLC in order to proactively utilize targeted anticancer therapy.

Keywords

ARMS; BEAMing; ddPCR; EFIRM; liquid biopsies; NSCLC; targeted therapy

I INTRODUCTION

In the past few decades, cancer has grown incrementally and has become the second leading cause of death in the United States. Although the incident rate of breast and prostate cancer has increased, lung cancer remains to be the leading cause of cancer-related deaths in the United States. Lung cancer alone accounts for approximately 221,200 new cases and 158,040 deaths in 2015 alone. There are fewer deaths from prostate, breast, and colon cancer combined compared to the staggering 27% from lung cancer. [1] Although commonly referred colloquially as just “lung cancer,” there exist two types: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). More than 85% of cases are NSCLC that can be separated into three different categories: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma.[2] It is a predominant form of lung cancer in nonsmoking patients.

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Although conventional therapies continue to be used in the treatment of NSCLC, the five-year survival rate between 2004 and 2010 is still 18%, only surpassed by pancreatic cancer at 7%.[1] These statistics suggest the need for new novel technologies and treatments to be able to improve early detection and care.

Unfortunately, early stages of these cancers can be asymptomatic, therefore making it difficult for an early diagnosis. Early symptoms tend to be easily misinterpreted and dismissed immediately. Current diagnostic procedures include a variety of tests that can either be invasive or noninvasive. Chest X-rays, CT scans, low-dose CT scans, and positron emission tomography (PET scans) are all used as non-invasive techniques to diagnose NSCLC. Although exposure to radiation is relatively small per exam, the constant monitoring of high-risk patients poses a risk of radiation-induced cancers.[3,4] Although there are other invasive methods such as bronchoscopies and needle biopsies, there has been a shift from traditional procedures to more advanced technologies that utilize biomarkers in biofluids. With the advances in the sensitivity of different methods of PCR and sequencing, circulating DNA has been identified as an extremely efficient approach to detecting tumors. Levels of cell-free DNA (cfDNA) have been observed to be elevated in patients with systemic irregularities such as myocardial infarction, trauma, neoplasia, and cancer.[5–7] Cancer patients often experience a relatively high concentration of cfDNA in their plasma/serum. This is often known as circulating tumor DNA (ctDNA). ctDNA often reflects modifications of normal genomic DNA that is indicative of the systemic disease such as single-nucleotide polymorphisms (SNPs), methylation, deletions, and many others.[7,8] It has been shown that these ctDNA sequences do not come from circulating tumor cells, but often from necrotic cancer cells.[9] ctDNA has become important as a direct diagnostics tool because a conclusion can be made from abnormally high concentrations of ctDNA in patients with neoplasia or cancer.[9] Technology has limited the usefulness of ctDNA because the oncogenic DNA fragments are at a very low concentration relative to the wild-type DNA. These mutant DNA fragments are often only 0.1% of all cfDNA.[8,10] With recent advancements in next-generation sequencing (NGS), polymerase chain reaction (PCR, ddPCR, BEAMing), and electric field-induced release and measurement (EFIRM), the task of detecting small concentrations of these mutant DNA fragments has become more realistic. The biofluids of NSCLC patients have been identified as containing different mutant ctDNA reflecting the oncogenes responsible for the development of NSCLC. This paper will focus on the detection methods of these ctDNA in biofluids as well as showcase their application in the use of target anticancer therapy.

II. RESULTS

A. Liquid Biopsy Versus Tissue Biopsy

The detection of NSCLC has always been done by tissue biopsy, and although new methods are being developed, characterization of histology has always been the gold standard. The introduction of liquid biopsies has changed the view of clinicians and researchers regarding the future of cancer detection, diagnoses, prognosis, monitoring, and therapeutic decision making. If the development of liquid biopsies continues, it will become more effective and

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practical than existing biopsies of today. This review will concentrate on the mutational aspect of the subject.

Tissue biopsies have been used successfully for many decades now, but recent advances in sequencing have allowed for complete sequencing of different regions of tumors to compare their genetic compositions. In a recent study, different exons of tumors were sequenced. It was found that there was a high degree of heterogeneity among cells in different regions of the tumor.[11] This poses a problem for targeted biopsies because they can only target one specific region of the tumor. Liquid biopsies offer a more complete analysis of mutations present in tumors. Cancer cells secrete ctDNA that are often indicative of their genetic makeup. There are many ways for ctDNA to enter circulation. Apoptosis and necrosis are relatively straightforward, but the secretion of ctDNA from active tumor cells is an intriguing observation worth future investigation. These tumors release their small, fragmented DNA in the form of extracellular vesicles including microvesicles and exosomes.[12,13] Some of the sources of these vesicles are illustrated in Fig. 1.[14] These vesicles containing proteins, lipids, and nucleic acids circulate in the microenvironment and have been shown to be absorbed by other cells. This suggests that tumor cells may be communicating via the secretion of these ctDNA, which often include various mutated sequences.[12,13]

