Cell-Free DNA in Dermatology Research

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In various diseases, particularly cancer, cell-free DNA (cfDNA) has been widely studied as a marker of disease prognosis or to facilitate the detection of therapeutic targets. In dermatology, most studies have focused on melanoma; other skin diseases such as vascular malformations and psoriasis have also been examined. Genetic alterations unique to the tissue of origin such as sequence variations, copy number alterations, chromosomal rearrangements, differential DNA methylation patterns, and fragmentation patterns can be identified in circulation providing information on patient disease status. These alterations can be detected either by PCR-based methods or next-generation sequencing depending on the target of interest. In this article, we discuss the origins of cfDNA, the most common methods of detection, current studies assessing cfDNA as a biomarker, and cfDNA’s potential clinical applications in melanoma and other skin diseases. In addition, we provide important factors to consider during blood processing and DNA extraction as well as limitations for each assay.

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INTRODUCTION

Cell-free DNA (cfDNA) refers to nonencapsulated fragments (~166 base pair [bp]) of double-stranded (free-floating) DNA emerging from any cell type in the body (Figure 1). First detected in late 1940s, cfDNA is derived primarily from cellular debris released into the circulation after necrosis, apoptosis, or other mechanisms of cell death (Oliveira et al., 2020; Watanabe et al., 2021). In healthy individuals, low levels of cfDNA result from normal cell turnover and can be detected in any bodily fluid, including blood, saliva, urine, and cerebrospinal fluid (Cirmena et al., 2021; Mondelo-Macia et al., 2021; Watanabe et al., 2021) (Figure 1). For the purposes of this article, we will focus on the cfDNA extracted from blood plasma.

WHAT INFORMATION CAN cfDNA PROVIDE?

Aside from clinical applications in prenatal diagnosis, most studies of cfDNA focus on identifying point mutations in cancer. Total cfDNA quantity, copy number alterations (CNA), fusions, differential DNA methylation patterns, and fragmentation patterns have also been analyzed. In the context of nononcological skin diseases, studies of cfDNA are less advanced.

The quantity of cfDNA in the plasma may fluctuate after physiological changes in the body such as excessive physical activity, stress, inflammation, and/or malignancy. (Mondelo-Macia et al., 2021). In healthy individuals, the normal concentration of cfDNA is around 7 ng/ml. In metastatic melanoma, these levels may rise above 1,000 ng/ml (Syeda et al., 2020). Cancer cells may actively secrete DNA into the circulation (Watanabe et al., 2021), which may also contribute to higher levels.

Circulating tumor DNA (ctDNA) (not to be confused with circulating tumor cells) is a subset of cfDNA characterized by tumor-associated genetic and epigenetic alterations. Detection of ctDNA, often called liquid biopsy, was first described in the late 1970s. Technological innovations have greatly accelerated progress in this field and enabled the detection of different cancer-associated genetic/genomic changes. ctDNA tends to be shorter (~134–144 bp) in length than nontumor derived cfDNA (~166 bp) owing to differential nucleosome phasing (Watanabe et al., 2021). Tumor-associated structural genomic changes such as CNAs and chromosomal rearrangements, including fusions, can also be detected in ctDNA. Most applications have focused on the detection of point mutations. The first Food and Drug Administration (FDA)-cleared liquid biopsy clinical assay for solid tumors was the cobas EGFR Mutation Test, version 2 (Roche Molecular Systems, Branchburg, NJ). This plasma-based RT-PCR test is designed to recognize 42 mutations in the EGFR gene to identify patients with nonsmall cell lung cancer eligible for treatment with EGFR inhibitors, without the need for a lung biopsy. Other non–FDA-cleared assays that test for multiple mutations are available for a variety of cancers. In addition, a ctDNA test to analyze differentially methylated CpG sites between cancer and normal cells has been developed into the Galleri blood test (GRAIL, Menlo Park, CA), marketed to screen for occult malignancies. This test uses machine learning to distinguish between different cancer-associated methylation patterns (using approximately 1 million CpG

