Introducción

A pesar de los avances recientes en medicina, el cáncer se considera el principal problema de salud en el mundo, aunque los índices de mortalidad y morbilidad siguen siendo altos. La heterogeneidad del tumor, particularmente la heterogeneidad intertumoral, es una de las principales causas del fracaso terapéutico, lo que da pie a problemas del tratamiento y una variedad de estrategias terapéuticas, incluyendo terapias dirigidas a los genes. Los avances recientes en secuenciación massively parallel e intentos de genómica digital sugieren que el DNA circulante del tumor (ctDNA) se puede usar como un “biopsia líquida.” En el caso del cáncer de mama, se ha sugerido que el ctDNA en plasma puede servir como una herramienta no invasiva para escanear los genomas tumorales y evaluar el volumen del tumor.

El ctDNA en plasma puede usarse para identificar anomalias genéticas importantes, rastrear respuestas al tratamiento, descubrir resistencia a medicamentos y detectar progresión del cáncer antes de la confirmación clínica y radiográfica. Además, el ctDNA se puede utilizar para identificar heterogeneidad tumoral y mutaciones específicas de metástasis, proporcionando información que puede ayudar en el tratamiento del paciente.

En este artículo, nos enfocamos en el estado actual de ctDNA circulante, incluyendo la biología del ctDNA, los métodos de detección recientemente desarrollados, así como los métodos de detección específicos de mama, con un énfasis en las aplicaciones clínicas de los biomarcadores basados en ctDNA en el cáncer de mama.

**Palabras clave:** DNA no-codificante, diagnóstico del cáncer, biomarcador, biopsia líquida, cáncer de mama.
complete tumor cells\textsuperscript{5}.

In this article, we will provide a brief overview of the most prominent circulating biomarkers, known as cfDNAs, which are now being used for the prognosis, diagnosis, and treatment management of breast cancer.

**Circulating Nuclear DNA**

In cancer patients, circulating tumor DNA (ctDNA) is a subset of total cell-free DNA. The vast majority of cfDNAs in a cancer patient are released by normal cells, with ctDNA accounting for only a minor fraction of cfDNA in plasma. cfDNA levels in the blood can range from 13 ng/mL in healthy people to around 180 ng/mL in cancer patients\textsuperscript{13}.

ctDNAs are deoxynucleic acid fragments that are highly detectable in breast cancer patients’ body fluids such as serum, plasma, urine, and CSF\textsuperscript{49,50}. This increase in circulating free DNA amount in the blood is not cancer-specific and could be caused by a variety of disorders such as systemic lupus erythematosus, autoimmune disease, diabetic patients, and cardiovascular diseases\textsuperscript{15}.

The most obvious difference between circulating tumor DNA and healthy DNA is that ctDNA fragments have distinct genetic and epigenetic changes while retaining their integrity\textsuperscript{90}. In general, DNA released from tumor cells maintains its wholeness in length, which is primarily determined by the type and stage of the tumor, which can yield longer DNA fragments in advanced disease and metastatic breast cancer compared to low stage tumors and healthy people\textsuperscript{46}. The usual length of cfDNAs in the blood of healthy persons is 70-200bp. However, ctDNAs from people with malignant tumors range from 200 to over 1 kb in length, indicating a necrotic origin\textsuperscript{17,18}.

Tumor necrosis occurs in the core areas of solid tumors, where glucose and oxygen levels are typically low, resulting in cell membrane disintegration and tumor cell enlargement. Subsequently, cells release their contents, including proteins and nucleic acids\textsuperscript{99}. Increasing cfDNA in breast cancer patients may serve as a potential minimally invasive tool for diagnosis, prognosis, monitoring the dynamic course of cancer and response to therapy. This also may help with first therapy decisions, aiding in the initiation of adjuvant therapy to prevent recurrence and identification of drug sensitive/resistance to prevent cytotoxicity\textsuperscript{20,21}. The biological importance of the released tumor DNA from breast cancer cells is debatable, but it is thought to play a significant role in carcinogenesis because free DNA is absorbed by sensitive cells and transformed ontogenically by integration into their genomes\textsuperscript{22} or by binding to hormone receptor positive (HR+) breast cancer cells, which trigger bio-effects such as proliferation, invasion, and metastasis via activating the TLR9/ NF-kB/cyclin D pathway\textsuperscript{23}.