Lung cancer tumors shed these DNA fragments into the surrounding area creating a condition of abnormal fluid called malignant pleural effusion.[15] This eventually spreads into other bodily fluids including both plasma and saliva.[16,17] In liquid biopsies, these ctDNA can offer a better understanding of the mutations because the selection is not location sensitive, unlike the specific region sampled in a needle biopsy. The use of tissue biopsies is also limited because they are invasive and expensive. Physicians will never order biopsies on asymptomatic patients with no preexisting illnesses. Cancer is treated best if discovered early, but if biopsies can only be performed once a patient is symptomatic and in a later stage, their usefulness is greatly reduced. If the technology to perform liquid biopsies becomes widespread, the test for ctDNA can be routinely performed in a blood test. This will allow for earlier detection of tumors that are asymptomatic at first. The ability of liquid biopsies to overcome these two limitations proves their effectiveness. The benefits of liquid biopsies should be evident because it can be a relatively inexpensive, practical, and noninvasive procedure that can be carried out. Oncologists will be able to monitor the disease posttreatment. Therefore, although still currently in development, liquid biopsies have the potential to dethrone tissue biopsies as the gold standard.

B. Detectable Mutations in NSCLC

The importance and urgency of NSCLC have resulted in many studies and observations. Although the discovery of the mutations responsible for the development of NSCLC are not new, only recently have researchers begun to grasp the biochemical importance of each individual mutation. Pharmaceuticals have begun to produce targeted anticancer therapy, which usually involves a kinase inhibitor specific for the mutated enzyme. These new treatments require an understanding of the individual mutations and their biochemical mechanisms. Although there exist many different mutations in NSCLC, this review will

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focus on the three important mutations involved in targeted anticancer therapy: EGFR, KRAS, and ALK-EML4.

1. EGFR—Epidermal growth factor receptor (EGFR) mutations have been studied extensively in NSCLC patients. In the United States, about 10–20% of cases are associated with EGFR mutations with a higher incidence rate in nonsmoking women.[18–21] These EGFR mutation cases have been studied because they are often asymptomatic until the late stages and often develop drug resistances to tyrosine kinase inhibitors (TKIs).[22] In normal cells, EGFR belongs to a family of receptor kinases that when bound by ligands, such as epidermal growth factor (EGF) and transforming growth factor alpha, induce a conformational change that results in the kinase activity.[23] This kinase activity includes many cellular processes involving cellular proliferation.[24] Exons 18–21 are most vulnerable to EGFR mutations; ~90% of the mutations involve a deletion in exon 19 or a point mutation in exon 21 (L858R).[25] In 2009, Soh et al. studied both NSCLC mutations *EGFR* and *KRAS* and found that they are both heterozygous oncogenes and undergo what they call mutant allele specific imbalance (MASI). The mutant allele undergoes an amplification over the wild-type allele.[26] All these mutations affect the total EGFR function in three possible mechanisms: overexpression of EGFR ligands, amplification of EGFR production, and constant activation of EGFR.[22,27–29]

2. KRAS—KRAS mutations in NSCLC are changes in the genetic sequence of the V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog in humans. It is part of the Ras family of proteins and in normal cells acts as a GTPase regulator of signal transduction.[30] Like EGFR, KRAS has been studied extensively due to its involvement in many different cancers including colon, lung, and pancreatic cancer.[31,32] Being in the same family as other GTPases, KRAS switches between its active bound state and its inactive state depending on its ligation with either GTP or GDP. This regulates cell growth, differentiation, and apoptosis depending on which effector it interacts with. This can include mitogen-activated protein kinase (MAPK), signal transducer and activator of transcription (STAT), and phosphoinositide 3-kinase (PI3K) signal cascades.[33–35] In NSCLC, around 10–15% of patients have KRAS mutations with 97% of them involving exon 2 and 3. This includes G12, G13, and Q61.[36] These mutations in the *KRAS* gene eventually cause an increase in the activated form of the GTPase. RAS signaling is sustained without a switch to turn it off.[37] Since EGFR and KRAS share many similar functions in the development of cancer, it has been observed that they are mutually exclusive.[38]

3. EML4-ALK—Anaplastic lymphoma kinase (ALK) is another receptor tyrosine kinase that has also been identified to be a mutation involved with NSCLC. ALK rearrangements create mutant proteins with completely different biochemical characteristics. The formation of these ALK variants involves echinoderm microtubule-associated proteinlike 4 (EML4).[39,40] Nine different EML4-ALK fusion variants have been sequenced and identified in NSCLC.[39,40] These variants arise from the rearrangement of chromosomes.[41] Patients in the EML4-ALK cohort often have EGFR tyrosine kinase inhibitors (TKIs).[39] Although not as prominent in NSCLC as the other two mutations, ALK fusion proteins still comprise 3–

