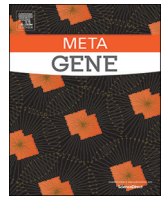




ELSEVIER

Contents lists available at ScienceDirect

Meta Gene

journal homepage: www.elsevier.com/locate/mgene

Circulating tumor DNA, liquid biopsy, and next generation sequencing: A comprehensive technical and clinical applications review



Sarah Abou Daya, Rami Mahfouz*

Department of Pathology and Laboratory Medicine, American University of Beirut Medical Center, Beirut, Lebanon

ARTICLE INFO

Keywords:

ctDNA
NGS
Review

ABSTRACT

Circulating tumor DNA (ctDNA) represents a small fraction of the total circulating free DNA and its analysis is increasingly used for diagnostic, prognostic and treatment purposes of cancer.

ctDNA is released into the bloodstream from tumor cells through different mechanisms including apoptosis, necrosis, autophagy, and necroptosis. Liquid biopsy is a method used to detect specific cancer mutations in ctDNA from the plasma fraction of a standard blood draw and has numerous applications. Adoption of this newly introduced method has many advantages for detecting mutations in blood where it is an alternative to the direct sampling of tissue through resection and biopsy, as the genetic mutations present in a patient may change following treatment, and conducting additional biopsies and resection may present risk to the health status of the patient since it is an invasive technique. In addition, the information acquired from a single biopsy of a tumor is limited and might fail to reflect its heterogeneity, leading to a false negative reading, and finally, an alternative to direct tissue sampling may also lessen the fiduciary and resource strain on caregivers and patients. Next Generation Sequencing (NGS) is gaining more presence in diagnostic molecular laboratories and is nowadays very close to embrace the field of liquid biopsies with an ultimate sensitivity and range of clinical applications.

This review article is a first comprehensive technical overview of the ctDNA detection using NGS as a state-of-the-art technology.

1. Introduction

A biomarker is designated as “a substance or activity that can be objectively measured and evaluated as an indicator for a normal biological process, pathogenic process, or pharmacological responses to a therapeutic intervention” (Wu and Qu, 2015). Cancer biomarkers encompass a wide variety of molecules, including DNA, mRNA, enzymes, metabolites, transcription factors, and cell surface receptors that are present in the blood together with molecules released from normal cells. The importance of the cancer biomarker is the indication of cancer risk, early detection of cancer, and tumor classification in order to develop reliable, cost-effective, powerful detection, treatment and monitoring approaches for disease progression, regression and recurrence (Wu and Qu, 2015). In this review article, our focus will be on DNA biomarkers.

cfDNA is the circulating free DNA circulating in blood that is not necessarily from tumor origin while ctDNA are those DNA fragments that reflect the tumor burden. Circulating free DNA with tumor specific alterations (ctDNA) represents a small fraction of the total circulating free DNA and its analysis is increasingly used for diagnostic, prognostic

and treatment purposes of cancer. ctDNA is released into the bloodstream from tumor cells through different mechanisms including apoptosis, necrosis, autophagy, and necroptosis. ctDNA strands are small fragments measuring approximately 180–200 base pairs in length, containing tumor-specific alterations in tumor suppressor genes or oncogenes, microsatellite instability, and DNA hypermethylation (Fan et al., 2017).

Liquid biopsy is a method used to detect specific cancer mutations in ctDNA from the plasma fraction of a standard blood draw, and it has numerous applications. In addition to the importance of biomarkers detection mentioned above, these detected mutations in blood have a major role in reducing the need for an invasive tissue biopsy specimen. First, longitudinal, quantitative monitoring of cancer mutations in ctDNA provides information about the general tumor burden for patients during treatment. Thus, elevated levels of detectable ctDNA mutations are associated with the presence of higher tumor burden. Second, ctDNA is a gold standard method especially after surgical resection of the tumor where liquid biopsy replaces the tumor biopsy and helps in measuring and quantitatively tracking the abundance of cancer mutations (or what is known as Tumor Mutational Burden). Third, this

* Corresponding author.

E-mail address: rm11@aub.edu.lb (R. Mahfouz).

method can identify mutations that can help in treatment decisions like the presence of hotspot *KRAS* mutations and the negative predictive response to *anti-epidermal growth factor receptor* therapy (Wood-Bouwens et al., 2017). Fourth, it is desirable to have an alternative to the direct sampling of tissue through resection and biopsy, as the genetic mutations present in a patient may change following treatment, and conducting additional biopsies and resection may present risk to the health status of the patient since these are invasive techniques (Tu et al., 2015). For example, serious side effects related to biopsy from metastatic breast cancer patients were reported in a study done by Lebofsky et al. and included pneumothorax, pain, hematoma, and hemorrhagic shock. These complications can be eliminated or reduced by a simple and affordable blood draw (Lebofsky et al., 2015). Fifth, information acquired from a single biopsy of a tumor is limited and might fail to reflect its heterogeneity, leading to a false negative reading. Finally, an alternative to direct tissue sampling may also lessen the fiduciary and resource strain on caregivers and patients (the cost of a fine needle aspiration biopsy has been estimated to be approximately \$1300 per procedure, while many companies have been marketing the ability to perform liquid biopsy panel tests for half the cost from 20 ml of blood (Tu et al., 2015).

However, relying on cancer-associated alleles in blood is sometimes impossible since the majority of circulating free DNA (cfDNA) is often not of cancerous origin and due to its high degree of fragmentation (Crowley et al., 2013) Figs. 1 and 2.

1.1. Pre-analytical steps for ctDNA detection

Variations in the genetic results are caused by different factors mainly the limited ctDNA amount recovered from the sample, high PCR error rates especially the intrinsic ones, limited sequencing coverage for lower range ctDNA and bias in the enrichment steps (Ignatiadis et al., 2015). To increase ctDNA coverage and improve sensitivity, pre-analytical steps should be done correctly.

Plasma is preferred to serum because the clotting process increases the release of wild-type DNA from leukocytes which may mask the small quantities of ctDNA in the sample in addition to the higher sensitivity for plasma mutations analysis (Normanno et al., 2017). Anticoagulants such as ethylenediaminetetraacetic acid (EDTA) and citrate can be used for ctDNA analysis, but heparin should be avoided because it interferes with polymerase chain reaction (PCR). Besides, 2 ml of blood are needed to cover a suitable amount of ctDNA for analysis (Normanno et al., 2017).

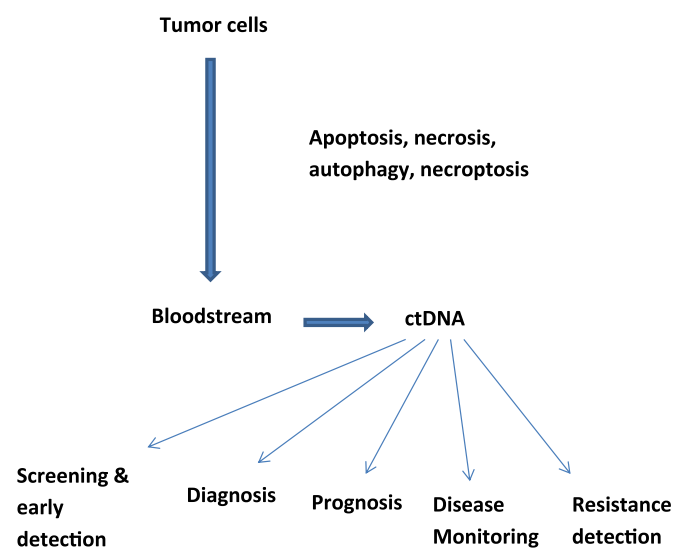


Fig. 1. The mechanisms for ctDNA release from tumor into blood and importance of ctDNA detection,

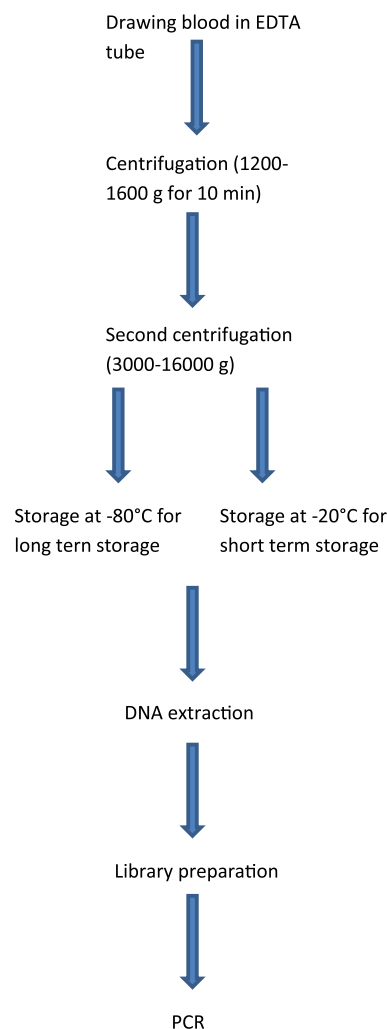


Fig. 2. Pre-analytical steps for ctDNA detection.

In addition to that, the plasma should be isolated within 4 h of blood drawing using EDTA tubes, as an increase in total DNA (indicative of white cell degradation) occurs if blood remains unprocessed for longer periods. If blood processing cannot be done within 4 h, usage of specific fixatives, such as cell-free DNA BCT[®] [Streck, Omaha, NE, USA] and cfD[™] [Ariosa Diagnostics, San Jose, CA, USA], is needed in order to stabilize blood in collection tubes for up to 7 days. Blood separation is done by centrifugation of the blood sample at 1200–1600 g for 10 min and isolating the supernatant. A second, higher-speed centrifugation between 3000 and 16,000 g in a microcentrifuge or filtration through a 0.2 μm filter is preferred to remove all cellular contamination (either before or after the freeze/thaw). Concerning plasma storage, fresh plasma should be stored at –80 °C in the long-term (for up to 9 months) and DNA concentrations decrease over time) and at –20 °C for a period of 1 month. In addition, if shipping is needed, it should be on dry ice to avoid sample degradation. Of note, repeated freeze/thaw cycles should be avoided with 2 to 3 cycles being considered acceptable (Normanno et al., 2017).

Next, DNA extraction occurs. If the amount of blood taken is < 2 ml, several kits are used to isolate smaller fragments, including QIAamp[®] circulating nucleic acid kit (Qiagen) (Sorber et al., 2017). DNA purity and concentration are measured by a spectrophotometer (Newman et al., 2014).