Longitudinal analysis of genetic variants in plasma samples has been shown to provide information of tumor heterogeneity on a scale not generally achieved by evaluating tumor tissue samples alone\textsuperscript{44}. Namely, ctDNA assessment of tumor clonal heterogeneity provides insights into the molecular profile in the tumor cell population and enables the development of molecularly-guided techniques that aid in improving cancer diagnosis, forecast, and monitor of tumor responses to treatment\textsuperscript{49}.

As previously stated, the key genetic modifications that occur in cancer cells, such as methylation, as well as mutations, polymorphisms, amplifications, and rearrangements, are all traceable through ctDNA. Due to the importance of the issue, we explore each of these molecular alterations and their subsequent studies in ctDNA.

**Methylation**

The most prevalent epigenetic modification is DNA methylation, which is thought to be a tumor-specific change that affects gene expression without changing the DNA sequences and is a common feature of many malignancies\textsuperscript{5,26}. Methylation can be classified as hyper- or hypomethylation, both of which alter the expression of germline genes\textsuperscript{27}. Aberrant patterns of hypermethylation occur primarily within CpG dinucleotide reigns known as CpG islands in tumor-suppressor gene promoters, homeobox genes, and other sequences, and are thought to be an important mechanism of transcriptional inactivation of tumor suppressor genes and have been observed as an early event in tumorigenesis in many types of cancer\textsuperscript{28}. Promoter hypermethylation is typically observed in breast cancer to block the production of tumor suppressors such as BRCA1/BRCA2\textsuperscript{29}, P53, PTEN\textsuperscript{30}, RASSF1A\textsuperscript{31}. On the contrary, hypomethylation has been linked to the transcriptional activation of “cancer-germline” genes, which are typically inactivated by hypermethylation in normal somatic tissues. The activation of cancer-germline genes is associated with the stimulation of oncogenic pathways involved in cell proliferation, invasion, and migration\textsuperscript{32}. However, hypomethylation can also occur inside non-coding genomic areas, affecting autonomous transposons elements such as ALU and LINE1. This results in transposable element retrotransposition, chromosomal rearrangement, and, ultimately, genomic instability\textsuperscript{33,34}. Hypomethylation of non-coding genomic areas could be an early step of carcinogenesis in cancers such as breast\textsuperscript{34}, colon\textsuperscript{35}, pancreas\textsuperscript{36} and gastric cancer\textsuperscript{37}. Numerous investigations have been suggested that LINE1 hypomethylation and transcriptional activation is correlated with unintentionally proto-oncogene activation\textsuperscript{38} including c-MET, c-myc, RAS, RAB3IP, and, CHRM3 which activates a wide range of cell signaling pathways, suggesting an increased malignancy of these oncogenes in breast neoplasia\textsuperscript{39}. Aside from LINE1 methylation levels in cfDNA as a diagnostic biomarker for breast cancer, high copy numbers of LINE-1 in the
genome will be beneficial when the liquid biopsy sample amount is limited. However, the high homology of LINE-1 elements with sequence mutations may complicate data interpretation.

Because alterations in the methylation profile of DNA, are one of the first stages in the cancer formation process, circulating DNA provides a source of intact genomic and epigenomic information toward methylation profiles without the need for invasive methods, and it may provide accurate indicators for early diagnosis, prognosis, and surveillance of breast cancer, as well as a more personalized treatment strategy.