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7%.[42] Similarly to KRAS and EGFR, rarely are mutations in EGFR and KRAS identified in patients with EML4-ALK fusions.[43–45]

III. METHODS OF DETECTION

Because ctDNA is ~0.1% of cfDNA, the process of detecting point mutations in plasma has been limited by technology.[8,10] With the recent advancements in sequencing and PCR, the sensitivity and specificity of these newly developed assays are reaching acceptable levels. Although many variations of the aforementioned technologies exist, this review will focus on the main technologies that have been novel and innovative in the field of liquid biopsies. As seen in Fig. 2, direct sequencing methods are unable to reach the required sensitivity levels.[46] However, modifications in PCR have led to the development of a new class of PCR-based assays that have the ability to detect and genotype mutations in plasma. The methods that will be discussed include amplification-refractory mutation system (ARMS), digital PCR, droplet digital PCR (ddPCR), beads, emulsions, amplification, and magnetics (BEAMing), next-generation sequencing (NGS), and electric field-induced release and measurement (EFIRM).

A. Amplification-Refractory Mutation System (ARMS)

ARMS is a consistently reliable method developed to detect single base mutations in mixtures after agarose gel electrophoresis. This method was published in 1989 by Newton et al. when they utilized ARMS to detect mutations in the DNA of patients with alpha-1 antitrypsin (AAT) deficiency. [47] This technique revolves around Taq DNA polymerase's ability to differentiate mismatches on the 3′ end of the primers. This allows for differentiation between mutant and wild type by amplifying sequences that are completely matched with the added primer.[47] If one mismatched base pair is not enough, they proceeded to add an additional mismatch depending on the single nucleotide polymorphism.[47] This technique was used by two different validation groups to test its ability to differentiate wild type and mutation in EGFR for NSCLC patients.[48,49] Both groups compared ARMS to direct sequencing in plasma. In 2013, Chu et al. compared Adx-ARMS and found that more mutations were detected from using ARMS than direct sequencing, and that the concordance was ~83.3%. They concluded that ARMS was more sensitive and more reliable than direct sequencing in EGFR mutations.[49]

B. DIGITAL PCR

Digital PCR was originally a new idea based on the analog PCR reactions, but added the ability to directly quantify DNA sequences from one template. This was possible by diluting the number of copies per PCR reaction to either zero or just one copy. This ensures that the reading for each well or individual fragment of DNA would be independent of any other fragments in the sample. This is done by utilizing microwells or microfluidics chambers to split up the DNA fragments from the sample into nanoliter samples.[50–52] By partitioning each individual fragment of DNA, the microfluidics chip runs thousands of PCR reactions in parallel. Fluorescent probes are then added that have the ability to differentiate between wild type and mutant type. This is done by using MB probes that consist of a fluorophore and a quenching agent in a loop structure. The signal is quenched because the fluorophore is

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relatively close to the quenching agent. Through cycles of heating and cooling, the binding of a complimentary probe to the sequence separates the fluorescent dye from the quenching group, which results in increased fluorescence.[53] Yung et al. applied digital PCR to NSCLC by detecting EGFR mutations in plasma. After screening 35 plasma samples, their sensitivity and specificity were 92% and 100%, respectively.[54]

C. Droplet Digital PCR

Droplet digital PCR is an advancement on the concept of digital PCR. Instead of doing limiting dilutions in the microwell plates, they utilize a "water-in-oil droplet" technique.[52] Hindson et al.[52] developed an approach that has a higher throughput and lower cost by using the same concept. They load all of the reagents including template, ddPCR Mastermix, and Taqman reagents. Taqman reagents were designed by utilizing the 5′-3′ exonuclease activity of Taq polymerase.[55] There are two probes that are necessary for Taqman PCR: the primer and the fluorescent probe. The fluorescent probe binds downstream and acts as the reporter and is introduced in a quenched state by a quenching agent. If the target sequence is complementary to the introduced primer, the polymerase will begin amplifying the DNA until it reaches the fluorescent probe. Once the fluorescent probe is cleaved, the fluorophore and the quencher are separated. Fluorescence can now be detected once excited by an external light source.[55]

The process of ddPCR was outlined by Hindson et al. in 2011.[52] In order to avoid using an expensive plate to separate the strands, a disposable droplet cartridge and a small vacuum can generate droplets of oil in an aqueous phase. These droplets are transferred to a 96-well PCR plate and thermal cycling is performed. After completion, a droplet reader is used to move the droplets toward a detector that can perform simultaneous multiplex readings. By keeping the droplet volume constant, a correlation between positive droplets and concentration can be made.[52] This technology has also been applied to NSCLC in the detection of EGFR mutations. Zhu et al. were able to utilize ddPCR in a plasma screening of 86 NSCLC patients with ~90% concordance rate with ARMS.[56] The specific steps of ddPCR are outlined in Fig. 3.[57]