Moving to the step of library preparation, many sequencing platforms are used but the most common ones are Illumina HiSeq and MiSeq systems, in addition to Ion Torrent (Life Technologies). Steps of

Illumina include DNA shearing, end-repair, A-tailing, and index-specific adapter ligation. Then, the library is subjected to PCR to amplify the captured sequences and add indexes (Hagemann et al., 2013).

The enrichment step permits separating areas of interest from the genome by isolating some genomic segments and, thus, enhances the efficiency of next-generation sequencing (NGS). The two most common custom-capture methods are based on hybridization (either on array or in-solution) or on highly multiplexed PCR. In the method based on hybridization, probes present in high density microarrays are bound to the segments of interest. In the solution-based method, the segments of interest are bound to biotinylated DNA or RNA probes. Then, they are purified using streptavidin-labeled magnetic beads. The second method has many advantages compared to the first one including its easy accessibility since it does not require additional processing equipment (Bodi et al., 2013).

1.2. ctDNA detection methods

Due to its low sensitivity and specificity, ctDNA is not always a reliable marker for monitoring prognosis of cancer patients; thus, introducing biomarkers into routine clinical practice may be affected (Mäbert et al., 2014). However, the increased usage of PCR and Next Generation Sequencing techniques are facilitating novel high-sensitivity applications for ctDNA, the generation of large clinical data sets, and a better understanding of the origins of both cfDNA and ctDNA (Wan et al., 2017).

Numerous platforms have been developed to analyze ctDNA including real-time quantitative PCR (qPCR); the Scorpion Amplification-Refractory Mutation System (ARMS); Beads, Emulsion, Amplification and Magnetics (BEAMing); digital PCR (dPCR); and Next-Generation Sequencing (NGS) (Sorber et al., 2017).

Real-time qPCR is a quantitative method (Valasek and Repa, 2005), consisting of both TaqMan based and Scorpion ARMS assays which are the most widely used platforms for the quantification of nucleic acids. ARMS is a standard targeted PCR method for detection of single-base mutations and small deletions by amplifying the target DNA using sequence-specific primers that only trigger this amplification when the target allele is present within the sample. Because this method is not relatively very sensitive, most likely due to primer competition, it is often combined with Scorpion technology, thus the presence of a mutation can be detected by the Scorpion ARMS in a real-time PCR setting. Scorpions consist of a PCR primer covalently linked to a probe that interacts with a quencher. The presence of a mutation can be detected by the Scorpion ARMS in a real-time PCR setting (Sorber et al., 2017). This technique is sensitive and highly selective in detecting a low percentage of a mutant allele in a background of wild-type DNA (EGFR RGQ PCR Kit Handbook, 2010).

Although qPCR has many advantages including its low cost and straightforward techniques, it does not have the sensitivity that other newer techniques (such as ddPCR) can achieve (Sorber et al., 2017). These newer technologies with even greater sensitivity than existing ones reduce the false-negative rates observed with qPCR-based ctDNA testing methods. Other methods based upon emulsion PCR, such as digital droplet PCR (ddPCR) and BEAMing enhance the traditional PCR (Fan et al., 2017). The ddPCR approach is similar to real-time qPCR but instead of amplifying the whole sample, the DNA templates are partitioned into thousands of individual, parallel PCR reactions. Signal detection indicates the presence (positive) or absence (negative) of the target sequence. In this manner, ddPCR is considered highly sensitive by detecting the mutated ctDNA in a high background of wild type cfDNA. Several array-loading platforms exist where partitioning is based on capillary action, either microfluidic-chamber or microfluidic-well chip based (Thermo Fisher OpenArray and Fluidigm) and droplet-based dPCR such as Bio-Rad® and Raindance®, in which samples are dispersed into 20,000 and 10,000.000 droplets, respectively, and amplified and screened individually by flow cytometry for the presence of

signal (Sorber et al., 2017).

Other than the low time, cost and easy workflow, compared to the quantitative PCR, this technique has a high precision for better reproducibility for low-input target concentrations since it allows the sequencing of high quality libraries prepared from tens to hundreds of picograms of starting material, higher precision for higher copy number variation analysis, greater sensitivity for rare mutation detection and library quantification, and absolute measurement eliminating the need for standard curve so that no standard, calibration, or information is needed about the molecular weight distribution of the template molecules (Aarthy et al., 2015; White et al., 2009).

Although ddPCR only screens for known mutations, since it requires prior knowledge of the region of interest, several mutations can be assessed simultaneously (Perakis and Speicher, 2017; Sorber et al., 2017). The two most prominent examples of ddPCR-based methods are beads, emulsion, amplification, and magnetics (BEAMing) and droplet ddPCR. Both methods involve separation of template sample into thousands of tiny droplets that each represents isolated reaction chambers, which contain at most 1 template molecule (Chaudhuri et al., 2015). BEAMing is based on the same principle as emulsion ddPCR, during which streptavidin-biotin interactions bind DNA templates to magnetic microbeads before emulsion into droplets. After amplification, magnetics are employed to release and purify the PCR products; thus, known mutations can be detected in a high background of wild type cfDNA (Sorber et al., 2017). Clinically, this technique enabled the quantification of the mutant allele fraction of cancer mutations in the plasma of patients with various stages of cancer (Wan et al., 2017). However, in addition to only screening for known mutations, BEAMing also has a complex workflow and high cost per sample, making it difficult to be applied in routine clinical settings (Sorber et al., 2017).

Combining it with allele-specific PCR assays leads to an extremely precise enumeration of the number of total template molecules that carry a specific mutation and can achieve low detection limits (as low as 0.01%). Using these methods, ctDNA has been detected in a wide range of patients with cancer (Chaudhuri et al., 2015).

Due to the practical limitation of the number of genomic positions that can be interrogated by ddPCR, next-generation sequencing (NGS)-based approaches were developed for ctDNA detection by massively parallel sequencing of hundreds of millions of DNA fragments from a single sample thus genotyping many potential mutations simultaneously (Chaudhuri et al., 2015).

Moving from research to clinical applications, NGS is now helping in effectively guiding therapeutic decision making by improving molecular markers detection and analysis for diagnosis, prognosis and monitoring of cancer. With characterization of tumor mutation profiles, the patient is now involved in deciding on treatment by being able to make informed choices regarding choice of therapy since the treatment is targeted to specific genes or pathways activated by particular mutations. On the other hand, the DNA sequence data helps physicians move toward personalized medicine by understanding the genetic alterations at the DNA and RNA levels and correlate them with specific characteristics in each patient, and discover new actionable biomarkers (Wang and Wheeler, 2014).

Compared with ddPCR, individual DNA fragments in NGS are also isolated prior to sequencing, leading to an increased sensitivity, so that a small fraction of a mutated sequence can be detected. As such, allelic frequency can be determined by NGS with an accuracy comparable to that of ddPCR for ctDNA detection (99% accuracy) (Fan et al., 2017).

Highly sensitive and specific methods have been developed to detect ctDNA either through detecting single-nucleotide mutations in ctDNA or whole-genome sequencing (WGS) to establish copy-number changes. These technologies are divided into targeted methods that aim to detect mutations in a specified subset of the genome or untargeted approaches, like array-CGH, whole-genome sequencing, or whole exome sequencing, that aim to screen the genome and discover new genomic

aberrations such as those that confer resistance to a specific targeted therapy. In general, targeted approaches have a higher analytic sensitivity than untargeted approaches, despite strong efforts to improve detection limits (Alix-Panabières and Pantel, 2016).

Targeted approaches are used to analyze the allelic frequency of specific mutation in a sample. These mutations can be single nucleotide mutations or structural chromosomal rearrangements in the specified genomic regions of plasma DNA (Perakis and Speicher, 2017). In contrast, untargeted approaches do not depend on a predefined set of genes but aim at a comprehensive analysis of the whole genome. Whole exome sequencing (WES) can be adopted for sequencing of cfDNA for the identification of mutations that are clinically actionable and may help in diagnosis, prognosis or treatment. It has some similar approaches to genotyping and based on sequencing of all exons within the genome (Chaudhuri et al., 2015). Whole genome sequencing of plasma DNA is based on structural variations and somatic copy number alterations (SCNAs) detection (Perakis and Speicher, 2017). It was also used on cfDNA to identify chromosomal aberrations, focal amplifications and gene rearrangements, and hybrid-capture sequencing (Wan et al., 2017).

Although WGS can screen the whole genome but it cannot be adopted for routine use because of the high cost when detecting single nucleotide variants, whereas WES methods focuses on the protein coding sequences only, but is less sensitive to identifying copy number changes (Ma et al., 2015). Other than the high cost and dependence primarily on copy number variation analysis, untargeted sequencing has limited sensitivity to detect specific single nucleotide variations, insertions, deletions, or rearrangements (Chaudhuri et al., 2015).

Finally, using untargeted sequencing involves ethical concerns such as informing about variants irrelevant to the main purpose of testing or disease such as discovering hereditary genes. Thus, physicians should know what to report especially that most of these genes involve non-cancer-related syndromes and bring up unexpected inherited genetic issues (Gagan and Van, 2015).

Effectively, and in order to decide which approach should be used, many factors should be taken into consideration mainly the cost, genome coverage, and sample availability (i.e., quality and quantity). Among the three genotypic assays, targeted sequencing uses the least time, reagents, data storage and expert analysis in addition to being less expensive than either WGS or WES and permits sequencing with great depth (Lin et al., 2014).

The development of sensitive and accurate techniques such as Next-Generation Sequencing (NGS); Beads, Emulsion, Amplification, and Magnetics (BEAMing); and Digital PCR (dPCR), has helped in targeted therapy such as detecting *EGFR* mutations, *MET* amplifications, and *ALK* and *ROS1* translocations that already have available therapy against. Other than the help of detecting genetic alterations in targeted therapy, quantification of these changes improves the follow-up of tumor dynamics in real time (Sorber et al., 2017). It is important to mention that NGS approaches use digital PCR in the *template preparation step* (Uchida et al., 2015).