According to earlier studies, aberrant DNA methylation patterns are more widespread than genetic mutations and have been extensively researched in multiple cancer subtypes. With the emergence of high-performance methods for studying gene expression, such as microarray, breast cancer has been divided into six major different groups: luminal A 50–60%, luminal B 10–20%, luminal C HER+ positive 15–20%, basal-like (TNBC) 10–20%, and normal breast-like 7.8%. Breast cancer subtypes have unique methylation patterns that reflect distinct patterns of gene expression, resulting in a variety of therapy reactions. Furthermore, the prevalence of methylation state varies amongst breast cancer subtypes. For example, basal-like breast cancer (BLBC) or triple-negative breast cancer (TNBC) with a clinically aggressive character has the lowest frequency of methylation status as compared to ER+/luminal subtype. These breast cancer subtype classifications based on distinct methylation patterns reflect the variety of breast cancer and aid in the development of targeted treatments for certain subtypes.

In breast cancer, hypermethylation of the CpG island inside promoter regions of tumor suppressor genes such as CDH1, APC, MGMT, Tp53, BRCA1, BRCA2, RASSF1A, GSTP1, p16, and RAR-2 could be exploited as a possible biomarker for early diagnosis and prognosis. TET1 DNA demethylase, which converts 5 methylcytosine (5mC) primarily into 5 hydroxymethylcytosine (5hmC), is overexpressed in approximately half of TNBC patients, and is associated with hypomethylation in the CpG island of promoter region in TET1 gene, and facilitates the activation of cancer-specific pathways such as PI3K, AKT, mTOR, and EGFR, and could be used as a potential biomarker in earliest TNBC subtype detection. We highlighted the important genes that could be exploited as a possible biomarker in breast cancer in the Table 1.

### Rearrangement

Breast cancer subtypes have multiple chromosome rearrangements in their genome, which can be recognized in tumor biopsy and even in liquid biopsy and has higher specificity and sensitivity than other genetic alterations, such as point mutations, which can occur in many tumors. Nonetheless, chromosomal rearrangement serves as different genetic “fingerprints” for a specific tumor and allows clinical surveillance of little residual tumor after surgery often takes two forms: balanced and unbalanced. The genetic material has been retained and its genetic information has been transferred equally and phenotypically normal in balanced rearrangement. However, balanced chromosomal rearrangement can cause phenotypic symptoms and increase cancer risk by becoming juxtaposed to an alien promoter/enhancer site as a result of the translocation or fusing chromosomes at two breaks and forming hybrid gene with no loss of genetic information, resulting in chimeric protein. For example, MAGI3-AKT3 gene fusion as a balanced translocation is responsible for roughly 7% of TNBC subtype patients, and RIF1-PKD1L1, TAX1BP1-AHCY gene fusion has been reported in breast cancer patients.

Unbalanced chromosomal rearrangement, which may include gene-specific breaks, is more common in the early stages of cancer development and has a higher potential to cause tumorigenesis than balanced chromosomal rearrangement. This is known as a well-established pathway for oncogene activation in breast cancer. In contrast to balanced translocation, unbalanced translocation is the most common alteration of the genetic content of cancer cells, accounting for 30% of all inactivated tumor suppressor genes (LOHs) in the regions of interest. The breaks in the chromosome may involve more than two chromosomes and can interfere with multiple genes at breakpoints, resulting in loss of function, or merge genes, forming novel chimeric genes and altering the genetic content. It is accompanied by a clinical consequence such as cancer (which accounts for roughly 20% of human cancer mortality), including breast, AML, sarcoma, and lung cancer, and also have been identified in multiple disease such as renal disease, tuberous sclerosis, and epilepsy. Pathological gene fusions in breast cancer, such as ESR1-CCDC170, ETV6-NTRK3, and BCL2L14-ETV6, can be used to diagnose cancer less invasively and can be used as an encouraging positive strength by generating clinically targetable protein products, which can act as biomarkers for diagnosis and monitoring of breast cancer. The study of pathological gene fusions has