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Abbreviations: bp, base pair; cfDNA, cell-free DNA; CH, clonal hematopoiesis; CNA, copy number alteration; ctDNA, circulating tumor DNA; ddPCR, droplet digital PCR; FDA, Food and Drug Administration; LDH, lactate dehydrogenase; LoD, limit of detection; MAF, mutant allele fraction; NGS, next-generation sequencing; UMI, unique molecular index; WES, whole-exome sequencing; WGS, whole-genome sequencing

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SUMMARY POINTS

**Sample collection**
- Cell-free DNA (cfDNA) can be obtained from any bodily fluid, but the most commonly used source is plasma.
- Rare targets of interest may require higher fluid volume and DNA input.
- If plasma separation from whole blood cannot be accomplished within a few hours, specialized collection tubes with stabilizing reagents need to be used.

**Droplet digital PCR**
- Droplet digital PCR (ddPCR) has been used frequently in melanoma studies to detect mutated BRAF and/or NRAS alleles as potential biomarkers of disease burden and/or response to treatment.

**Advantages.**
- ddPCR provides absolute quantification of rare alleles.
- ddPCR has the highest sensitivity and specificity of available PCR-based methods.

**Limitations.**
- ddPCR generally requires previous knowledge of the target of interest.
- ddPCR is often limited to the detection of a single mutant and wild-type allele.

**Next-generation sequencing**

**Advantages.**
- Next-generation sequencing (NGS) methods are utilized to detect a wide array of targets, including multiple mutations, copy number alterations, chromosomal rearrangements, differential methylation, and fragmentation patterns (i.e., tumor vs. normal).
- NGS methods can be used to identify alterations in a small a panel of targets or for broad coverage such as whole-exome or whole-genome analysis.

**Limitations.**
- NGS methods typically do not provide precise quantification owing to library preparation and sequencing methodologies.
- NGS often has higher limits of detection than ddPCR.
- Mutational analysis of cfDNA may be confounded by nontumor mutations originating from aging normal lymphocytic cells (i.e., clonal hematopoiesis).

SAMPLE COLLECTION
Most studies use between 1 and 10 ml of plasma collected in EDTA tubes. Although attractive owing to their low cost, blood collected in EDTA tubes requires processing within 4–6 hours to avoid contamination from the lysis of mononuclear cells resulting in the release of cellular DNA. Specialized tubes containing proprietary reagents allowing for storage for up to 7–14 days at room temperature are available but are more expensive. A list of collection tubes is available (Cirmena et al., 2021).

Sample processing involves initial centrifugation to separate plasma from the buffy coat and erythrocytes. The plasma is transferred to a tube for second centrifugation to pellet residual cell debris. Storing separated plasma at −80 °C, and avoiding freeze-thaw cycles will preserve DNA integrity (Cirmena et al., 2021). The low concentration of cfDNA in plasma and its presence as small fragments result in low extraction yields using traditional methods designed for high molecular weight genomic DNA. Commercially available kits specially designed for cfDNA either use magnetic beads or silica-membrane columns and are generally recommended for their reproducibility and purity (Cirmena et al., 2021; Devonshire et al., 2014; Diefenbach et al., 2018). Although a modified version of the traditional phenol–chloroform extraction has been optimized for cfDNA, it yielded a higher proportion of larger fragments (>202 bp) than a commercial kit (Yuan et al., 2012). A detailed list of available kits and techniques is available for further review (Cirmena et al., 2021).

DETECTION METHODS
After DNA extraction, several methods are available to assess cfDNA for mutations (Figure 2). When the intended target mutation is known (e.g., as a result of genetic analysis of a patient’s tumor), PCR-based methods can be used to identify whether the specific mutation of interest is present in ctDNA (Cirmena et al., 2021). For the purposes of this article, we will focus on droplet digital PCR (ddPCR), which has been frequently used in melanoma. Of the available targeted PCR-based methods, ddPCR achieves the highest sensitivity and specificity and provides accurate and precise quantification of rare alleles. Its limits of detection (LoDs), expressed as the ratio between the mutant allele and the wild-type allele at a given locus, called mutant allele fraction (MAF), can typically be as low as 0.005% (Syeda et al., 2020). Importantly, there is an association between LoD and DNA input quantity. The ultralow LoDs (i.e., MAFs) observed using ddPCR are often a result of large amounts of DNA input (Syeda et al., 2020). Although the high sensitivities, specificities, and quantitative output of ddPCR are favorable characteristics, ddPCR is generally limited to the detection of a single or few mutation(s) in the same gene (Oliveira et al., 2020).