Newman et al. developed a new approach that combines the benefits of targeted and untargeted sequencing, and it is called *Cancer Personalized Profiling by Deep Sequencing* (CAPP-Seq). It is very sensitive and has the potential to detect specific single nucleotide variations, insertions or deletions, somatic copy number alterations, and gene rearrangements in a single assay. CAPP-Seq uses a multiphase bioinformatics method that enriches recurrently mutated regions of interest via hybrid capture. In addition to that, it can be applied to any cancer type, thus there is no need for personalization of assays (Chaudhuri et al., 2015).

On the other hand, Forshew et al. developed another method called tagged-amplicon deep sequencing (Tam-Seq) that is based on the amplification and deep sequencing of genomic regions covering thousands of bases, even from individual copies of fragmented DNA. This technique has great sensitivity and specificity (> 97%) and ability to detect

mutations at very low allele frequencies (as low as 2%) (Sorber et al., 2017). It can be used for targeted sequencing such as a panel of commonly mutated exons or can be used for untargeted sequencing. This method can quantify mutant DNA at many loci in each patient but it has a limitation regarding sensitivity that is low for mutations below approximately 1%–2% (Volik et al., 2016). They also developed the Safe-Sequencing System (Safe-SeqS), an amplicon-based approach, to improve the sensitivity of NGS and increase its ability to identify rare variants. Each template corresponds to a unique identifier (UID) and then it is amplified and sequenced. The importance of this method is that it can detect ctDNA in many tumor types (Chaudhuri et al., 2015).

These improved NGS techniques are characterized by high sensitivity, decreased sequencing error rate and PCR errors in addition to broad genomic coverage (Ignatiadis et al., 2015). But they remain expensive, time intensive, and require sophisticated data analysis systems with patient-specific optimization of amplicons to be able to detect alterations (breakpoints, rearrangements, somatic copy number alterations, etc.) (Chaudhuri et al., 2015).

Usage of ctDNA for NGS analysis has increased and demonstrated that ctDNA could be used for alterations detection with high sensitivity and ability to detect low-frequency mutations. For example, Dawson and colleagues reported that ctDNA in patients with metastatic breast cancer using whole-genome or targeted sequencing had superior sensitivity to other circulating biomarkers and helped in treatment decision. On the other hand, Murtaza and his colleagues demonstrated that whole-exome sequencing for ctDNA in patients with advanced breast, ovarian, and lung cancers reflected tumor genome alterations and analysis of these mutant alleles was associated with treatment resistance (Wang and Wheeler, 2014). Moreover, using ctDNA for panels of tagged amplicons by deep sequencing of multiple genes has enabled analysis of multiple mutations in a single assay.

However, analysis of ctDNA mutations has several limitations. CtDNA is highly fragmented in blood and constitutes a low fraction of the total circulating cell-free DNA even in patients with metastatic tumors. In addition, the half-life of ctDNA is short ranging from several minutes to hours. Furthermore, the preamplification step used in some techniques increases polymerase-based errors and PCR bias which decrease accuracy (Wood-Bouwens et al., 2017). Finally, as already mentioned, the process of sequencing from sample preparation to computational construction of the sequence takes days and even weeks (Shen et al., 2016).

To overcome these issues, a single-color digital droplet PCR (ddPCR) assay has been developed. It involves usage of a double-stranded DNA intercalator dye, such as EvaGreen, and paired allele-specific DNA primer sets. It involves partitioning one or several DNA molecules into many droplet reactions, where a specific PCR amplicon product is generated. The intercalator dye binds to double-stranded DNA. After droplet partitioning and thermal cycling, the droplet-specific fluorescence generated is measured which is directly proportional to the size of the amplicon product. This method has a lot of benefits including owing a flexible design, high sensitivity (detects a 0.10% mutation fraction), high specificity for single-nucleotide alterations detection, no requirements for a preamplification step, small amount of starting material is required (only several nanograms of cell-free DNA), and ability to detect as few as three mutation-bearing molecules in a single reaction, in addition to the acceptable time required for completion within few hours (Wood-Bouwens et al., 2017). Recently, PCR-based digital approaches have been modified and supplemented with techniques that use NGS to identify rare mutant variants in complex mixtures of DNA. These methods have allowed detecting a single point mutation, and multiple genes of interest in one sample (Diaz Jr and Bardelli, 2014).

Moreover, gene methylation can also be analyzed based on ctDNA. DNA methylation is silently expressed because it occurs in CpG dinucleotides in the promoter region of tumor suppressor genes. There are numerous ways to methylation analysis that are mainly divided into 3

categories. The first category contains (for methylation content) high-performance liquid chromatography (HPLC) or high-performance capillary electrophoresis (HPCE). The second category includes (for candidate gene) methylation-sensitive restriction endonuclease-PCR/Southern (MSRE-PCR/Southern), bisulphite sequencing, methylation-specific PCR (MS-PCR), MethyLight, etc. Finally, the third category covers (for methylation pattern and methylation profiling) restriction landmark genomic scanning (RLGS), amplification of inter-methylated sites (AIMS), Methylated CpG-island amplification (MCA), etc. Of note, the most commonly used method is usually MS-PCR. Detection of ctDNA methylation alterations help in diagnosis, prognosis, and monitoring disease for cancer patients and created many improvements especially in colorectal cancer, lung cancer, breast cancer, pancreatic cancer as well as some other types of carcinoma (Ma et al., 2015).

1.3. Examples of NGS application using ctDNA

1.3.1. Acute myeloblastic leukemia

Acute myeloblastic leukemia (AML) is an active white blood cells cancer characterized by increased cytogenetic abnormalities and genetic mutations. Next-generation sequencing techniques have the potential to influence the diagnosis and prognosis of AML patients and allow for more personalized therapy. In order to reduce sequencing cost, study epigenetic alterations, understand changes across various stages, increase the availability of these techniques and easily adapt them in the future, whole-genome, exome, and targeted gene sequencing studies have been conducted successfully in AML (Rao and Smith, 2013).

Traditional cytogenetic testing methods have been used as standard procedures for the detection of both balanced chromosomal rearrangements and copy number variants (CNVs) in myeloid malignancies for years. Compared to these conventional methods, a new technology, single nucleotide polymorphism (SNP) microarray (SNPa) technology, has emerged for genome wide CNV detection. In addition, this method has better resolution for an enhanced diagnosis. Using SNPa, 50% of AML patients and 70% of MDS patients present CNVs, which may be important for risk stratification. For example, these recurrent submicroscopic deletions involving genes have been encountered in MDS and AML patients: *TET2* on 4q, *EZH2* on 7q, *ETV6* on 12p, *TP53* on 17p and *RUNX1* on 21q. However, next generation sequencing (NGS) methods can detect somatic mutations in a number of recurrently mutated genes in myeloid cancer patients. These mutations include *ASXL1*, *TET2*, *DNMT3A*, *IDH1* and *IDH2*, *CALR*, spliceosome genes such as *SF3B1* and cohesion complex genes, and are single-nucleotide variants (SNVs), short insertions and deletions (indels) which are too small to be detected by methods such as SNPa. Moreover, NGS has the potential to detect mutations with single base resolution; thus, unlike SNPa, gene level or sub-gene level CNVs can be detected. Overall, NGS are superior to SNPa due to having a better resolution and ability to detect small-scale intragenic CNVs (Shen et al., 2016).

CNV analysis by whole exome and genome sequencing is not routinely adopted for clinical testing due to being expensive, laborious, and having low sequence coverage which may limit the detection of lower somatic CNVs. But using this technique offered the opportunity to analyze CNVs using a single testing method. The most frequent mutations and CNVs detected were in the genes *LUC7L2* (7q deletion), *EZH2* (7q deletion), *TP53* (deletion), *ETV6* (deletion), *RAD21* (possible trisomy 8), *NSD1* (5q deletion) and *NPM1* (5q deletion). Furthermore, *KMT2A*-PTDs, exon-level duplications, predicting a worse outcome, were found in 6–10% of AML patients. Thus, identifying simultaneously both gene mutations and targeted CNVs helped in finding associations between CNVs and somatic mutations. For example, in a study for Shen and his colleagues, *ETV6* deletion was strongly associated with *TP53* alterations; noting that *TP53* was either deleted or mutated in 5 out of 8 *ETV6* deleted cases. On the other hand, 7q deletion in *LUC7L2* and *EZH2* genes was associated with mutations in *TP53*, *KRAS* and *IDH1*. As

previously analyzed in other studies, *TP53* mutations were associated with targeted CNVs in MDS and AML cases. Finally, *NPM1* mutations were highly present in AML patients, and were inversely-associated with detection of targeted CNVs. In concordance with SNPa, these CNVs detected by untargeted NGS have influenced patient prognosis. For example, AML patients harboring 17p deletion including the *TP53* gene, 7q deletion and 5q deletion had poor prognosis (Shen et al., 2016).

Other than detecting CNVs with somatic driver mutations, NGS assays were also able to detect CNVs alone in the targeted genes, in order to provide additional information concerning patient prognosis that mutation analysis alone cannot provide (Shen et al., 2016). However, for routine clinical testing of cancer, targeted sequencing panels are more commonly adopted because they are more sensitive and yield a high read depth. Most myeloid cancers are characterized by harboring more than one mutation which this is the reason behind adopting panel-based testing, unlike primary methods that used to combine few single gene tests. Usage of panel-based testing helps in diagnosis, prognosis and monitoring of AML cases, in addition to better patient management. For example, concurrent *DNMT3A/NPM1* mutations were detected in most AML patients. In addition, *DNMT3A* missense mutations indicate shorter overall survival and higher relapse when stratified by *NPM1* mutation status, whereas *DNMT3A* truncation mutations are not associated with clinical outcomes (Shen et al., 2016). Therefore, numerous NGS methods have been developed targeting a specified region of the genome using ctDNA, not one or few mutations (Perakis and Speicher, 2017).

1.3.2. Lung cancer

Usage of ctDNA in lung cancer patients has many applications and purposes. As a tool for early diagnosis for lung cancer, DNA methylation detection methods are useful and essential. There are numerous hypermethylated genes related to lung cancer (> 80) including *APC*, *RARB*, *RASSF1A*, *CDH13*, *SHOX2*, *SHP-1*. Other than early diagnosis, DNA methylation plays a major role in determining the prognosis of patients and their survival. For example, in a study done by Salazar et al., the methylation status of the gene *CHFR* was correlated with the use of second-line chemotherapy or EGFR TKIs in 179 of 366 patients. Thus, using ctDNA for methylation status testing is a very encouraging and promising method for patient diagnosis, prognosis and monitoring (Ma et al., 2015).