### Table 1

| Gene               | Methylation     | Reference |
|--------------------|-----------------|-----------|
| BRCA1              | Hyper-methylation | [31, 47] |
| BRCA2              | Hyper-methylation | [31, 47] |
| Tp53               | Hyper-methylation | [31, 47] |
| MGMT               | Hyper-methylation | [31, 47] |
| CDH1               | Hyper-methylation | [31, 47] |
| APC                | Hyper-methylation | [31, 47] |
| TET1 DNA Demethylase | Hypo-methylation | [48] |
Table 2  Circulatory Gene Fusion DNAs Detected in Breast Cancer Patient Blood Samples

| Gene Fusion          | Subtype            | Reference |
|---------------------|--------------------|-----------|
| Esrc1-Ccdc170       | Luminal B          | 60        |
| Etv6-Ntrk3          | Secretory BC       | 61        |
| Bel2H16-Etv6        | Basal-Like (TNBC)  | 59        |
| Rps6k1-Akt3         | Er+ (Luminal)      | 60        |
| Sec16a-Notch1       | Basal-Like (TNBC)  | 62        |
| Mpyb-Nfib           | Ez-                | 63        |

not only enhanced the treatment of breast cancer patients, but has also enabled the determination of the specific subtype of breast cancer. Patients with ESR1-CCDC170 fusion are more responsive to combination endocrine medications with tyrosine kinase inhibitors in the realm of targeted therapy (TKIs) Lapatinib and RPS6K1-AKT3 fusion is linked to endocrine therapy resistance, and Abemaciclib as a CDK4/6 inhibitor combined with estrogen withdrawal resulted in a significant reduction in tumor formation.

A summary of circulatory gene fusion DNAs detected in different breast cancer subtypes is shown in Table 2. With the introduction of innovative sequencing technologies for detecting underlying genomic rearrangements such as chromosomal translocations and fusion genes, new features of therapeutic intervention and complete recurrence monitoring of cancer patients have been demonstrated.

**Mutation**

The majority of published research on the potential use of ctDNA in oncology focuses on the identification of specific mutations from liquid biopsy samples. Several studies have demonstrated the efficacy and great benefit of ctDNA in detecting tumor-specific mutations, specifically driver mutations. Detection of ctDNA mutations, which reflects the main tumor mutational signatures, has sparked a lot of interest in using it to monitor early cancer diagnosis, prognosis, and prediction. After surgery, evaluating possible therapy resistance, and predicting relapse risk. Furthermore, the identification of driver mutations causing tumor progression, leads to improved prognosis and more personalized and well-planned targeted therapies. According to the FDA, Olaparib (Lynparza) and Talazoparib (Talzenna) are primarily administered in HER2-negative patients with BRCA mutations and PIK3CA gene mutations, respectively, and are PARP inhibitors that cause cell death in cancer cells. However, in older people who do not have cancer, a process known as clonal hematopoiesis can induce somatic mutations in ctDNA that should be taken into account during clinical evaluation.