Another approach to analyzing cfDNA is next-generation sequencing (NGS) (Figure 2). These assays can simultaneously detect a multitude of genetic mutations, amplifications/deletions, rearrangements, and fragmentation patterns. NGS methods can be divided into three categories: targeted sequencing, whole-exome sequencing (WES), or whole-genome sequencing (WGS). Although WES and WGS have been successfully used for ctDNA detection and enable a broad coverage of the genome, they have relatively high LoDs, ranging between 1 and 10% MAF, and require a relatively
Targeted sequencing (i.e., sequencing of a specified list of genes) has lower LoDs and is the preferred sequencing-based method for liquid biopsies (Cirmena et al., 2021; Watanabe et al., 2021). This approach employs high sequencing coverage and uses unique molecular indexes (UMIs) to reduce the sequencing error rate of NGS. Although the general sensitivity of targeted UMI-based NGS methods ranges from 0.1 to 5%, Guardant360 (Guardant Health, Redwood City, CA) and Cancer Personalized Profiling by deep Sequencing (CAPP-Seq; Roche, Basel, Switzerland) have reported the highest sensitivities among NGS methods with LoDs of 0.04 and 0.01%, respectively (Watanabe et al., 2021). To identify CNAs, either low-pass WGS or targeted deep sequencing can be used. In general, CNAs require a higher tumor fraction (5%) and multiple reference genes to achieve detection compared with mutational analyses (Cirmena et al., 2021). Differential fragmentation patterns can be identified using low-coverage WGS. One analysis technique, DNA evaluation of fragments for early interception (DELFI; DELFI Diagnostics, Baltimore, MD), has been successfully employed to distinguish patients with cancer from healthy individuals using genome-wide analysis of cfDNA and machine learning (Watanabe et al., 2021).

The detection of epigenetic changes, such as differential methylation patterns, is also measured using sequencing-based methods. However, additional steps are required to ensure that methylated sites remain traceable. The main technique uses bisulfite treatment to chemically modify unmethylated cytosines so that they are

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**Figure 2. Methods to detect ctDNA in the plasma.** After cfDNA extraction, DNA alterations can be assessed through two main detection methods: ddPCR or NGS. ddPCR requires the preparation of an amplification master mixture with allele-specific probes. The sample is partitioned into ~20,000 droplets. The targets of interest undergo end-point amplification within each droplet. Each droplet is analyzed by the droplet reader, providing absolute quantification. The workflow for NGS requires ligation of adaptors for each target and a library preparation followed by an amplification step. Targets of interest are sequenced and analyzed using various bioinformatics tools. cfDNA, cell-free DNA; ctDNA, circulating tumor DNA; ddPCR, droplet digital PCR; NGS, next-generation sequencing.
converted to thymines during PCR amplification. The resulting sequence variations are evidence of methylation (or lack of it) at particular sites. Losses in DNA quantity after bisulfite treatment are substantial, so bisulfite sequencing typically requires a minimum input of 100 ng. Alternatively, a relatively new genome-wide non-bisulfite conversion method for plasma DNA, called cell-free methylation DNA immunoprecipitation and high-throughput sequencing (cfMe-DIP-seq), only uses 1–10 ng of cfDNA. It is very sensitive and shows a high correlation with methylated regions published in The Cancer Genome Atlas (Shen et al., 2018; Watanabe et al., 2021).

**CLINICAL APPLICATIONS OF cfDNA IN DERMATOLOGY**

**Melanoma**

In general, clinical applications of cfDNA in cancer have focused on blood-based molecular diagnosis, minimal residual disease detection, and patient monitoring for response to treatment and development of resistance. In dermatology, melanoma has been the most widely studied cancer (Tarhini and Kudchadkar, 2018), in part, because there are no validated predictive biomarkers currently implemented in the clinic. Serum lactate dehydrogenase (LDH) is the only marker used to monitor patients with advanced melanoma. Although LDH is part of the staging system for melanoma, as a liquid biopsy biomarker, it lacks sensitivity and specificity to detect disease progression. Conveniently, for ctDNA analysis, cutaneous melanoma is an ideal tumor because over 80% of cases carry hot spot, point mutations in *BRAF* or *NRAS* or in the promoter region of *TERT* (Chang et al., 2020).