In order to study disease progression, prognosis and drug resistance, different studies were conducted to analyze ctDNA EGFR receptor mutations by NGS methods. EGFR receptor mutations are activated in about 15% of lung adenocarcinomas, and 90% of these mutations are either the *L858R* point mutation or in-frame deletions of exon 19. These adenocarcinomas are often treated with tyrosine kinase inhibitor (TKI) drugs which are mostly erlotinib, gefitinib, or afatinib. However, during treatment with these agents, most lung cancer patients develop drug resistance within one year, and in around 50% of the cases the resistance is due to the appearance of EGFR *T790M* mutation (Ansari et al., 2016). However, some studies have shown that *T790M* may be present before starting TKI drugs therapy. ctDNA has the potential to detect *T790M* in blood and thus proves a possibility to monitor drug resistance by a non-invasive method. Other than detection of drug resistance, *T790M* detection is important because third-generation inhibitors have shown activity in the presence of this mutation and using a combination of drugs provides a promising response (Crowley et al., 2013). As already mentioned, using ctDNA and NGS for *T790M* detection has been adopted in many studies to prove its importance in clinical settings. Wang et al. used ddPCR and ARMS to analyze *T790M* cfDNA mutations in 135 TKI-treated lung cancer patients who had progression-free survival on the TKI therapy for over six months. *T790M* was identified in 31.5% using ddPCR and 5.5% using ARMS of these patients prior to TKI therapy, demonstrating that ddPCR was a more sensitive test. Post TKI therapy, *T790M* was identified in 43.0% of patients by ddPCR, and in 25.2% by ARMS. *T790M* levels had a poorer

progression-free and overall survival, demonstrating the importance and prognostic value of *T790M* detection (Ansari et al., 2016).

On the other hand, Watanabe et al. examined 373 NSCLC patient tumor samples with EGFR-activating mutations for *T790M* using ultra-sensitive ddPCR. They found *T790M* in 79.9% of the patients and it was associated with larger tumor volumes. Thus, these reported results confirm that ctDNA analysis for *T790M* in lung cancer patients have a great value in monitoring disease burden, disease progression, and drug resistance emergence (Ansari et al., 2016).

Other than *T790M*, *MET* amplification is also one of the resistance mechanisms associated with *EGFR* mutated NSCLC. Paweletz et al. have reported the detection of *MET* amplification in ctDNA of NSCLC patients using a targeted NGS technique. In a patient harboring an *EGFR* exon 19 deletion, acquired resistance to erlotinib and afatinib was developed due to *MET* amplification. On the other hand, amplification was also detected in an advanced NSCLC patient with a high clinical risk of developing a targetable genomic alteration using ctDNA. These two cases of NSCLC had concordance for *MET* amplification between plasma and tumor tissue. Thus, testing for *MET* amplification at diagnosis is important for predicting the prognosis of patients and their response to targeted therapy (Sorber et al., 2017).

In a study done by Thierry et al., NGS methods were used on ctDNA to screen for a range of tumor biomarkers and found that between 68 matched tumor and plasma samples of patients with metastatic NSCLC, mutation concordance (including *BRAF*, *EGFR*, *ERBB2*, *KRAS*, and *PIK3CA* amplicons) was 68%, with a sensitivity of 58% and specificity of 87% (Thierry et al., 2014).

Uchida et al. concluded an NGS “deep sequencing” using ctDNA for genotyping of *EGFR* in lung cancer patients, after doing it on tissue biopsy samples as a reference. With the high specificity results and concordance that they got, they proved that plasma samples can be used for *EGFR* mutation analysis in order to improve TKI therapy (Fan et al., 2017).

More recently, Rachiglio et al. also performed NGS analysis of plasma sampled from 44 patients with metastatic lung cancer to detect *EGFR* mutations. Out of 22, 17 patients were positive knowing that their tumor samples were also positive (sensitivity 77%). In addition, 2 patients presented with positive *EGFR* mutations having their tumor tissues negative; of note, both were confirmed via ddPCR in plasma and tissue samples (Fan et al., 2017).

Similarly, Yao et al. performed targeted DNA sequencing to screen for driver gene mutations in 39 NSCLC Chinese patients with matched ctDNA and tissue samples. The gene mutation concordance was 78.2% between plasma and tissue samples, and a sensitivity of *EGFR*, *KRAS*, *PIK3CA* mutations, and gene rearrangements detected in ctDNA at 70.6%, 75%, 50%, and 60%, respectively (Fan et al., 2017).

As in the case of AML patients, using a panel of multiple genes is better than using a unique biomarker in order to increase specificity and sensitivity (Ma et al., 2015). In their turn, Villafior et al. assessed a targeted NGS gene panel in 31 NSCLC patients which matched tissue and blood samples. 7 samples presented activating *EGFR* mutation and had a concordance rate of 71% (Fan et al., 2017).

In order to confirm the NGS results using ctDNA, Thress and his colleagues developed a droplet digital PCR (ddPCR) assay for the detection of other *EGFR* mutations such as *C797S* in plasma and their association with treatment resistance. ddPCR was applied on serial plasma specimens of one subject. Exon 19 deletion and *T790M* mutation were highly present in plasma before treatment initiation with no presence of *C797S* mutation. After 6 weeks, quantity of exon 19 deletion and *T790M* mutation were reduced 100-fold compared to their concentration before treatment. Of note, the concentration of those 2 alterations also increased with systemic cancer progression. In addition, a newly acquired *EGFR C797S* mutation was noticed at progression. In order to confirm and analyze those findings, they then performed serial ddPCR profiling on plasma of 19 advanced lung cancer subjects with acquired resistance to a third EGFR TKI drug. *EGFR* activating

mutations were present in all subjects before treatment, *T790M* mutations were detected in 15 subjects while *C797S* mutations were not shown in any subject. Among the 15 patients who were positive for *T790M*, 6 patients (40%) presented simultaneously *C797S* at progression with *T790M* and exon 19 deletion. On the other hand, among those 15 patients, 5 of them (33%) did not present *C797S* mutation with the *T790M*. Additionally, among the 15 patients that were *T790M* positive before treatment, 4 of them (27%) did not show *T790M* anymore in their blood at progression; thus, in contrast, *EGFR* activating mutations did not increase at cancer progress. Finally, four patients who were negative for *T790M* before treatment, have not presented *C797S* or *T790M* mutations in plasma at progression (Thress et al., 2015).

Now, in patients with acquired *C797S* mutation, NGS using ctDNA was performed for further analysis. First, they reported that *T790M* and *C797S* were present on same allele because individual sequencing reads in one case contained the *T790M* and *C797S* mutations together. Second, another case at progression showed by NGS on ctDNA that *T790M* and *C797S* mutations are present on different alleles, thus patients without *T790M* can acquire the *C797S* mutation. In both cases, targeted NGS on tumor biopsies after treatment confirmed that *C797S* mutation was acquired and not identified in the pretreatment tumors, and no other acquired mutations. NGS of plasma showed concordance with tumor results and also identified a second DNA alteration encoding the *C797S* mutation, suggesting that multiple mutations occurring at the same time can be acquired and lead to resistance development. Besides, no other acquired mutations were detected using plasma NGS. Therefore, in the presence of *T790M*, acquired *C797S* mutation mediates resistance to certain therapy in NSCLC (Thress et al., 2015).

Also, the presence of *KRAS* mutations in different types of cancer patients showed bad prognosis. For example, the plasma of 246 patients with advanced NSCLC showing *KRAS* mutations predicted poor prognosis in those receiving first-line chemotherapy. On the other hand, *KRAS* mutations in plasma of 44 patients with pancreatic cancer, despite the poor sensitivity of the test (27%), showed a significantly reduced life survival compared to those negative for *KRAS* mutations (17% in *KRAS* positive versus 41% in wild type at 6 months, and 0% in *KRAS* positive versus 24% in wild type at 12 months) (Crowley et al., 2013).

Other than the mutations presented above, *ALK* (anaplastic lymphoma kinase) mutation *L1196M* or *C1156Y* was related to resistance development to crizotinib in NSCLC patients with *ALK* rearrangement (Polivka et al., 2015).

As already discussed, ctDNA usage has many advantages compared with the traditional usage of tissue biopsies and has the potential to become the gold standard for detection, prognosis and monitoring of cancer in a non-invasive way. However, the available methods for ctDNA analysis suffer from limited sensitivity and insufficient patient coverage for clinical usefulness. Thus, Diehn and his colleagues developed a quantitative, economical and ultrasensitive method which is cancer personalized profiling by deep sequencing (CAPP-Seq). They applied CAPP-Seq on NSCLC patients with a design covering several classes of somatic alternations that recognized mutations in > 95% of cases. They reported that ctDNA was detected in 100% of patients with stage II–IV and 50% of those with stage I, with 96% specificity. Therefore, CAPP-Seq can be applied in routine clinical applications of patients and plays a role in accelerating the detection, personalized therapy, and monitoring of disease (Wu and Qu, 2015). Furthermore, Chabon et al. employed NGS (CAPP-Seq) to study resistance mechanisms of ctDNA samples in 43 NSCLC patients treated with rocletinib (Fan et al., 2017).

Effectively, NGS assays were also used as confirmatory tools for other techniques being the dominant detection method with the highest resolution. As an example, it has been used to validate the *T790M* results detected by MALDI-TOF MS in NSCLC patients (Su et al., 2012). Finally, Narayan et al. developed an amplicon-based method that

incorporates an error-suppression application and showed that they could detect *EGFR*, *BRAF*, and *KRAS* mutations in the plasma of some patients with lung cancer (Chaudhuri et al., 2015).

1.3.3. Breast cancer

Bettegowda and colleagues detected ctDNA in > 75% of breast cancer patients with advanced stage and 50% of patients with localized breast cancer using whole-genome sequencing (Alix-Panabières and Pantel, 2016).

Murtaza and colleagues also tracked genomic evolution on ctDNA of metastatic breast cancer patients in response to therapy, and demonstrated that ctDNA mutation levels increased in association with acquired drug resistance, including activation of *PIK3CA* mutation following treatment with paclitaxel, a truncating mutation in the Estrogen Receptor (ER) coactivator mediator complex subunit 1 (MED1) following treatment with tamoxifen and trastuzumab, and, a splicing mutation in *GAS6*, the ligand for the tyrosine kinase receptor *AXL*, following subsequent treatment with lapatinib (Alix-Panabières and Pantel, 2016).