**Circulating Mitochondrial DNA**

Many studies have found a link between substantially damaged mitochondrial DNA and the onset and progression of numerous malignancies, including bladder, colon, kidney, liver, lungs, stomach, and prostate breast cancer. Although the majority of our genomic material is stored in the nucleus, mitochondria have their own genome (Mitogenome) in the form of a double-stranded circular structure. It’s about 16.7 Kbp that consists of 37 genes responsible for essential cell functions, specifically, providing energy through aerobic respiration and apoptosis. In general, mutations such as point mutations and deletions in the mitochondrial genome or even nuclear genes encoding proteins of the oxidative phosphorylation system have been shown to play critical roles in breast tumorigenesis and metastasis. Mitochondria are the primary locations for the production of Reactive Oxygen Species (ROS), which are produced in small quantities in the oxidative phosphorylation pathway. Because mitochondrial DNA lacks histones and introns, and has a poor DNA repair system, mitochondrial DNA is more likely to be altered by both internal and external degrading triggers such as ROS, which can serve as a potential sensor of cellular DNA damage and an indication of cancer initiation and progression. This mitochondrial genomic alteration frequently affects the D-loop region and the genes involved in the oxidative phosphorylation process. These mutations are found in a variety of inherited and acquired diseases. Methods for diagnosis and prognosis of breast cancer utilizing circulating mitochondrial DNA as a biomarker in clinical application differ depending on the types of mutations that arise in the mitochondrial DNA. For example, genetic alteration in D-loop region decreases mitochondrial DNA replication and transcription due to its significant role in the initiation of this process. It also increases the levels of reactive oxygen species (ROS) and their detrimental effect on mitochondrial DNA, making DNA more vulnerable to digestion into smaller fragments and leaking out of the mitochondria into the cytosol or the peripheral circulation. Interestingly, in patients with early stage (III, IV) breast cancer and ER, PR, or Her2 positive, the plasma content of mitochondrial DNA is higher than in patients with low stage (I, II) breast cancer or healthy individuals, with more fragmented and this fragments Cl-mtDNA associated with tumor load inversely. Because of the heteroplasmy in cancer cells, all mitochondrial fragments produced by these cells must be diagnosed. These fragments carry critical information about the biology underpinning cancer cells and have been employed as an effective biomarker for new diagnostic and prognostic approaches, and assisting in breast cancer classification without the use of invasive methods. Elevated levels of mt-DNA are not cancer specific and have been observed in either healthy individual or noncancerous diseases such as diabetes, trauma, neurodegenerative disease (Alzheimer, Huntington, Parkinson).
Methods for ctDNA detection and analysis

Due to extremely fragmented DNA and low levels, detecting circulating tumor DNA is difficult. The first techniques for evaluating ctDNA in the circulation were sanger sequence or pyrosequencing, but they were limited due to low ctDNA concentrations, intrinsic low sensitivity, and disproportionate amounts of wild alleles and mutants in the bloodstream. Currently, the most widely used techniques for detecting ctDNA mutations include real-time quantitative PCR (qPCR) and droplet digital PCR (dPCR), which have higher sensitivity, specificity, and are less expensive. These strategies, however, are inapplicable for high-throughput sequencing and unknown mutations.

The dPCR technology is a novel approach that improves the identification of genetically modified tumor drivers to remarkable accuracy levels (detects mutation rate at 0.001%) from sources such as plasma, CSF, and urine, and, in comparison to real-time PCR, shows significant advantages in detecting uncommon mutations and allelic disproportion in a background of wild-type alleles. The recent presentation of several different platforms based on dPCR have significantly increased utilization of this technology, such as Droplet digital PCR, RainDrop dPCR, Crystal dPCR, and considered as third-generation platforms that have enabled absolute quantification of genetic imbalances, or Copy Number of targeted genes. Multiplexing dPCR has recently been created as an alternate technique to address the present limitations in digital PCR. Multiplexing dPCR Assays discover numerous clinically valuable targets in a single experiment by utilizing various fluorescing dye tagged probes in a little amount of ctDNA extracted and so increasing dPCR analysis accuracy and sensitivity. In multiplexed dPCR, distinguishing between target sequences is based not only on different fluorescent colors, but also on different fluorescence intensities and the total number of targets in their combination.

The development of next-generation sequencing (NGS) has transformed our understanding by providing considerably more data points than previously stated approaches, such as the discovery of numerous gene mutations, the measurement of mutational load, and the analysis of mutational burden. Accurate differentiation of primary and metastatic breast cancers, breast cancer detection in the early and late stages, detection of unusual somatic mutations implicated in carcinogenesis with high discovery power, reduced analysis time, and possibility for tailored cancer therapy. The NGS method's high efficiency and accuracy make it ideal for developing NGS-based panels for identifying many particular target genes in both tissue biopsies and ctDNA, such as the MammaSeq panel for diagnosis, prognosis, and therapeutic options.