Most studies in melanoma have examined patients with unresectable and metastatic disease, seeking to assess the potential utility of ctDNA as a disease biomarker. Given the short half-life of ctDNA in circulation (~1–2 hours), it may provide real-time information on a patient’s disease (Mondelo-Macía et al., 2021; Oliveira et al., 2020). Baseline and/or on-treatment plasma samples have been analyzed for the presence of *BRAF* or *NRAS* mutations using ddPCR and assessed for their associations with treatment response, development of resistance/disease progression, and/or survival endpoints (Tarhini and Kudchadkar, 2018). In one of the largest studies to date, our group used analytically validated *BRAF*V600E or *V600K* ddPCR assays to show the potential of baseline and on-treatment ctDNA measurements to serve as predictive biomarkers in patients with advanced melanoma enrolled in a randomized phase III trial of targeted therapy. We detected ctDNA in 93% of the patients before initiating therapy and found that the quantity of ctDNA was associated with survival. Interestingly, patients whose ctDNA became undetectable 4 weeks into treatment had double the survival of those who still had detectable ctDNA (Syeda et al., 2021). Although this approach had markedly reduced sensitivity to detect isolated brain metastases, the results suggest that ctDNA monitoring may be useful for predicting patient outcomes early in treatment. Other reports from mostly smaller retrospective cohorts have yielded generally similar findings. Some studies found much lower detection rates and reduced associations with disease activity. The variability in results is likely due to a lack of standardized analytical approaches (Tarhini and Kudchadkar, 2018).

Lesser studies have focused on patients with resected stage III/IV or high-risk stage II melanoma. Measuring ctDNA to detect minimal residual disease after complete resection (but before radiographic recurrence) could be used for patient management decisions such as adjuvant therapy. However, patients with radiographically undetectable minimal residual disease are likely to have ultralow tumor fractions that limit the amount of ctDNA alleles present in a single blood draw. As such, detection rates in these contexts have been lower than in the metastatic setting (Lee et al., 2017). Ongoing studies are focused on implementing the simultaneous use of multiple markers to improve sensitivity and potentially detect the emergence of treatment resistance (e.g., *NRAS* mutations) (Gray et al., 2015; Watanabe et al., 2021).

**Nonmelanoma clinical applications**

Outside of melanoma, cfDNA has been examined in vascular malformations and psoriasis. Vascular malformations are characterized by an abnormal growth of underdeveloped vessels that may result in high blood pressure, reduced gas exchange, and possibly rupture. The majority of arteriovenous malformations possess an activating oncogenic mutation (e.g., *MAP2K1, BRAF, KRAS*), some of which may be good candidates for targeted therapies. Because tissue biopsies for molecular analysis may be associated with significant morbidity from bleeding, liquid biopsies from plasma and/or cyst fluid could be attractive for personalizing therapies while reducing patient risks and advancing our understanding of the disease at a molecular level (Zener et al., 2021).

Psoriasis is a chronic inflammatory disease in which elevated levels of cfDNA were detected. Using *TNF-α* DNA as a probe for total cfDNA, patients with severe psoriasis had higher cfDNA (copies/ml) than healthy controls (Sakamoto et al., 2020). Other studies have shown that cfDNA levels decrease in patients with clinical improvement from phototherapy (Coimbra et al., 2014). These findings suggest that measurements of total cfDNA may be a surrogate marker of disease activity in other inflammatory and autoimmune skin diseases. In particular, cfDNA quantity and cell-free mitochondrial DNA are being actively investigated in rheumatoid arthritis and lupus as biomarkers of disease activity and potentially as an indicator of treatment response (Duvvuri and Lood, 2019).