D. Beads, Emulsions, Amplification, and Magnetics (BEAMing)

Similar to ddPCR, BEAMing was developed to perform a large number of parallel single-molecule amplifications simultaneously. This method was only made possible after the development of emulsion PCR, because it utilizes the same method to isolate individual templates for amplification. BEAMing starts with a pre-amplification step of the target sequence in plasma. The product of this reaction is emulsified with beads precoated with the primer. Each amplicon should be in an emulsion with one bead. A second step of PCR is performed, increasing the number of identical DNA molecules bound to the same bead. After the beads are recovered by either centrifugation or a magnetic holder, multiple detector probes with different fluorophore are added. The probes should be complementary to wild type or mutant amplicon thus allowing for hybridization. The detection of these molecules is performed by flow cytometry, which allows for multiplexing. The method claims to have high signal-to-noise ratio due to amplification of each individual DNA molecule attached to the beads, as illustrated in Fig. 4.[58] Taniguchi et al. in 2011 utilized BEAMing with plasma

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DNA for EGFR mutations in NSCLC. Their results at best indicate that their detection range was in the 0.1–1% range.[59]

E. Next-Generation Sequencing (NGS)

Over the past decade, there has been a steady shift away from the traditional Sanger sequencing to a more robust platform. The NGS platform is extremely powerful due to its ability to provide not only the frequencies, but also the actual nucleotide sequence of mutations. Second-generation NGS systems are dependent on polymerase-based clonal replication of single DNA molecules and cyclic sequencing chemistries.[60] The benefits of NGS include the ability to completely sequence the genome, allowing for analysis of all regions, including the region of interest. The recent streamlining of NGS in commercial solutions (target panels and equipment) has made it possible for an efficient, cost-effective method in detecting mutations. The potential of NGS has been proven by detecting and monitoring EGFR and KRAS mutations.[61] This robust assay is important for diagnosis, prognosis, and therapeutic decision making of NSCLC patients.[60,62,63]

F. Electric Field-Induced Release and Measurement (EFIRM)

The search for ctDNA in biofluids does not have to be limited to serum. Wei et al. have created a quick, efficient method to detect ctDNA in biofluids.[17] This method involves an electrochemical sensor that is multiplexible. Figure 5 illustrates the simplistic protocol required to quantitate the concentration of mutant DNA fragments in biofluids.[17] This platform relies on an electrochemical sensor coated with gold connected to a potentiostat. Each step of the assay utilizes the potentiostat to apply different potentials. Wei et al. decided on developing a biosensor that utilizes a square wave, with different potentials for polymerization, hybridization, and protein interactions. Polypyrrole is used as the conducting polymer and is formed by applying an electric potential to its monomer.[64] Embedded in this conducting polymer is a capture probe. This probe is rooted in the conducting polymer and is complementary to the target sequence. This probe is typically designed to bind to the mutant target sequence. Saliva or plasma from patients is treated by a strong electric field and added into the reaction mixture. After each step, a wash is required to remove all free-floating fragments and nonspecific interactions. A detector probe is added that is also complementary to the target sequence starting from where the capture probe ends. The capture probe should bind to the initial mutation region while the detector probe binds downstream. Fluorescein isothiocyanate (FITC) is in the detector probe for further detection. Both these hybridization steps are performed. Anti-FITC antibodies conjugated to horseradish peroxidase are added to the mixture last as the reporter enzyme. Tetramethylbenzidine (TMB) is a liquid substrate that is added to the reaction mixture. The flow of electrons between the electrode and the redox reaction produces a current that can be measured on the potentiostat. By assuming that there is a correlation between the number of HRP molecules and the flow of electrons, a quantitative measurement can be made to determine the relative concentration in the sample.[17]

The greatest advantage of EFIRM is the detection time. While PCR and other similar techniques take hours if not days to perform, EFIRM requires only 10 min. By applying EFIRM to ctDNA, they developed a system termed SABER (saliva-based EGFR mutation

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detection) to detect mutations in EGFR using only 20–40 ml of sample. By using SABER, they were able to produce a positive linear relationship between the readout current and the tumor size (R = 0.86, 0.95, and 0.98).[17] To further validate their technology, they performed a blinded test using saliva from 40 late-stage NSCLC patients. In the EGFR exon 19 deletion, the area under the curve (AUC) was 0.94, whereas in L858R their AUC was 0.96. The similarities between the ctDNA in plasma and saliva may play a key role in the search for a pathway between tumors and saliva.

