Guideline
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Clinical Practice Guideline for Blood-based Circulating Tumor DNA Assays

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Circulating tumor DNA (ctDNA) has emerged as a promising tool for various clinical applications, including early diagnosis, therapeutic target identification, treatment response monitoring, prognosis evaluation, and minimal residual disease detection. Consequently, ctDNA assays have been incorporated into clinical practice. In this review, we offer an in-depth exploration of the clinical implementation of ctDNA assays. Notably, we examined existing evidence related to pre-analytical procedures, analytical components in current technologies, and result interpretation and reporting processes. The primary objective of this guidelines is to provide recommendations for the clinical utilization of ctDNA assays.

Key Words: Cell-free nucleic acid, Circulating tumor DNA, Guideline, High-throughput nucleotide sequencing

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INTRODUCTION

In recent years, there has been increasing interest in circulating tumor DNA (ctDNA) as a minimally invasive tool for various clinical applications, including early diagnosis, therapeutic target identification, treatment response and prognosis evaluation, and minimal residual disease detection [1-3]. ctDNA represents a subset of cell-free DNA (cfDNA) derived from apoptotic and necrotic tumor cells as well as viable tumor cells [4]. Notably, cfDNA released by apoptotic cells typically spans approximately 167 base pairs, whereas ctDNA tends to be shorter than cfDNA [5]. The detection of ctDNA primarily relies on the identification of somatic variants within cfDNA. This review delves into the clinical utilization of ctDNA assays, drawing upon existing evidence related to pre-analytical procedures, analytical considerations for current technologies, and result interpretation and reporting processes. Our main aim with the development of these recommendations was to establish a consensus grounded in evidence-based practices for clinical laboratories.

DEVELOPMENT PROCESS

The development of the recommendations adhered closely to the methodology outlined in the Adaptation Process for Developing Korean Clinical Practice Guidelines v. 2.0 [6]. This collaborative effort involved two teams: 1) the Clinical Practice Guidelines Committee of the Korean Society of Laboratory Medicine and 2) an expert panel of six laboratory medicine physicians. The clinical pathologists were responsible for reviewing evidence-based recommendations and providing their expert insights via commentary. The Clinical Practice Guidelines Committee first drafted the scope of this guideline and then performed literature searches in PubMed, KoreaMed, and Google Scholar for articles in English or Korean.

For the pre-analytical procedures, a comprehensive literature search was performed using a set of predefined keywords between January 2000 and July 2022. The following combinations of keywords were used: “cell-free DNA” AND “pre-analytical,” “ctDNA” AND “pre-analytical,” and “circulating DNA” AND “pre-analytical.” The Clinical Practice Guidelines Committee meticulously screened the titles and abstracts of the 268 initially retrieved literature sources. After the exclusion of duplicate records, 77 articles related to the pre-analytical phase of ctDNA testing using cfDNA in humans were retained. The content of these articles was comprehensively reviewed according to the evaluation criteria outlined in the Scottish Intercollegiate Guidelines Network and Korean Appraisal of Guidelines for Research and Evaluation (K-AGREE-II) evaluation tools [6]. Articles that were appropriately designed and exhibited a low risk of bias were included in the analysis. Ultimately, 27 articles were deemed suitable for inclusion in the guidelines for pre-analytical procedures (Table 1).

For the analytical aspects of current technologies, a comprehensive literature search was performed in PubMed, using the following combination of keywords: “analytical” AND “ctDNA,” and “analytical” AND “cell-free DNA.” Of the 527 literature sources initially retrieved, 503 were excluded based on a screening of their titles and abstracts. Ultimately, 23 articles were deemed suitable for a detailed review.

For result interpretation and reporting, an extensive literature search was conducted in PubMed, using the following keywords: (“ctDNA” OR “tumor NGS”) AND (“reporting” OR “interpretation”) AND (“guideline” OR “consensus” OR “recommendation”). Among the initially retrieved 273 literature sources, 258 were excluded after evaluating their titles and abstracts. Two were excluded after a thorough review of the remaining 15 articles. Finally, 13 articles were selected for the final review process.