Other uses and research interests involving cfDNA include prenatal testing and aging. During pregnancy, maternal blood cfDNA can be used as a noninvasive prenatal test to assess for

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**Table 1. DNA Alterations Studied through Liquid Biopsy in Dermatological Conditions**

<table>
<thead>
<tr>
<th>Cutaneous Disease and Conditions</th>
<th>Current Analytes Studied through Liquid Biopsy</th>
</tr>
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<tbody>
<tr>
<td>Melanoma</td>
<td>BRAF, NRAS, and TERT mutations</td>
</tr>
<tr>
<td>Arteriovenous malformations</td>
<td>MAP2K1, BRAF, and KRAS mutations</td>
</tr>
<tr>
<td>Psoriasis</td>
<td>Total cfDNA</td>
</tr>
<tr>
<td>Lupus</td>
<td>Total cfDNA and mtDNA</td>
</tr>
<tr>
<td>Aging</td>
<td>Chromatin changes (local and global)</td>
</tr>
</tbody>
</table>

Abbreviations: cfDNA, cell-free DNA; mtDNA, mitochondrial DNA
MULTIPLE CHOICE QUESTIONS

1. Cell-free DNA (cfDNA) can be found in healthy individuals at concentrations around 7 ng/ml. What can cause cfDNA to rise under non-pathological conditions?
   A. Diet
   B. Inflammation and exercise
   C. Hypertension

2. If your research budget for blood sample collection is insufficient for specialized collection tubes, what steps should you take to ensure cfDNA stability in your sample?
   A. Collect blood in an EDTA tube and store it at –80 °C
   B. Collect blood in a red top tube, centrifuge it to separate serum, and store it at 4 °C
   C. Collect blood in an EDTA tube, centrifuge it to separate plasma, aliquot it, and store it at –80 °C

3. If you want to simultaneously detect mutations in multiple genes using a cfDNA sample with a low tumor fraction, which of the following is the preferred method?
   A. Targeted sequencing
   B. Droplet digital PCR (ddPCR)
   C. Whole-exome sequencing

4. Your laboratory successfully extracted >100 ng of DNA from a plasma sample. To identify differential methylation patterns within the sample, you need to perform which of the following steps?
   A. Send your extracted cfDNA for sequencing
   B. Treat your cfDNA sample with bisulfite and then sequence your sample
   C. Assess your extracted cfDNA by ddPCR

5. Most melanomas (>80%) present with point mutations in BRAF, NRAS, or TERT, making them good candidates as biomarkers of disease. In what stages of the disease is ctDNA currently being studied?
   A. Unresectable/metastatic disease and resected stage III/IV or high-risk stage II melanoma
   B. Stages I and II melanoma
   C. All of the above

chromosomal abnormalities, CNAs, microdeletions, and sequence variants in the fetus using commercially available targeted sequencing panels. Although unpublished, some laboratories have been studying its potential in genodermatoses, such as the detection of mutations in COL7A1 in epidermolysis bullosa. With respect to aging, one study measured local and global chromatin changes in cfDNA and found differential nucleosomal landscapes associated with healthy centenarians compared with those with more medical comorbidities (Teo et al., 2019).

A summary of the DNA alterations associated with the diseases and conditions discussed earlier is presented in Table 1.

LIMITATIONS

When planning experiments using cfDNA, a few considerations should be kept in mind. The detection of rare targets may require a relatively high volume of fluid. In addition, the distance between collection and processing sites may require either onsite freezing and sample storage or a specialized tube to stabilize the sample for transport. In the analytical phase, the detection of genetic mutations may be confounded by clonal hematopoiesis (CH). CH is defined as the age-related clonal expansion of hematopoietic lineage cells in healthy individuals (Cirmena et al., 2021; Watanabe et al., 2021). These noncancer clones may acquire driver mutations (e.g., KRAS) that are detected in cfDNA, suggesting the presence of cancer (Watanabe et al., 2021). Analysis of the lymphocytic fraction to identify the same mutations provides evidence of CH.