IV. DEVELOPMENTS IN MUTATION-SPECIFIC THERAPY

Treatment for NSCLC has typically been a combination of surgery, radiation therapy, chemotherapy, immunotherapy, and targeted therapy. This combination depends on the type of the cancer, the stage, and the current health of the individual. Surgery has typically been the most effective treatment in early stages of cancer, but it is not without consequences. Incomplete resection may allow for recurrence.[65] Surgery is also very limited in its application because only healthier individuals are candidates for surgery. Although radiation and chemotherapy are used routinely, their effectiveness is limited by the lack of a complete mechanism. This has led to a rather bleak prognosis as lung cancer continues to have the largest number for cancer-related deaths.[1] Recently, however, researchers have begun to understand certain mechanisms that allow for cancer growth and sustainment. This prompted the development of new and improved drugs that are specific antagonists to biochemical pathways involved in cancer development and metastasis. Two categories of targeted therapy have been created: those that inhibit mutant proteins and those that prevent blood vessel growth. These drugs are often used in more advanced cases and coupled with other forms of treatment to maximize the efficiency while having less severe side effects. Though angiogenesis inhibitors are being used, this review will focus on drugs that function to inhibit protein kinases.

A. Erlotinib/Gefitinib

Because EGFR mutations are very common in NSCLC, much research has been devoted to understanding the biochemical mechanism. The mutant tyrosine kinase has become an important target for the development of new drugs to combat NSCLC. Erlotinib and gefitinib were designed to be reversible and highly specific inhibitors of tyrosine kinase. These small molecules are able to block the adenosine triphosphate from binding to the tyrosine kinase region of EGFR. This inhibition of autophosphorylation prevents the downstream signaling cascade that certain tumors depend on for continued growth and metastasis.[66–68] In 2005, Shepherd et al. performed a randomized, placebo-controlled, double-blind clinical trial in patients that had undergone unsuccessful chemotherapy. In 731 patients, the response rate was 8.9% in the erlotinib group while the placebo group had less than 1% response. Erlotinib was clearly superior after analyzing progression-free survival ($p < 0.001$).[66,69] Similarly, a random study conducted in Japan involving 230 and 177 patients sought to compare the response rate between gefitinib and traditional first-line chemotherapy. Gefitinib also prevailed with a progression-free survival ($p < 0.001$) and a better quality of life.[66,70–72] Although approved by the FDA for use, these drugs are not without side effects. A reduction in the dosage is necessary due to toxic effects such as a severe rash or

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debilitating diarrhea.[66] These side effects of erlotinib and gefitinib, along with a monthly cost of $4000 and $1800, respectively, make tyrosine kinase inhibitors imperfect in early stage treatment. [66] Another key issue with these drugs is the short life span in which these drugs are effective. Patients with exon 19 deletions and L858R point mutations respond to treatment well,[71] but after a few months they develop an acquired resistance. This is believed to happen due to the tumor's acquisition of another EGFR mutation (oftentimes T790M).[73]

B. Crizotinib

ALK mutations or translocations have been identified in various human cancers, but EML4-ALK is found in NSCLC. Although uncommon, patients with ALK rearrangements can also utilize the new forms of targeted anticancer therapy. Crizotinib is another small-molecule inhibitor of ALK tyrosine kinase. Similarly to other kinase inhibitors, crizotinib binds to the ATP-binding pocket of the kinase preventing downstream signaling.[74] Initial tests were done on 82 patients that had confirmed advanced NSCLC with ALK-rearranged mutations. ALK mutations were verified using fluorescence *in situ* hybridization. A total of 70 patients were confirmed to have the mutations although 76 patients had had at least one previous therapy. The overall response rate was relatively high being 57% with a 95% confidence interval from 46 to 68.[43] These targeted therapy solutions when compared to chemotherapy have significantly less adverse effects. These included 63% vision disorder, 54% nausea, 49% diarrhea, and 40% vomiting. These adverse effects are easily managed and, therefore, less taxing on patients.[74]

V. DISCUSSION

Although there have been major advancements in the diagnosis and treatment of NSCLC, it remains to be the leading cause of cancer-related deaths. Surgery, radiation therapy, and chemotherapy are used in combination to help combat the fast-paced onset of lung cancer. With the innovation in the detection of ctDNA, liquid biopsies have become a real competitor to tissue biopsies when it comes to diagnosing and genotyping tumors. Droplet digital PCR has proven in many studies that it has the sensitivity to detect low concentrations of circulating tumor DNA while having the specificity to differentiate it from wild type. The limitation of ddPCR comes from the difficulty in performing the assay. Because primary care providers do not commonly perform these types of assays, the technology is very much limited to a research tool used in validation studies. Even though EFIRM has only proven to work in one study, it shows promise in becoming a point of care solution. The simplicity of the reaction and the time scale make it the most hopeful solution for physicians. Liquid biopsies have the ability to revolutionize screening, monitoring, and treatment due to them being relatively affordable, noninvasive, and efficient. With all these methods being performed in blood, a system where every blood test includes screening for ctDNA may not be too far off in the future. No matter in what cancer or illness, early detection has always been pivotal to treatment and progress-free survival. Perhaps the targeted therapies are much more effective when used before patients become symptomatic. Without an efficient and affordable method for detection for asymptomatic patients, it may be difficult to reduce the number of cancer-related deaths.