Triple negative breast cancer (TNBC) cases are defined by the absence of both estrogen and progesterone receptors expression, and the lack of *HER2* amplification. *TP53* mutations are candidate biomarker targets in TNBC. Madic et al. used NGS techniques to detect *TP53*-specific mutations in TNBC patients with metastatic disease (Madic et al., 2015).

HER2-positive breast cancer patients might benefit from *HER2* targeted therapeutics such as trastuzumab, even if in metastasized or advanced stage. Thus, assessing the *HER2* status is crucially important for treatment decisions and prognosis. Current methods for the assessment of the *HER2* status in tumors, primarily immunohistochemistry and fluorescence in situ hybridization, have restrictions. Therefore, a liquid biopsy has considerable clinical importance. Gevensleben et al. have recently described the optimization of a digital PCR method to detect *HER2* amplification in ctDNA of patients with metastatic breast cancer (Crowley et al., 2013).

Newer sequencing-based platforms that aim to decrease sequencing error rate and PCR errors during library preparation include SafeSeq, TAM-Seq, CAPP-Seq, and Ampli-Seq. These technologies are increasing analytic sensitivities with PCR and maintaining broader genomic coverage at the same time (Ignatiadis et al., 2015).

The Tagged-Amplicon Sequencing (TAM-Seq) method was developed by Forshew and his colleagues to detect mutations in plasma samples from patients with advanced ovarian cancer, and showed that this technique can achieve a detection limit of ~2% (Chaudhuri et al., 2015).

Beaver et al. discussed the possibility of identifying *PIK3CA* mutations in plasma samples from 29 patients with early-stage breast cancer. There was concordance between tumor and plasma in these patients; the mutations identified in primary tumors were also detected in pre-surgery plasma samples by ddPCR with high sensitivity and specificity of 93.3 and 100%, respectively. In addition to that, residual disease was successfully identified by detection of the mutations in cfDNA from postoperative plasma samples. In another study done by Rothé et al., for 17 patients with metastatic breast cancer, the concordance between tumor and plasma samples after analysis for mutations in 50 selected genes by NGS was 76% (Beaver et al., 2014; Polivka et al., 2015; Rothé et al., 2014).

Other than disease detection and therapy response, disease recurrence could also be monitored. Some data suggested that persistence of *TP53* mutation in plasma cfDNA of breast cancer patients with stage II or III that was in remission was associated with higher risk for recurrence (Polivka et al., 2015). Numerous studies were conducted aiming to find novel methylated genes and assess the changing aspects of methylation in order to monitor the different forms of treatment for breast cancer, mostly using the candidate gene testing. This means a majority of the makers being the well-established genes, such as *cyclin*

D2, *RARβ2*, *ESR1* and so on. Dulaimi et al. have found that at least one hypermethylation of *APC*, *RASSF1A* or *DAP*-kinase could be found in 94% serum samples of all the breast cancer patients. Scholars from All India Institute of Medical Sciences have conducted prospective studies including 100 invasive ductal breast cancer patients were methylation status of multidrug resistance 1 (MDR1), Stratifin, ERα and PR, DNA repair genes-*BRCA1*, *MGMT* and *GSTP1* were tested. Although a significant correlation was found between methylation status of the promoter of the above genes in tumor tissue and paired serum, the sensitivity was not high: *MDR1* 50%, *Stratifin* 56%, *ERα* 55%, *PRB* 55%, *BRCA1* 22%, *MGMT* 26%, *GSTP1* 22% (Ma et al., 2015).

Besides, hypermethylated promoter regions of *BRCA1* in serum cfDNA of primary invasive ductal breast cancer patients had influence on prognosis: poor disease free survival as well as poor overall survival. Similarly hypermethylated promoter regions of *GSTP1* in serum of these patients had poor disease free survival. Other studies showed worse overall survival rate for primary invasive breast cancer patients with serum cfDNA hypermethylation in promoter regions of *GSTP1*, *PITX2*, *RASSF1A* and *RARβ2* compared to negative patients. Of note, only *RASSF1A* showed prognostic significance for distant disease free survival. These hypermethylation regions were associated with poor prognosis also in cfDNA of colorectal cancer, gastric cancer, hepatocellular carcinoma and other tumor types (Polivka et al., 2015).

1.3.4. Pancreatic cancer

Pancreatic cancer is the first solid tumor associated with a specific mutation in ctDNA due to many factors. One of the causes is that the *KRAS* gene is frequently mutated and easy to detect.

Detection of ctDNA in primary pancreatic cancer is mostly 30% to 50% whereas the specificity is generally higher, approximately 90%. Numerous detection methods have been developed including restriction digestion and single-stranded conformational polymorphism. In one study, sensitivity was enhanced when CA19-9 was measured in combination with DNA measurements. However, few pancreatitis cases also showed *KRAS* gene mutations but at a lower frequency, 5% to 15%, than adenocarcinoma. Most studies have focused on *KRAS* mutations in pancreatic cancer due to many advantages such as their prevalence. For example, Sorenson et al. used allele-specific amplification to assay for mutations in codon 12 in the plasma or serum of pancreatic adenocarcinoma patients. The usage of higher-throughput methods, such as NGS and digital PCR, have really affected and improved this field. For example, a recent study using this method showed that pancreatic duct cancer had a higher level of ctDNA than other types of cancer, especially in metastatic disease. In summary, ctDNA is an ideal candidate for pancreatic cancer diagnosis and prognosis (Ma et al., 2015).

1.3.5. Colorectal cancer

Presence of *KRAS*, *APC*, and *TP53* mutations in blood has influence on colorectal cancer diagnosis, prognosis, and treatment response (Ma et al., 2015).

Thierry et al. assessed in their study the mutation status of *KRAS* and *BRAF* by using allele-specific quantitative PCR of cfDNA in 106 plasma samples from patients with metastatic colorectal cancer and compared it to the mutations detected in tissue (primary or metastatic) tested by a standard method. The cfDNA analysis showed 100% specificity and sensitivity for the *BRAF V600E* mutation and 98% specificity and 92% sensitivity for the *KRAS* mutations, with a concordance value of 96% (Polivka et al., 2015; Thierry et al., 2014).

Also, *KRAS* mutations in ctDNA have been reported to influence the disease stage where mutations were detected at the highest level in patients with more advanced stage. Moreover, *KRAS* mutations in ctDNA are also associated with a higher risk of recurrence after surgery. Besides, *KRAS* analysis could also help in monitoring response to monoclonal antibody therapy for colorectal cancer (Ma et al., 2015). Furthermore, Crowley et al. reported that the presence of high levels of *KRAS* mutant alleles in the plasma is a clear indicator of response to

treatment with third-line cetuximab and irinotecan in the metastatic colorectal cancer setting (Crowley et al., 2013). Polivka et al. showed that patients with higher *KRAS* ctDNA levels had shorter progression-free survival than patients with lower ctDNA levels (Polivka et al., 2015).

Other biomarkers played a major role also in colorectal cancer such as the well-known novel sequences of *APC*, *RASSF1A* and *E-cadherin* as well as novel markers in plasma. A German study done by Philipp et al. showed that methylation of helicase-like transcription factor (*HLTF*) and hyperplastic polyposis 1 (*HPP1*) in serum significantly correlated with tumor size, stage, and metastatic disease, and had prognostic value in metastasized colorectal cancer (Ma et al., 2015).

Exon 15 is the point of interest in the analysis of *APC* mutations in ctDNA, and it is a hotspot for *APC* mutations in colorectal cancer. The rate of *APC* mutation detection in primary ctDNA is approximately 45%. As for *TP53*, where most studies focused on exons 4 and 8 in colorectal cancer patients, the mutation rate has been identified in ctDNA in about 40% of cases (Ma et al., 2015).

Other than the importance of ctDNA in detection, prognosis and monitoring of disease in colorectal cancer patients, the clonal evolution during targeted therapies might also be detected. Acquired resistance to EGFR-specific antibodies is associated with the emergence of RAS pathway mutations that can be detected by liquid biopsy. Besides, the ctDNA might be able to detect these mutations before disease progression can be documented by standard imaging. These patients that acquired resistance to EGFR-specific antibodies displayed a heterogeneous pattern of mutation in *KRAS*, *NRAS*, *BRAF*, and *EGFR* noting that the proportion of *KRAS*-mutated alleles is dynamically affected by the anti-EGFR drug. On the other hand, whole-genome sequencing was performed on ctDNA in those patients and revealed several copy-number changes in all samples, including loss of the chromosomal 5q22 region harboring the *APC* gene and loss of chromosome arms 17p and 18q in addition to amplifications in known genes involved in the resistance to EGFR blockade, such as *MET*, *ERBB2*, and *KRAS* (Alix-Panabières and Pantel, 2016).

Unless targeting ctDNA alterations in hotspots of certain genes, a panel targeting mutations of the *KRAS*, *TP53*, and *APC* genes enabled the detection of at least one gene mutation from approximately 75% of colorectal cancer tissue and 45% of these patients plasma (Ma et al., 2015).

For the early diagnosis of colorectal sample, the promoter hypermethylation status of *SEPT9* is used as an easy and quick screening test. Sensitivity and specificity were 72–90% and 88–90% respectively for *SEPT9* promoter methylation using PCR-based retrospective trials. Another research from USA showed that methylated *SEPT9* DNA in plasma may help screening out 72% colorectal cancer with a high specificity of 93%. Of note, *SEPT9* methylation could be found in precancerous lesions of colorectal cancer (Ma et al., 2015).