After the development of the draft guidelines, an advisory committee of six experts in the molecular diagnostics field was formed. To gather feedback and refine the guidelines, two rounds of questionnaires were administered using the Delphi method [7]. Of the total of 17 recommendations obtained during the first survey, six exhibited a CV >15% when assessed using a grading system that ranged from 1 (strongly disagree) to 9 (strongly agree). These six recommendations were subjected to a second round of surveys to gather further opinions. Following input from the advisory committee, one recommendation regarding the discussion of therapeutic options with a multidisciplinary team of experts was ultimately removed, and the remaining 16 recommendations were finalized.

Table 1. Levels of evidence based on which papers were included in the literature analysis

Level	Description
I	Evidence from well-conducted studies and studies with a low risk of bias
II	Evidence from well-conducted studies and studies with a moderate risk of bias
III	Evidence from studies with limitations in design and studies with a high risk of bias
IV	Evidence from studies with limitations in design and studies with a significant risk of bias

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The grading of recommendations and determination of evidence levels (Table 1) were conducted considering existing clinical practice guidelines and grading systems (Table 2) [8, 9].

PRE-ANALYTICAL PROCEDURES

Timing of blood sampling

As ctDNA concentrations depend on the response to cancer treatment, the timing of blood collection for ctDNA analysis should be carefully selected according to the test purpose [10]. Collecting blood before surgery, radiotherapy, or chemotherapy is recommended to identify actionable molecular alterations at cancer diagnosis or disease progression. If blood is collected when the tumor responds to therapy or is non-progressive, ctDNA concentrations can be lowered, which may result in false-negative results [10]. Tissue injury from surgery or chemotherapy can increase cfDNA concentrations, which may result in a ctDNA fraction below the assay detection limit [10-13]. Therefore, to detect residual disease and predict relapse, blood should not be collected immediately after treatment [10, 11]. Depending on the extent of tissue damage and recovery time, blood collection is recommended at least 1–2 weeks after surgery [10].

Blood collection, storage, and transport

Optimal sample for ctDNA analysis

For ctDNA analysis, plasma is more suitable than serum. DNA concentrations in serum samples are higher than those in plasma samples because of leukocyte degradation during the clotting process during serum preparation [14-17]. Therefore, the ctDNA fraction in total DNA is higher in plasma than in serum, increasing the detection sensitivity of ctDNA analysis [18, 19].

Blood collection tube and storage

Anticoagulants in blood collection tubes prevent blood from clotting before plasma separation. Among anticoagulants, K2- or K3-EDTA is suitable for ctDNA analysis because it inhibits DNase activity, protects cells from degradation, and does not inhibit PCR [17, 20, 21]. At 4–6 hrs after blood collection in EDTA tubes, the total DNA concentration is increased because of leukocyte lysis [20-22]. Therefore, plasma separation should be performed as soon as possible when using EDTA tubes and not be delayed for more than 4–6 hrs to minimize normal DNA contamination [20-22]. There is no significant difference in sample stability when blood is stored in EDTA tubes at 4°C or at room temperature (18–25°C) for 4–6 hrs [14, 22]. However, when plasma separation is delayed inevitably for more than 6 hrs, the sample can be stored at 4°C for up to 1 day [20-22]. The time interval from blood collection to plasma separation can be extended by using cell preservation tubes [16, 20, 22]. If cell preservation tubes are used, blood should be processed and stored according to the manufacturer's instructions [22]. Generally, blood collected in cell preservation tubes can be stored for 5–7 days at room temperature [20, 22]. After collecting blood in a blood collection tube, the tube has to be gently inverted 8–10 times to adequately mix the blood and additives [20, 22].

Blood volume

For optimal performance, blood should be collected at the volume specified for the blood collection tubes to maintain an appropriate ratio with the additives [22]. As the input DNA quantity is directly proportional to the plasma volume and correlates with the sensitivity of ctDNA analysis, additional blood collection tubes can be used to increase the amount of blood collected for tests requiring high sensitivity, such as minimal residual disease analysis [10, 23].