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The recent surge in targeted anticancer therapy has given new hope to oncology. Although chemotherapy and radiation therapy have been relatively effective, the adverse effects have become a burden to all patients. Targeted therapies, like those discussed in this review, are not free of adverse effects, but the severity is significantly less. Liquid biopsies will become indispensable for the further development and use of these targeted therapies. This will allow for constant monitoring, pre- and posttreatments, and perhaps be indicative of prognosis. The concentration of circulating tumor DNA has been linearly correlated to the severity and size of the tumor.[17] Liquid biopsies can also be applied to targeted therapy because of acquired resistances. All discussed previously, ALK fusion genes, KRAS mutations, and EGFR mutations are commonly mutually exclusive. Researchers have shown that the detection of a KRAS mutant in the circulating tumor DNA can be indicative of a patient's nonresponse to anti-EGFR therapy.[75] NSCLC patients that were wild type for EGFR but mutant for KRAS participated in a study that involved treatment with an EGFR tyrosine kinase inhibitor. The results revealed that those patients had the lowest response rate due to an increased acquired resistance.[76]

Targeted anticancer therapy is looking to be the next generation of treatments for cancers developed from gene mutations. The next step would be to develop a point of care machine that is usable by not only researchers, but also physicians. As the technology improves and treatments become more effective, the future of cancer treatment can be less trial and error and more of a standard operating procedure.

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ABBREVIATIONS

ALK	anaplastic lymphoma kinase
ARMS	amplification-refractory mutation system
CtDNA	circulating tumor DNA
ddPCR	droplet digital PCR
EFIRM	electric field-induced release and measurement
EGFR	epidermal growth factor receptor
KRAS	Kirsten rat sarcoma viral oncogene homolog
NGS	next-generation sequencing
NSCLC	non-small cell lung cancer
PCR	polymerase chain reactions

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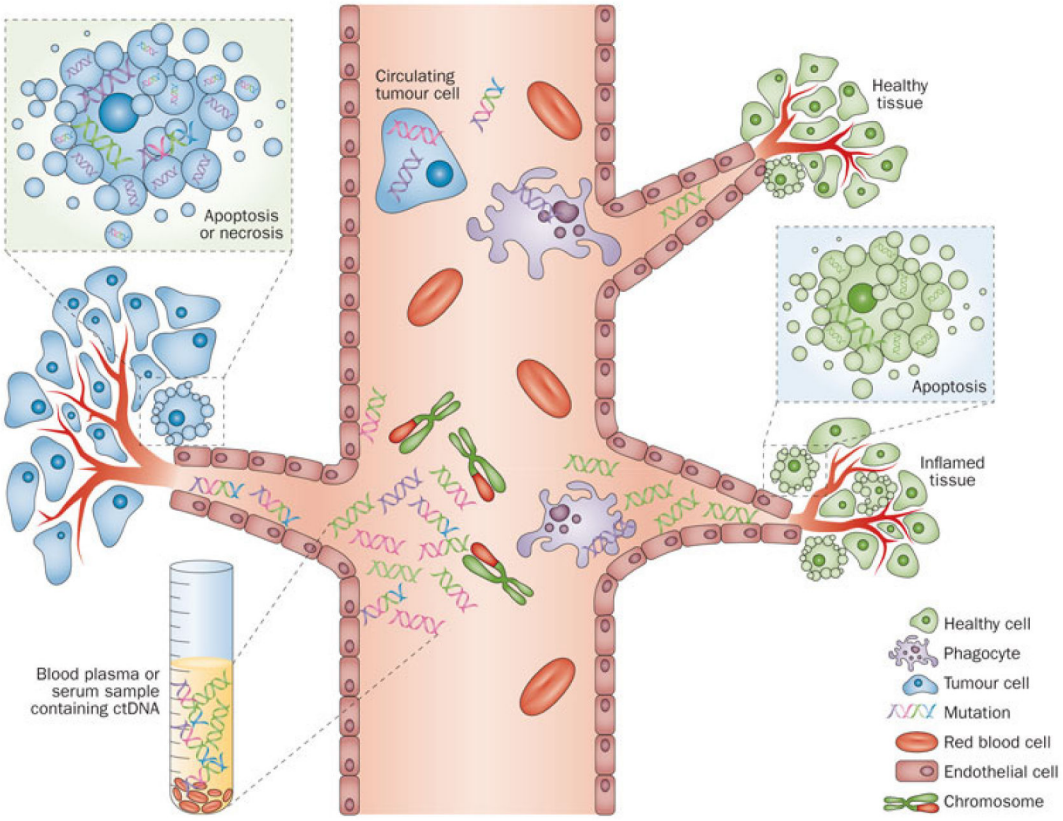