1.3.6. Prostate cancer

ctDNA is able to detect multiple copy-number aberrations in prostate cancer patients using genome-wide profiling. Similar to those detected in prostate tumors, losses in 8p and gains in 8q were detected. For treatment, patients with castration resistant prostate cancer (CRPC) demonstrated high-level copy-number gains in the *AR* locus. In addition, Azad and colleagues found that *AR* amplification was significantly more common in patients progressing on enzalutamide than on abiraterone or other agents. Besides *AR* gene amplification, Joseph and colleagues showed that the presence of a missense mutation in the ligand-binding domain of the *AR*, *AR* F876 L, which is detected by ctDNA from ARN-509-treated patients with progressive CRPC, might be a relevant mechanism of second-generation anti-androgen resistance that can be potentially targeted with next-generation anti-androgens. Thus, Romanel and colleagues developed a targeted next-generation sequencing approach using ctDNA, covering all *AR* coding bases and genomic regions that are highly informative in prostate cancer. On the other

hand, whereas no changes were observed in *AR* copy number from before treatment to progression and no mutant *AR* alleles were present for acquired gain, emergence of T878A or L702H *AR* amino acid changes in 13% of tumors at progression on abiraterone was demonstrated. Patients with *AR* gain or T878A or L702H before abiraterone had a significantly worse overall and progression-free survival. Overall, ctDNA detection for *AR* gene aberrations has proved to predict outcome of anti-androgen therapy (Alix-Panabières and Pantel, 2016).

1.3.7. Other cancer types

Forshev et al. used the TAM-Seq method for identification and monitoring of oncogenic mutations in plasma ctDNA. They screened 5995 genomic bases in coding regions of *TP53* and *PTEN* (phosphatase and tensin homolog) and looked for low-frequency mutations in *EGFR*, *BRAF*, *KRAS* and *PIK3CA* regions. The assay sensitivity and specificity were of > 97%. At relapse, *TP53* mutation was originally found in the ovarian primary tumor, whereas the bowel-associated mutations were not detected (Forshev et al., 2012; Polivka et al., 2015).

On the other hand, Zill et al. assessed the mutation status of 54 genes by NGS in the tumor tissue and corresponding ctDNA in plasma samples from 26 patients with pancreaticobiliary carcinomas (18 pancreatic ductal adenocarcinoma cases and 8 biliary cancer cases). 90.3% of tumor mutations were also detected in plasma. The sensitivity and specificity of *KRAS*, *TP53*, *APC*, *FBXW7* and *SMAD4* mutations detection in plasma were 92.3% and 100% respectively, and the diagnostic accuracy was 97.7%; noting that these five mutations are the most frequently mutated genes in tumor tissue biopsies (Polivka et al., 2015; Zill et al., 2015).

Panka et al. developed blood-based assay for the detection of *BRAF* V600E in 128 patients with stage II–IV melanoma. For the subset of 42 stage IV patients, the assay showed 96% sensitivity and 95% specificity with an excellent ability to discriminate *BRAF*-mutant melanoma patients (Panka et al., 2014; Polivka et al., 2015).

Pupilli et al. examined the plasma of 103 patients with papillary thyroid carcinoma (PTC) to analyze the presence of *BRAF* V600E-mutated allele and its role in diagnosis and follow up. PTC patients demonstrated a higher level of circulating *BRAF* V600E mutation compared to those with benign histology and healthy controls with assay diagnostic sensitivity and specificity of 80 and 65%, respectively (Polivka et al., 2015). Furthermore, *BRAF* V600E somatic mutation is also present in other cancer types such as melanoma and colorectal adenocarcinoma (Pupilli et al., 2013).

Amplification of the gene *MYC*-related oncogene (*MYCN*) in neuroblastoma cases indicated disease aggressiveness. Even though *MYCN* status is unknown in around 29% of all patients, genetic testing is recommended since *MYCN* status helps in treatment options. Unlike standard therapy, new treatment approaches have been developed, such as immunotherapy, to significantly increase event-free and overall survival in patients with high risk *MYCN* amplified neuroblastomas. Liquid biopsy can accurately detect *MYCN* amplification in stage III to IV neuroblastoma patients with a sensitivity and specificity of 75–85% and 100%, respectively (Crowley et al., 2013).

Melanoma cases are commonly known to have metastasis rapidly and resistance occurs in most treated patients. Targeted therapies have started to change the prognosis of these patients especially inhibitors therapy for *BRAF* gene that may guide *BRAF*-directed therapies in the future. Detecting levels changes of *BRAF* mutations in blood are also important predictors of sensitivity to therapy (Alix-Panabières and Pantel, 2016). Furthermore, in a study for Crowley et al., improvements were shown in the treatment of acquired and de novo resistance in melanoma cases to *BRAF* inhibitor dabrafenib and the *MEK* inhibitor trametinib that were used in combination (Crowley et al., 2013).

Effectively, the role of ctDNA in indicating drug resistance is very important. For example, mutations in *NRAS* (neuroblastoma RAS viral oncogene homolog), *MEK* and *BRAF* amplification indicate resistance to *BRAF* inhibitor vemurafenib in *BRAF*-mutant melanoma (Polivka et al.,

2015). ctDNA is also useful for acquired resistance detection that occurs in patients with *BRAF V600E* mutant melanoma who are treated with vemurafenib by several mechanisms, including *MAP3K8* overexpression, activation of a *MAPK*-redundant survival pathway, *PDGFR β* upregulation, *BRAF* truncation or amplification and *NRAS Q61K* mutation. Consequently, it is possible to monitor the acquisition of resistance to vemurafenib by the appearance of genetic aberrations (same as *KRAS* mutations monitoring in colorectal cancer) or gene amplifications (like *HER2* amplification in breast cancer) (Crowley et al., 2013).

1.4. Minimal residual disease

As already discussed, ctDNA detection has influenced in a great way the field of cancer diagnosis, prognosis and monitoring especially in treatment resistance prediction. Besides, detection of ctDNA plays a major role also in indicating the presence of minimal residual disease (MRD) even in the absence of symptoms or other clinical signs of disease especially after surgery or treatment. Therefore, presence of ctDNA could recognize patients at higher risk of relapse. It is very important to classify patients of higher risk of relapse in order to benefit from combined therapy options and separating them from those of low risk to avoid unneeded side effects from this therapy (Wan et al., 2017). For example, Tie et al. analyzed ctDNA in stage II colorectal cancer treated with surgery. They reported that 6 patients developed recurrence and 5 of them had already ctDNA detected. However, 72 patients did not relapse and only 5 out of them had detectable ctDNA (Chaudhuri et al., 2015; Tie et al., 2014).

Cured cancer patients are monitored after ending treatment through regular follow up to check signs of residual disease and undergo radiological imaging. But these methods are expensive especially if requiring contrast media, have limited sensitivity for detection of micrometastasis, in addition to the side effect after exposing patients to doses of radiation, and thus using such techniques for frequent monitoring is not efficient (Crowley et al., 2013).

In their turn, Diehl et al. monitored patients with colorectal cancer post-surgery and analyzed their plasma for tumor-specific aberrations including *APC*, *TP53* and *KRAS*. When detected, these mutations presence was related with recurrence with almost 100% sensitivity and specificity. Other studies for breast and lung cancer, and oral squamous cell carcinoma, confirmed that disease recurrence was highly related with the reappearance of certain tumor aberrations, including *KRAS*, *APC* and *TP53* mutations and allelic imbalances (Crowley et al., 2013).

Other than the importance of ctDNA in minimal residual disease for predicting the disease recurrence, it plays an important role for determining dormant disease in patients. Dormancy is common in many cancers such as breast, melanoma, renal cancer and non-Hodgkin lymphoma, and cannot be detected by standard methods. Plasma of breast cancer patients was studied for tracking copy-number aberrations before and after surgery, and it was proved that copy-number aberrations stay up to 12 years post diagnosis. DNA is cleared from circulation in 30 min, thus its presence indicates that dormant cancer cells are existing (Crowley et al., 2013).

Lymphoid malignancies are characterized by clonal immunoglobulin (Ig) or T-cell receptor (TCR) gene rearrangements which are highly specific biomarkers. Thus, using these biomarkers for MRD has become widely adopted especially in acute lymphoblastic leukemia (ALL). Around 80–95% of adult ALL patients and > 95% of pediatrics achieve complete remissions (CR) after chemotherapy but some patients express recurrence. Therefore, MRD detection in ALL patients has great influence on patient risk stratification and early therapeutic response monitoring. Currently, adopted MRD detection techniques include flow cytometry, allele-specific oligonucleotide (ASO) qPCR of Ig/TCR gene rearrangements, and qRT-PCR of fusion transcripts. All of these methods are helping in clinical settings, but present many limitations like relying on bone marrow (BM) sampling which is invasive

and inability to capture clonal heterogeneity. Modern approaches are able to bypass those restrictions by combining universal PCR primers for rearranged Ig/TCR regions with next generation sequencing. Using this technique to PB and BM samples demonstrated high sensitivity between 0.001 and 0.0001% and high concordance with standard methods (ASO-qPCR and flow cytometry) for oligoclonality detection at diagnosis and disease monitoring over time using non-invasive samples. Besides, targeted approaches are preferred over whole genome or exome sequencing in general due to current costs and sequencing error rates (Scherer et al., 2017).

More recently, a high-throughput DNA sequencing method was developed by Bohers et al. which is LymphoSIGHT[®], to detect quantitatively ctDNA as minimal residual disease to reflect treatment failure and relapse in patients with newly diagnosed blood cancer patients such as diffuse large B-cell lymphoma, chronic lymphocytic leukemia or acute lymphoblastic leukemia (Bohers et al., 2015).

On the other hand, AML is characterized by certain gene rearrangements, such as *PML-RARA*, *CBFB-MYH11*, *RUNX1-RUNX1T1*, *BRC-ABL1*, and mutations, such as *NPM1*, *FLT3-ITD*, *RUNX1*, *CEBPA*, in BM or PB which are important for diagnosis, patient risk assessment and therapeutic decision making. In addition, the goal is testing for MRD and achieving complete remission without MRD presence. PCR-based technologies for MRD detection are highly sensitive (between 0.1 and 0.001%) and have standardized monitoring of treatment response over time. But detection limits of those techniques are variable among biomarkers, mainly due to difference in expression levels of fusion genes and mutant alleles analyzed, in addition to the time of MRD testing that is still unclear depending on treatment, methods, and markers tested (Scherer et al., 2017).

MRD detection by high-throughput technology (HTS) in AML/MDS is a relatively new field. Thol et al. used amplicon HTS to study *FLT3-ITD* and *NPM1* mutations in BM and PB samples of AML patients. High concordance was seen with other method such as qRT-PCR and robust detection of MRD, including the identification of developing mutations during relapse. Most recently, Yeh et al. showed that ctDNA analysis by targeted HTS accurately reflects tumor genes and clonal evolution over time, and predicts treatment resistance in patients, showing a potential for noninvasive disease monitoring (Scherer et al., 2017).