Blood transport

Agitation and temperature fluctuation should be avoided when

Table 2. Grades for recommendations

Grade	Description
A	Recommended. There is sufficient evidence to recommend clinical practice.
B	May be considered. There is moderate to sufficient evidence to recommend clinical practice. Selective application of clinical practice to specific patients based on professional judgment is deemed appropriate.
C	Not recommended. There is sufficient evidence for the adverse effects in clinical practice.
I	No evidence for a recommendation. There is insufficient evidence regarding the benefits or adverse effects in clinical practice to make a recommendation. Further studies are required.

transporting collected blood tubes to the laboratory to prevent hemolysis and cellular damage [20, 22]. When requesting the test from an external laboratory, the use of cell preservation tubes for blood collection and adherence to the proper time duration and temperature requirements for blood storage are recommended [20, 24, 25].

Plasma preparation, QC, and storage

Plasma preparation

For EDTA tubes, we recommend a two-step centrifugation protocol to remove remnant cells and debris and obtain cell-free plasma [15, 20, 26]. We recommend a first centrifugation at 800–1,600×*g* at 4°C for 10 mins and a second centrifugation at 14,000–16,000×*g* at 4°C for 10 mins [20-22]. When separating the first plasma supernatant, caution must be exercised to avoid buffy coat contamination [20]. For cell preservation tubes, centrifugation protocols should follow the manufacturer's recommendations.

Plasma QC

Common interference in clinical testing, such as hemolysis, lipemia, and icterus, can affect ctDNA analysis [20, 27]. Therefore, visual inspection of plasma color after plasma separation is recommended [20]. Orange or red plasma suggests hemolysis and accompanying leukocyte lysis [20]. Icteric plasma (dark yellowish or greenish color) with a high bilirubin concentration or opaque plasma indicating hyperlipidemia may have a lower cfDNA concentration [27].

Plasma storage

Plasma should be immediately cooled to 4°C and stored frozen until DNA extraction to minimize nuclease activity [20]. As cfDNA continues to degrade *ex vivo*, extraction of cfDNA immediately after plasma separation is recommended. For short-term storage, plasma can be stored at 4°C for 3 hrs or at −20°C for a more extended duration [21]. Several studies have examined the influence of storage temperature and duration on cfDNA stability in plasma [21, 28, 29]. For long-term storage, we recommend that plasma be stored at −80°C. The allowable period for long-term storage varies depending on the purpose of cfDNA analysis [20, 21]. We recommend dividing plasma into small aliquots for downstream analysis to avoid multiple freeze–thaw cycles.

cfDNA extraction, QC, and storage

cfDNA extraction

The main methods for cfDNA extraction are the spin column-based method (e.g., QIAamp Circulating Nucleic Acid Kit [Qiagen, Hilden, Germany], Quick-cfDNA Serum & Plasma Kit [Zymo Research, Irvine, CA, USA], and Plasma/Serum Cell-free Circulating DNA Purification Midi Kit [Norgen Biotek Corp., Thorold, ON, Canada]) and the magnetic bead-based method (e.g., QIAamp minElute ccfDNA Mini Kit [Qiagen], Maxwell RSC ccfDNA Plasma Kit [Promega, Madison, WI, USA], MagMAX cell-free DNA Isolation Kit [Thermo Fisher Scientific, Waltham, MA, USA], NextPrep-Mag cfDNA Isolation Kit [Bioo Scientific, Austin, TX, SA], and Magnetic Serum/Plasma Circulating DNA Kit [Dxome, Seoul, Korea]). Multiple studies have evaluated the performance of the various cfDNA extraction kits [18, 30-34]. Each laboratory should select the most appropriate extraction method considering yield and purity for low-molecular-weight DNA isolation. In addition, a manual or automated workflow can be considered, depending on platform performance and the laboratory's required throughput.