FIG. 1.

ctDNA and cfDNA in blood can come from a variety of sources. They can come from the necrosis or apoptosis of cancer or normal cells. Circulating tumor cells also contribute to the diversity of nucleic acids in blood (reprinted with permission from Macmillan Publishers Ltd., Copyright 2013).[14]

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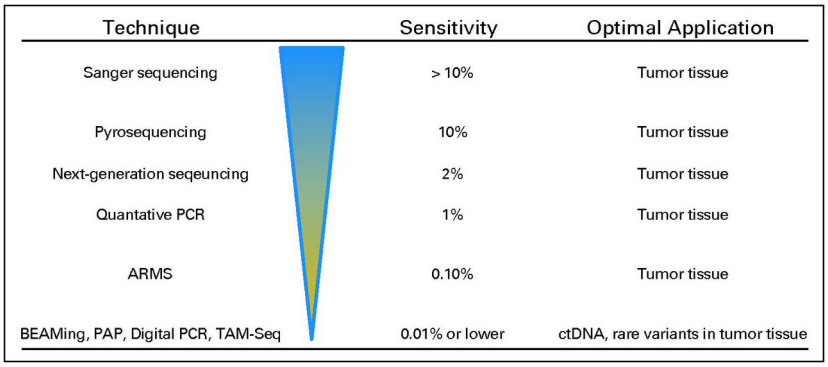

FIG. 2.

The relative sensitivity of ctDNA of different techniques. Lower concentrations can be achieved by using the new technologies BEAMing, PAP, Digital PCR, and TAM-Seq. These tests claim to be better than existing tests in tumor tissues (reprinted with permission from American Society of Clinical Oncology, Copyright 2014).[46]

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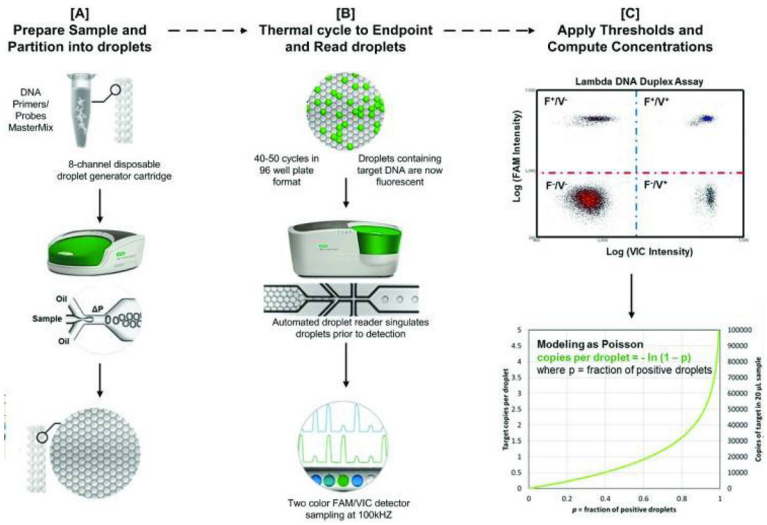

FIG. 3.

The general protocol for ddPCR. An emulsion is formed to separate each individual DNA fragment. The thermal cycling is performed to produce a readout. Statistical analysis is then performed for a relative readout (reprinted with permission from American Chemical Society, Copyright 2011).[57]