However, adopting high-throughput technology into routine clinical uses involves comprehensive calibration, validation and taking into consideration the challenges and limitations that this application might present (Kotrova et al., 2017).

2. Conclusion

Next Generation Sequencing is the promising technology within almost all molecular diagnostic laboratories and will be shouldering liquid biopsies to the best sensitivity of application. This state-of-the-art technology is providing ultimate options for the detection of ctDNA both upon diagnosis, follow-up and minimal residual disease monitoring of patients. The applications are variable and indeed progressing fast for full incorporation into routine clinical and diagnostic work-up especially of cancer patients.

References

- Aarthy, R., Mani, S., Velusami, S., Sundarsingh, S., Rajkumar, T., 2015. Role of circulating cell-free DNA in cancers. *Mol. Diagn. Ther.* 19 (6), 339–350.
- Alix-Panabières, C., Pantel, K., 2016. Clinical applications of circulating tumor cells and circulating tumor DNA as liquid biopsy. *Discovery* 6 (5), 479–491.
- Ansari, J., Yun, J.W., Kompelli, A.R., Moufarrej, Y.E., Alexander, J.S., Herrera, G.A., Shackelford, R.E., 2016. The liquid biopsy in lung cancer. *Genes & Cancer* 7 (11–12), 355–367.
- Beaver, J.A., Jelovac, D., Balukrishna, S., Cochran, R.L., Croessmann, S., Zabransky, D.J., Wong, H.Y., Valda Toro, P., Cidado, J., Blair, B.G., Chu, D., Burns, T., Higgins, M.J., Stearns, V., Jacobs, L., Habibi, M., Lange, J., Hurley, P.J., Lauring, J., Van Den Berg, D.A., Kessler, J., Jeter, S., Samuels, M.L., Maar, D., Cope, L., Cimino-Mathews, A., Argani, P., Wolff, A.C., Park, B.H., 2014. Detection of cancer DNA in plasma of

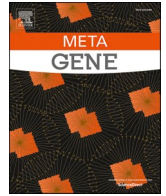
- patients with early-stage breast cancer. *Clin. Cancer Res.* 20 (10), 2643–2650.
- Bodi, K., Perera, A.G., Adams, P.S., Bintzler, D., Dewar, K., Grove, D.S., Kieleczawa, J., Lyons, R.H., Neubert, T.A., Noll, A.C., Singh, S., Steen, R., Zianni, M., 2013. Comparison of commercially available target enrichment methods for next-generation sequencing. *J. Biomol. Tech.* 24 (2), 73–86.
- Bohers, E., Viailly, P.J., Dubois, S., Bertrand, P., Maingonnat, C., Mareschal, S., Ruminy, P., Picquenot, J.M., Bastard, C., Desmots, F., Fest, T., Leroy, K., Tilly, H., Jardin, F., 2015. Somatic mutations of cell-free circulating DNA detected by next-generation sequencing reflect the genetic changes in both germinal center B-cell-like and activated B-cell-like diffuse large B-cell lymphomas at the time of diagnosis. *Haematologica* 100 (7), e280–e284.
- Chaudhuri, A.A., Binkley, M.S., Osmundson, E.C., Alizadeh, A.A., Diehn, M., 2015. Predicting radiotherapy responses and treatment outcomes through analysis of circulating tumor DNA. *Semin. Radiat. Oncol.* 25 (4), 305–312.
- Crowley, E., Di Nicolantonio, F., Loupakis, F., Bardelli, A., 2013. Liquid biopsy: monitoring cancer-genetics in the blood. *Nat. Rev. Clin. Oncol.* 10, 472–484.
- Diaz Jr., L.A., Bardelli, A., 2014. Liquid Biopsies: Genotyping Circulating Tumor DNA. *J. Clin. Oncol.* 32 (6), 579–586.
- EGFR RQV PCR Kit Handbook, 2010. For qualitative measurement of 29 somatic mutations in the EGFR oncogene, for use with the Rotor-Gene® Q 5plex HRM® Instrument. *July 2010*.
- Fan, G., Zhang, K., Yang, X., Ding, J., Wang, Z., Li, J., 2017. Prognostic value of circulating tumor DNA in patients with colon cancer: systematic review. *PLoS One* 12 (2), e0171991.
- Forshev, T., Murtaza, M., Parkinson, C., Gale, D., Tsui, W.Y.D., Kaper, F., Dawson, S.J., Piskorz, M.A., Jimenez-Linan, M., Bentley, D., Hadfield, J., May, P.A., Caldas, C., Brenton, D.J., Rosenfeld, N., 2012. Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. *Sci. Transl. Med.* 4 (136), 136ra68.
- Gagan, J., Van Allen, E.M., 2015. Next-generation sequencing to guide cancer therapy. *Genome Med.* 7 (80).
- Hagemann, S.I., Cottrell, E.C., Lockwood, C.M., 2013. Design of targeted, capture-based, next generation sequencing tests for precision cancer therapy. *Cancer Genet.* 206 (12), 420–431.
- Ignatiadis, M., Lee, M., Jeffrey, S.S., 2015. Circulating tumor cells and circulating tumor DNA: challenges and opportunities on the path to clinical utility. *Clin. Cancer Res.* 21 (21), 4786–4800.
- Kotrova, M., Trka, J., Kneba, M., Brüggemann, M., 2017. Is next-generation sequencing the way to go for residual disease monitoring in acute lymphoblastic leukemia? *Molecular Diagnosis & Therapy* 17, 1–12.
- Lebofsky, R., Decraene, C., Bernard, V., Kamal, M., Blin, A., Leroy, Q., Rio, F.T., Pierron, G., Callens, C., Bieche, I., Saliou, A., Madic, J., Rouleau, E., Bidard, F.-C., Lantz, O., Stern, M.-H., Le Tourneau, C., Pierga, J.-Y., 2015. Circulating tumor DNA as a non-invasive substitute to metastasis biopsy for tumor genotyping and personalized medicine in a prospective trial across all tumor types. *Mol. Oncol.* 9 (4), 783–790.
- Lin, E., Chien, J., Ong, F.S., Fan, J.B., 2014. Challenges and opportunities for next-generation sequencing in companion diagnostics. *Expert. Rev. Mol. Diagn.* 15 (2), 193–209.
- Ma, M., Zhu, H., Zhang, C., Sun, X., Gao, X., Chen, G., 2015. “Liquid biopsy”—ctDNA detection with great potential and challenges. *Ann. Transl. Med.* 3 (16), 235.
- Mäbert, K., Cojoc, M., Peitzsch, C., Kurth, I., Souchelnyskiy, S., Dubrovska, A., 2014. Cancer biomarker discovery: Current status and future perspectives. *Int. J. Radiat. Biol.* 90 (8), 659–677.
- Madic, J., Kiiäläinen, A., Bidard, F.-C., Birzele, F., Ramey, G., Leroy, Q., Frio, T.R., Vaucher, I., Raynal, V., Bernard, V., Lermine, A., Clausen, I., Giroud, N., Schmucki, R., Milder, M., Horn, C., Spleiss, O., Lantz, O., Stern, M.-H., Pierga, J.-Y., Weisser, M., Lebofsky, R., 2015. Circulating tumor DNA and circulating tumor cells in metastatic triple negative breast cancer patients. *Int. J. Cancer* 136 (9), 2158–2165.
- Newman, A.M., Bratman, S.V., To, J., Wynne, J.F., Eclow, N.C.W., Modlin, L.A., Long Liu, C., Neal, J.W., Wakelee, H.A., Merritt, R.E., Shrager Jr., J.B., Loo, B.W., Alizadeh, A.A., Diehn, M., 2014. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. *Nat. Med.* 20 (5), 548–554.
- Normanno, N., Denis, M.G., Thress, K.S., Ratcliffe, M., Reck, M., 2017. Guide to detecting epidermal growth factor receptor (EGFR) mutations in ctDNA of patients with advanced non-small-cell lung cancer. *Oncotarget* 8 (7), 12501–12516.
- Panka, D.J., Buchbinder, E., Giobbie-Hurder, A., Schalck, A.P., Montaser-Kouhsari, L., Sephr, A., Lawrence, D.P., McDermott, D.F., Cohen, R., Carlson, A., Wargo, J.A., Merritt, R., Seery, V.J., Hodi, F.S., Gunturi, A., Fredrick, D., Atkins, M.B., Iafrate, A.J., Flaherty, K.T., Mier, J.W., Sullivan, R.J., 2014. Clinical utility of a blood-based BRAF (V600E) mutation assay in melanoma. *Mol. Cancer Ther.* 13 (12), 3210–3218.
- Perakis, S., Speicher, M.R., 2017. Emerging concepts in liquid biopsies. *BMC Med.* 15. Polivka Jr., J., Pesta, M., Janku, F., 2015. Testing for oncogenic molecular aberrations in cell-free DNA-based liquid biopsies in the clinic: are we there yet? *Expert. Rev. Mol. Diagn.* 15 (12), 1631–1644.
- Pupilli, C., Pinzani, P., Salvianti, F., Fibbi, B., Rossi, M., Petrone, L., Perigli, G., De Feo, M.L., Vezzosi, V., Pazzagli, M., Orlando, C., Forti, G., G., 2013. Circulating BRAFV600E in the diagnosis and follow-up of differentiated papillary thyroid carcinoma. *J. Clin. Endocrinol. Metab.* 98 (8), 3359–3365.
- Rao, A.V., Smith, B.D., 2013. Are results of targeted gene sequencing ready to be used for clinical decision making for patients with acute myelogenous leukemia? *Curr. Hematol. Malig.* 8 (2), 149–155.
- Rothé, F., Laes, J.-F., Lambrechts, D., Smeets, D., Vincent, D., Maetens, M., Fumagalli, D., Michiels, S., Drisis, S., Moerman, C., Detiffe, J.-P., Larsingmont, D., Awada, A., Piccart, M., Sotiriou, C., Ignatiadis, M., 2014. Plasma circulating tumor DNA as an alternative to metastatic biopsies for mutational analysis in breast cancer. *Ann. Oncol.* 25 (10), 1959–1965.
- Scherer, F., Kurtz, D.M., Diehn, M., Alizadeh, A.A., 2017. High-throughput sequencing for noninvasive disease detection in hematologic malignancies. *Blood* 130 (4), 440–452 (Jul 27).
- Shen, W., Szankasi, P., Sederberg, M., Schumacher, J., Frizzell, K.A., Gee, E.P., Patel, J.L., South, S.T., Xu, X., Kelley, T.W., 2016. Concurrent detection of targeted copy number variants and mutations using a myeloid malignancy next generation sequencing panel allows comprehensive genetic analysis using a single testing strategy. *Br. J. Hematol.* 173 (1), 49–58.
- Sorber, L., Zwaenepoel, K., Deschoolmeester, V., Van Schil, P.E.Y., Van Meerbeeck, J., Lardon, F., Rolfo, C., Pauwels, P., 2017. Circulating cell-free nucleic acids and platelets as a liquid biopsy in the provision of personalized therapy for lung cancer patients. *Lung Cancer* 107, 100–107.
- Su, K.Y., Chen, H.Y., Li, K.C., Kuo, M.L., Yang, J.C.H., Chan, W.K., Ho, B.C., Chang, G.C., Shih, J.Y., Yu, S.L., Yang, P.C., 2012. Pretreatment Epidermal Growth Factor Receptor (EGFR) T790M Mutation Predicts Shorter EGFR Tyrosine Kinase Inhibitor Response Duration in Patients With Non-Small-Cell Lung Cancer. *J. Clin. Oncol.* 30 (4), 433–440.
- Thierry, A.R., Moulriere, F., El Messaoudi, S., Mollevi, Caroline, Lopez-Crapez, E., Rolet, F., Gillet, B., Gongora, C., Dechelotte, P., Robert, B., Del Rio, M., Lamy, P.J., Bibeau, F., Nouaille, M., Loriot, V., Jarrousse, A.S., Molina, F., Mathonnet, M., Pezet, D., Ychou, M., 2014. Clinical validation of the detection of KRAS and BRAF mutations from circulating tumor DNA. *Nat. Med.* 20 (4), 430–435.
- Thress, K.S., Pawelczak, C.P., Felip, E., Cho, B.C., Stetson, D., Dougherty, B., Lai, Z., Markovets, A., Vivancos, A., Kuang, Y., Ercan, D., Matthews, S.E., Cantarini, M., Barrett, J.C., Jänne, P.A., Oxnard, G.R., 2015. *Nat. Med.* 21 (6), 560–562.
- Tie, J., Kinde, I., Wang, Y., Wong, H.L., Skinner, I., Wong, R., Steel, M., Diaz, L.A., Papadopoulos, N., Kosmider, S., Yip, D., Underhill, C., Haydon, A.M., Christie, M., Strausberg, R., Kinzler, K.W., Vogelstein, B., Gibbs, P., 2014. Circulating tumor DNA (ctDNA) as a marker of recurrence risk in stage II colon cancer (CC). *J. Clin. Oncol.* 35 (27), 11015.
- Tu, M., Chia, D., Wei, F., Wong, D., 2015. Liquid biopsy for detection of actionable oncogenic mutations in human cancers and electric field induced release and measurement liquid biopsy (eLB). *Analyst* 141, 393–402.
- Uchida, J., Kato, K., Kukita, Y., Kumagai, T., Nishino, K., Daga, H., Nagatom, I., Inoue, T., Kimura, M., Oba, S., Ito, Y., Takeda, K., Imamura, F., 2015. Diagnostic accuracy of noninvasive genotyping of EGFR in lung cancer patients by deep sequencing of plasma cell-free DNA. *Clin. Chem.* 61 (9), 1191–1196.
- Valasek, M.A., Repa, J.J., 2005. The power of real-time PCR. *Adv. Physiol. Educ.* 29, 151–159.
- Volik, S., Alcaide, M., Morin, R.D., Collins, C., 2016. Cell-free DNA (cfDNA): clinical significance and utility in Cancer shaped by emerging technologies. *Mol. Cancer Res.* 14 (10), 898–908.
- Wan, J.C.M., Massie, C., Garcia-Corbacho, J., Moulriere, F., Brenton, J.D., Caldas, C., Pacey, S., Baird, R., Rosenfeld, N., 2017. Liquid biopsies come of age: towards implementation of circulating tumour DNA. *Nat. Rev. Cancer* 17 (4), 223–238.
- Wang, L., Wheeler, D.A., 2014. Genomic Sequencing for Cancer Diagnosis and Therapy. *Annu. Rev. Med.* 65 (1), 33–48.
- White, R.A., Blainey, P.C., Fan, H.C., Quake, S.R., 2009. Digital PCR provides sensitive and absolute calibration for high throughput sequencing. *BMC Genomics* 10, 116.
- Wood-Bouwens, C., Lau, B.T., Handy, C.M., Lee, H.J., Ji, H.P., 2017. Single-color digital PCR provides high-performance detection of Cancer mutations from circulating DNA. *J. Mol. Diagn.* 19 (5), 697–710.
- Wu, L., Qu, X., 2015. Cancer biomarker detection: recent achievements and challenges. *Chem. Soc. Rev.* 44 (10), 2963–2997.
- Zill, O.A., Greene, C., Sebisano, D., Siew, L.M., Leng, J., Vu, M., Hendifar, A.E., Wang, Z., Atreya, C.E., Kelley, R.K., Van Loon, K., Ko, A.H., Tempero, M.A., Bivona, T.G., Munster, P.N., Talasz, A.A., Collisson, E.A., 2015. Cell-free DNA next-generation sequencing in pancreaticobiliary carcinomas. *Cancer Discov.* 5 (10), 1040–1048.