cfDNA QC

QC of cfDNA in terms of quantity and molecular size is essential for ctDNA analysis. Spectrophotometry (e.g., NanoDrop [Thermo Fisher Scientific]), fluorometry (e.g., [Qubit Thermo Fisher Scientific] and Quantus [Promega]), real-time PCR, and digital PCR approaches can be used for cfDNA quantification [35]. Fluorometric quantification is more accurate than spectrophotometric analysis for low cfDNA concentrations [15, 36]. Electrophoresis-based methods (e.g., Bioanalyzer [AgilentTechnologies] and TapeStation [Agilent Technologies]) allow quantification and size measurement of cfDNA (Supplemental Data Fig. S1).

cfDNA extract storage

Extracted cfDNA is generally more stable than cfDNA in plasma [20]. When cfDNA is not immediately used for downstream analysis, we recommended storing it at −80°C and in multiple aliquots to avoid repeated freeze–thaw cycles. Storage conditions for cfDNA should adhere to the recommendations provided by the cfDNA isolation kit manufacturer [22].

The recommendations for pre-analytical procedures are summarized in Table 3.

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Table 3. Recommendations for pre-analytical procedures

Recommendation	Grade of recommendation	Level of evidence
It is recommended to use plasma rather than serum for ctDNA analysis.	A	I
It is recommended to separate plasma immediately when collecting blood in an EDTA tube and not delay plasma separation more than 4–6 hrs.	A	I
It may be considered to use cell preservation tubes if plasma separation is delayed more than 4–6 hrs.	B	I
It may be considered to avoid agitation and temperature fluctuation when transporting samples to the laboratory.	B	I
It may be considered to conduct two-step centrifugation for plasma isolation.	B	I
It is recommended to avoid buffy coat contamination when separating plasma.	A	I
It is recommended to analyze cfDNA in terms of quantity and quality before downstream analysis.	A	I
For long-term storage, it may be considered to store plasma or cfDNA extracts at −80 °C and in aliquots to avoid repeated freeze–thaw cycles.	B	I

Abbreviations: ctDNA, circulating tumor DNA; cfDNA, cell-free DNA.

ANALYTICAL ASPECTS

Target genes

The Cancer Genome Atlas and the International Cancer Genome Consortium Data Portal harbor extensive cancer genome research data, which have allowed the identification of key driver genes in various solid tumors [37, 38]. This groundbreaking work has paved the way for the development of effective treatment and diagnostic strategies and the integration of next-generation sequencing (NGS) into clinical practice to inform treatment decisions. The OncoKB database of the Memorial Sloan Kettering Cancer Center, NY, USA, is valuable for pinpointing Food and Drug Administration (FDA)–approved therapies tailored to patients with advanced solid tumor cancers exhibiting specific biomarkers [39, 40]. An up-to-date compilation of clinically significant genetic alterations associated with FDA approvals as of January 2023 is provided in Table 4.

NGS for ctDNA detection

Current molecular technologies for detecting ctDNA encompass PCR-based methods and NGS technologies. PCR-based techniques encompass real-time quantitative PCR, digital PCR, and BEAMing (beads, emulsion, amplification, and magnetics). These methods target specific mutations based on prior knowledge of the genetic alterations within the tumor, such as *KRAS* G12D. In contrast, NGS technologies are employed to identify a broader spectrum of mutations, offering comprehensive genomic profiling of tumors, including single-nucleotide variants, structural variants, and copy number variations [41, 42]. The ensuing discussion primarily focuses on the ongoing development of NGS technologies for sensitive ctDNA detection.

On average, 1 mL of plasma contains approximately 2,000 genome equivalents of cfDNA [43]. Detecting tumor-derived cfDNA, typically found in low fractions (with variant allele frequencies [VAFs] in ctDNA typically being < 1%), poses a significant challenge because of the limited analytical sensitivity of standard NGS. This sensitivity is typically limited to VAFs of 2–5% because of errors that arise during library preparation and sequencing, obfuscating true-positive variants [44-46]. Recent advancements in NGS technologies have enhanced sensitivity by implementing strategies such as molecular barcoding and *in silico* error suppression. These innovations enable the reliable differentiation of genuine mutations with VAFs < 1% from background artifacts.