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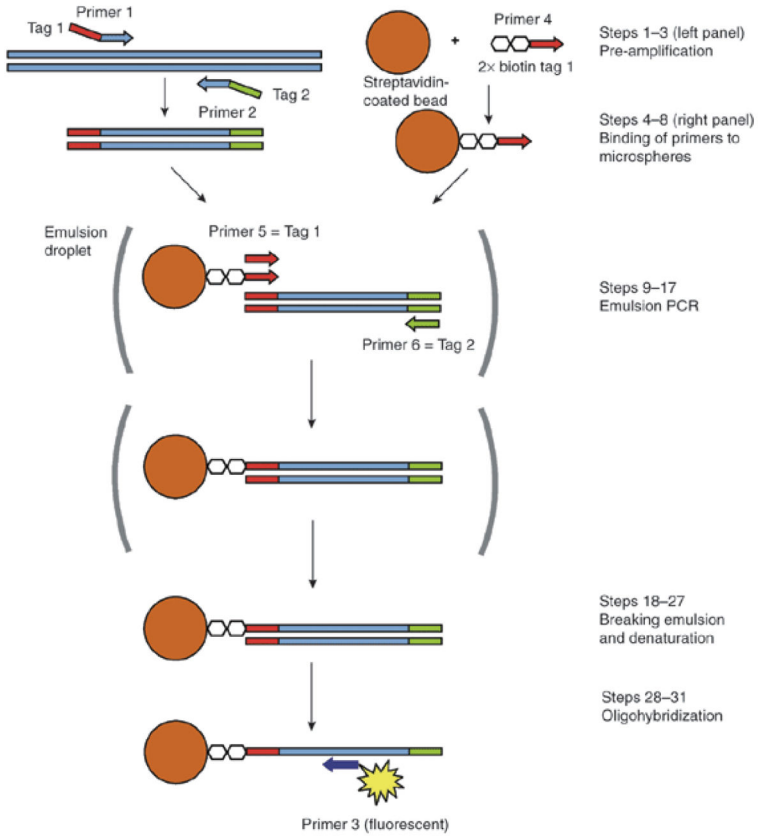

FIG. 4.

The basic concept of BEAMing is illustrated step by step. Initial amplification is performed before emulsifying each fragment with a bead coated with primers. A second amplification is performed in the emulsion followed with the isolation of these beads. A fluorescent reporter is used to bind to each bead to allow for flow analysis (reprinted with permission from Macmillan Publishers Ltd., Copyright 2006).[58]

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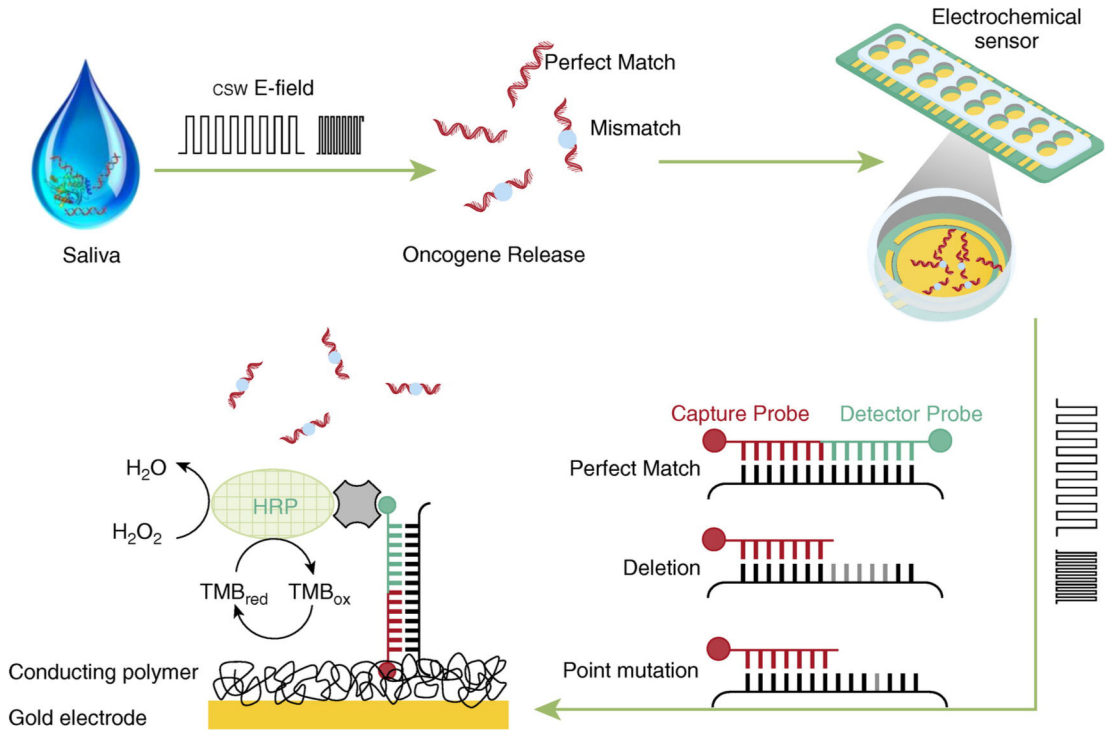

FIG. 5.

A simplified diagram of the EFIRM technology. Saliva or other biofluid is treated with an electric field to release oncogenes. The electrochemical sensor is prepared using pyrrole and a capture probe. Biofluids are added and an electric current is applied to foster hybridization. After the detector and reporter steps, a signal readout is performed (reprinted with permission from the American Thoracic Society, Copyright 2015).[17]