The MammaSeq panel may be used to investigate copy number variation in clinical interventions, and structural DNA variations accountable for the effects of gene dosage or cis-regulation. Common Copy number variant (CNV) modifications in breast cancer discovered by MammaSeq panels including CCND1, MYC, PVT1, HER2, and RUNX1 oncogenes, which are amplified in the majority of breast cancer patients.

Furthermore, due to the limited sensitivity of conventional NGS for detecting the very low amount of ctDNA in early stages of breast cancer, as well as the existing heterogeneous properties that harbor numerous modifications within tumor tissue, recent technology in molecular bar code (MB) has allowed NGS to simultaneously identify ctDNA with sufficient sensitivity in a variable allele frequency at around 0.1%. In patients with a high recurrence rate after surgery, MB-NGS may detect extremely small amounts of tumor in the patient's blood than regular NGS without MB.

Methylation

Recently, multiple approaches, including bead array, pyrosequencing, methylation-specific PCR (MSP), methylation-sensitive high-resolution melting (MS-HRM), and COLD-PCR, have been developed to identify and assess specific genes/regulatory areas of interest that are differentially methylated (via detection of unmethylated islands). The use of Bisulfite conversion as the initial step in examining DNA methylation is a trait shared by several approaches. Bisulfite conversion is a process in which cytosine is deaminated with sodium bisulfite and transformed into uracil while 5-methylcytosine remains intact. Because of the low frequency of conversion, this distinguishing reaction rate of 5-mC to C could be used to evaluate DNA methylation in targeted DNA specimens, followed by PCR and sequencing of cloned amplicon DNA. However, technologies such as pyrosequencing, which is regarded as a “gold standard,” are not accurate in high or low levels of DNA methylation, and its sensitivity is only around 5%. It also has drawbacks such as short read length and limited sample throughput. With the advent of new technology such as Next Generation Sequencing and its combination with bisulfate conversion-based methods, we were able to assess the methylation status of multiple regions of interest in the targeted genome concurrently or at a single-nucleotide resolution in a single cell, with high sensitivity, specificity, and capability.

Rearrangement

Despite the diversity of cancer-related genetic aberrations and their varied prevalence in breast cancer subtypes, gene fusions are more disease-specific. Previously acknowledged approaches for discovering...
gene fusions in tumor tissue includes CGH, FISH, spectral karyotyping and RT-PCR\(^9\). Recently, several NGS-based approaches have been introduced, including whole-genome DNA-sequencing (DNA-seq), massively parallel analysis of whole RNA transcriptome sequencing (RNA-seq), and chimeric whole transcriptome sequencing (RNA-seq), which significantly track genomic rearrangements and chimeric whole transcriptome respectively in plasma of a breast cancer patient\(^9\).

In contrast to DNA seq, which evaluates chimeric DNA resourced from both coding and noncoding regions, RNA-seq is the most commonly used method for identifying chimeric transcriptions that are actively expressed on the genomic area. More research on the capability of chimeric DNA to create fusion transcripts is required in order to evaluate chimeric DNA significance. However, RNA sequencing typically generates a large amount of input data from chimeric RNAs, the majority of which are clinically insignificant and are the result of library artifacts and mapping errors\(^9\).

### Mutation

Various methods have been proposed in recent advances in genome sequencing technologies to probe multiple driver mutations and particular variation in ctDNAs with low frequency (as low as 0.001%) in Droplet digital PCR (ddPCR) technologies. Real-time PCR, digital PCR (d-PCR), and next-generation sequencing (NGS) are currently the most commonly used techniques for detecting ctDNA mutations. These technologies have enabled us to accurately identify single, or small numbers of, mutations with high sensitivity and specificity at a relatively fast and cost-effective rate\(^9\). Several different platforms based on dPCR such as Droplet digital PCR, Rain Drop dPCR and Crystal dPCR have offered evaluating therapeutic resistance and prognosis by targeted deep sequencing of cancer-related driver gene mutations in ctDNAs such as EGFR, DNMT3A, TET2, BRAF, ESR1, KRAS, PIK3CA, and Tp53. Despite the fact that these regularly used dPCR techniques have a wide range of applications, they can only evaluate one or a limited number of unique somatic mutations in parallel and are ineffective in detecting previously unknown mutations\(^78,79\).