CONCLUSION

Plasma cfDNA is an attractive analyte for study. It is easy to collect, and there are several available technologies that enable measurements of multiple parameters relevant to health and disease. In dermatology, the use of cfDNA has been explored mostly in the context of melanoma as a predictive and prognostic marker in patients with metastasis. In noncancer dermatological diseases, the clinical use of cfDNA currently has limited applications because monitoring disease severity in patients with inflammatory/autoimmune diseases is easily accomplished by visual examination. However, using cfDNA as a biomarker for clinical trial selection for patients with vascular malformations shows promise. With further research, cfDNA measurements may eventually be incorporated into patient clinical management.

Data availability statement

No datasets were generated or analyzed during this study.

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CONFLICT OF INTEREST

The laboratory receives in-kind research support from Bio-Rad laboratories, the manufacturer of droplet digital PCR assays and systems, and research contracts with Novartis and Bristol-Myers Squibb. JW is supported by NIH grants T32 AR064184 and P50 CA225450; DP is supported by NIH grants U01 CA206124 and P50 CA225450; and Department of Defense grant ME200024.

AUTHOR CONTRIBUTIONS

Conceptualization: JW, DP; Funding Acquisition: DP; Investigation: JW, SA; Project Administration: DP; Supervision: DP; Validation: JW; Visualization: SA; Writing - Original Draft Preparation: JW, SA, DP; Writing - Review and Editing: JW, SA, DP

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SUPPLEMENTARY MATERIAL
Supplementary material is linked to this paper. Teaching slides are available as supplementary material.

REFERENCES


DETAILED ANSWERS

1. Cell-free DNA (cfDNA) can be found in healthy individuals at concentrations around 7 ng/ml. What can cause cfDNA to rise under nonpathological conditions?

CORRECT ANSWER: B. Inflammation and exercise

Circulating cellular materials, including DNA fragments, are derived from leftover debris after phagocytic clearance from necrosis, apoptosis, or other mechanisms of cell death. These levels may rise as a byproduct of inflammation or after excessive physical activity when the release of DNA exceeds the clearance rate.

2. If your research budget for blood sample collection is insufficient for specialized collection tubes, what steps should you take to ensure cfDNA stability in your sample?

CORRECT ANSWER: C. Collect blood in an EDTA tube, centrifuge it to separate plasma, aliquot it, and store it at −80 °C.

Blood collected in EDTA tubes requires processing within 4–6 hours after collection to avoid lysis of mononuclear cells. Freezing/thawing of whole blood will result in lysis of cells, contaminating the cfDNA preparation. Similarly, formation of a blood clot during the collection of serum will lyse cells.

3. If you want to simultaneously detect mutations in multiple genes using a cfDNA sample with a low tumor fraction, which of the following is the preferred method?

CORRECT ANSWER: A. Targeted sequencing

Targeted sequencing focuses on a specific list of genes, allowing the simultaneous interrogation of multiple loci. Targeted sequencing can generally achieve higher coverage at the loci of interest and achieve a lower limit of detection than whole-exome sequencing. Although droplet digital PCR methods typically achieve the lowest limits of detection, they are generally limited to an interrogation of a single mutation and its wild-type counterpart.

4. Your laboratory successfully extracted >100 ng of DNA from a plasma sample. To identify differential methylation patterns within the sample, you need to perform which of the following steps?

CORRECT ANSWER: B. Treat your cfDNA sample with bisulfite and then sequence your sample.

Bisulfite treatment chemically modifies unmethylated cytosines so that they are converted to thymines during PCR amplification. The resulting sequence variation is evidence of methylation at particular sites. Losses in DNA quantity after bisulfite treatment are substantial, so bisulfite sequencing typically requires a minimum input of 100 ng. If samples are not treated with bisulfite (or other methods), it is not possible to detect differential methylation using extracted cfDNA.

5. Most melanomas (>80%) present with point mutations in BRAF, NRAS, or TERT, making them good candidates as biomarkers of disease. In what stages of the disease is ctDNA currently being studied?

CORRECT ANSWER: A. Unresectable/metastatic disease and resected stage III/IV or high-risk stage II melanoma.

To date, only studies examining advanced melanoma have been published. Patients with the earlier-stage disease are very likely to be cured surgically, and those with radiographically undetectable minimal residual disease will have very low levels of ctDNA.