Update

Meta Gene

Volume 30, Issue , December 2021, Page

DOI: <https://doi.org/10.1016/j.mgene.2021.100918>



Erratum regarding missing Declaration of Competing Interest statements in previously published articles

Declaration of Competing Interest statements were not included in published version of the.

following articles that appeared in previous issues of Meta Gene. Hence, the authors of the below articles were contacted after publication to request a Declaration of Interest statement:

1. "Breast cancer susceptibility genes in estrogen metabolizing pathway in a southern Indian population" [Meta Gene, 2018; 19C: 225–234] <https://doi.org/10.1016/j.mgene.2018.12.009>
2. "Protective effect of high levels of TGF- β 21 cytokine and its corresponding allele at C-509T locus in Punjabi ESRD population" [Meta Gene, 2019; 21C: 100587] <https://doi.org/10.1016/j.mgene.2019.100587>
3. "In silico analysis of non-synonymous single nucleotide polymorphism in a human KLK-2 gene associated with prostate cancer" [Meta Gene, 2019; 21C: 100578] <https://doi.org/10.1016/j.mgene.2019.100578>
4. "Association of polymorphism in P16 and myeloperoxidase genes with susceptibility to oral lesions in North Indian population" [Meta Gene, 2018; 17C: 88–92] <https://doi.org/10.1016/j.mgene.2018.05.005>
5. "Circulating tumor DNA, liquid biopsy, and next generation sequencing: A comprehensive technical and clinical applications review" [Meta Gene, 2018; 17C: 192–201] <https://doi.org/10.1016/j.mgene.2018.06.013>
6. "DNA methylation and expression status of glutamate receptor genes in patients with oral squamous cell carcinoma" [Meta Gene, 2019; 20C: 100545] <https://doi.org/10.1016/j.mgene.2019.100545>
7. "Mutation screening of the BRCA1 gene in sporadic breast cancer in the Central of Iran" [Meta Gene, 2018; 17C: 23–27] <https://doi.org/10.1016/j.mgene.2018.04.005>
8. "Genetic diversity analyses for population structuring in *Channa striata* using mitochondrial and microsatellite DNA regions with implication to their conservation in Indian waters" [Meta Gene, 2018; 16C: 28–38] <https://doi.org/10.1016/j.mgene.2018.01.004>
9. "Expression of brain-derived neurotrophic factor (BDNF) and its naturally occurring antisense in breast cancer samples" [Meta Gene, 2018; 19C: 69–73] <https://doi.org/10.1016/j.mgene.2018.10.012>
10. "Single nucleotide polymorphisms of lncRNA H19 are not associated with risk of multiple sclerosis in Iranian population" [Meta Gene, 2019; 21C: 100592] <https://doi.org/10.1016/j.mgene.2019.100592>
11. "Single nucleotide polymorphisms of lncRNA H19 are not associated with risk of multiple sclerosis in Iranian population" [Meta Gene, 2019; 21C: 100592] <https://doi.org/10.1016/j.mgene.2019.100592>
12. "Identification of novel miRNAs with potential role in Gastric Cancer diagnosis: In silico procedure" [Meta Gene, 2018; 19C: 246–252] <https://doi.org/10.1016/j.mgene.2018.12.008>
13. "Association of interleukin-6 rs1800796 polymorphism with reduced cognitive performance in healthy older adults" [Meta Gene, 2018; 19C: 51–55] <https://doi.org/10.1016/j.mgene.2018.10.007>
14. "Genetic diversity of 18 male and 18 female accessions of *Jობа* [*Simmondsia chinensis* (link) Schneider] using EST-SSRs" [Meta Gene, 2018; 19C: 134–141] <https://doi.org/10.1016/j.mgene.2018.11.010>
15. "A novel allele frequency trajectories template to discriminate genetic similarity among populations" [Meta Gene, 2018; 19C: 42–50] <https://doi.org/10.1016/j.mgene.2018.10.002>

DOIs of original article: <https://doi.org/10.1016/j.mgene.2018.12.009>, <https://doi.org/10.1016/j.mgene.2019.100578>, <https://doi.org/10.1016/j.mgene.2018.06.013>, <https://doi.org/10.1016/j.mgene.2018.05.005>, <https://doi.org/10.1016/j.mgene.2018.10.002>, <https://doi.org/10.1016/j.mgene.2018.10.007>, <https://doi.org/10.1016/j.mgene.2019.100587>, <https://doi.org/10.1016/j.mgene.2018.01.004>, <https://doi.org/10.1016/j.mgene.2019.100545>, <https://doi.org/10.1016/j.mgene.2018.12.008>, <https://doi.org/10.1016/j.mgene.2018.11.010>, <https://doi.org/10.1016/j.mgene.2018.04.005>, <https://doi.org/10.1016/j.mgene.2018.10.012>, <https://doi.org/10.1016/j.mgene.2019.100592>.

<https://doi.org/10.1016/j.mgene.2021.100918>

Available online 6 July 2021

2214-5400/© 2021 Published by Elsevier B.V.