#### Circulating Mitochondrial DNA

There are several reliable methods for quantifying mitochondrial DNA, which are critical for improving its use as a clinical tool and providing insight into cf-mtDNA levels in cancer. Previous methods for investigating and evaluating the copy quantity and physical properties of circulating mitochondrial DNA involved direct sequencing of the mitochondrial genome in specific locations, such as the use of sanger sequencing to analyze hyper-variable regions (I, II, III). Despite being regarded as the gold standard, sanger sequencing has been limited due to a number of difficulties, including the inability to sequence virtually thousands of fragments simultaneously in order to provide a speedy test answer in an emergency circumstance. The identification of heteroplasmic mtDNA mutations is also expensive and inaccurate (below 10%)\(^93,94\). In the last decades accurate techniques, including NGS, quantitative real-time PCR, droplet digital PCR allow us to study in depth mtDNA heteroplasmacy and millions of mitochondrial DNA copy number in samples simultaneously\(^95,96\). The use of next-generation sequencing (NGS) techniques has transformed the study of mitochondrial DNA as a biomarker in cancer from exhausting, complex, and low-sensitivity methods to a high-sensitivity method for identifying mitochondrial disease-associated mutations (point mutations and deletions) in both common and uncommon cancers. This technique examines high risk reigns in a cost-effective manner and is capable of precisely detecting homoplasm and heteroplasmy with a high depth of coverage\(^94\).

### Conclusion

The discovery of a reliable cancer biomarker is critical to the development of practical personalized medicine. Targeted therapy and gene detection are inextricably linked and when compared to tissue biopsy, ctDNA examination has the advantages of less invasion, more convenience, and no interaction with radioactive substances. Prior to imaging, ctDNA analysis can be utilized to advise prompt management, elucidate drug resistance mechanisms, and detect limited residual diseases or recurrence. Tissue biopsy is a great method for obtaining static cancer information, whereas ctDNA detection is more rapid. The main advantage of ctDNA analysis is its high specificity, as ctDNA mutations are consistent with those found in tumor DNA. Although for non-invasive cancer detection as well as diagnosis, prognosis management, and treatment guidance, tumor-specific methylations and mutations in ctDNAs are prospective targets, and there are still limitations in the accurate detection of specific cell-free nucleic acids.

Notably, there is no universal detection standard. There is still no agreement on the typical concentrations of cfDNAs found in healthy people's blood. Because the processes involved in ctDNA extraction are not often explained in depth in published publications, the concentrations of detected ctDNA tend to vary a lot. Due to the low blood concentrations of ctDNAs, a lot of blood sample is required for detection at this time. The pace of purification of these samples must be considerably increased. Tumor DNA fragments are diluted with normal DNA in circulation, which may make further analysis difficult. Moreover, false-positives continue to occur due to the inadequacies of sensitivities and specificities of these techniques. To achieve better outcomes, technologi-
cal advancements and a deeper understanding of ctDNA biology are required. Because of their high costs, ctDNA-based methods are limited in the clinical context, and the clinical application of this technology is currently being debated due to the need to enhance the sensitivity and accuracy of ctDNA detection.

Nevertheless, based on current research development and the growth of the medical industry, we anticipate that ctDNA assays will be utilized in the future to personalize real-time treatments for cancer patients based on their individual ctDNAs or ctDNA methylation levels, and for diagnosis, prognoses, and treatment advices.

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