Molecular barcoding

Errors can arise during the NGS library preparation and sequencing steps, posing challenges in identifying true variants with low VAFs. These errors may originate from the PCR amplification process during DNA preparation or the use of hybridization capture techniques for the targeted enrichment of genomic regions. Additionally, the inherent error rate of the sequencing process is estimated to be approximately 0.1% [47]. The introduction of molecular barcodes, known as unique molecular identifiers (UMIs), has proven effective in mitigating these errors. UMIs comprise short oligonucleotide tags, typically consisting of 4–14 random nucleotides, designed to facilitate the identification of sequencing reads originating from the same DNA molecule. Errors identified in individual reads are eliminated, and variants present in all reads sharing the same UMI are retained and grouped into a single-strand consensus sequence (SSCS) [47].

Table 4. Level 1 therapeutic implications currently defined in OncoKB

Gene	Alterations	Cancer types	Drugs
ALK	Fusions	Inflammatory myofibroblastic tumor	Crizotinib
		Non-small cell lung cancer	Alectinib, brigatinib, ceritinib, crizotinib, lorlatinib
ATM, BARD1, BRCA1, BRCA2, BRIP1, CDK12, CHEK1, CHEK2, FANCL, PALB2, RAD51B, RAD51C, RAD51D, RAD54L	Oncogenic mutations	Prostate cancer (not otherwise specified), prostate cancer	Olaparib
BRAF	V600	Erdheim–Chester disease	Vemurafenib
		Melanoma	Vemurafenib+atezolizumab+cobimetinib
	V600E	All solid tumors	Dabrafenib+trametinib
		Colorectal cancer	Encorafenib+cetuximab
		Melanoma	Dabrafenib, vemurafenib, encorafenib+binimetinib, trametinib, vemurafenib+cobimetinib
	V600K	Melanoma	Encorafenib+binimetinib, trametinib, vemurafenib+cobimetinib, dabrafenib+trametinib
BRCA1, BRCA2	Oncogenic mutations	Ovarian cancer, ovary/fallopian tube, peritoneal serous carcinoma	Olaparib, olaparib+bevacizumab, niraparib, rucaparib
		Prostate cancer (not otherwise specified), prostate cancer	Rucaparib
EGFR	Exon 19 in-frame deletions, L858R	Non-small cell lung cancer	Erlotinib, erlotinib+ramucirumab, afatinib, dacomitinib, gefitinib, osimertinib
	Exon 20 in-frame insertions	Non-small cell lung cancer	Amivantamab, mobocertinib
	G719, L861Q, S768I	Non-small cell lung cancer	Afatinib
	T790M	Non-small cell lung cancer	Osimertinib
ERBB2	Amplification	Breast cancer	Ado-trastuzumab emtansine, lapatinib+capecitabine, lapatinib+letrozole, margetuximab+chemotherapy, neratinib, neratinib+capecitabine, trastuzumab+pertuzumab+chemotherapy, trastuzumab+tucatinib+capecitabine, trastuzumab deruxtecan, trastuzumab, trastuzumab+chemotherapy
		Colorectal cancer	Tucatinib+trastuzumab
		Esophagogastric cancer	Pembrolizumab+trastuzumab+chemotherapy, trastuzumab+chemotherapy, trastuzumab deruxtecan
	Oncogenic mutations	Non-small cell lung cancer	Trastuzumab deruxtecan
FGFR2	Fusions	Bladder cancer	Erdafitinib
	Fusions	Cholangiocarcinoma	Futibatinib, infigratinib, pemigatinib
FGFR3	Fusions	Bladder cancer	Erdafitinib
	G370C, R248C, S249C, Y373C	Bladder cancer	Erdafitinib

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Table 4. Continued

Gene	Alterations	Cancer types	Drugs
IDH1	R132	Intrahepatic cholangiocarcinoma, cholangiocarcinoma	Ivosidenib
KIT	D816	Mastocytosis	Avapritinib
	Oncogenic mutations	Gastrointestinal stromal tumor	Imatinib, regorafenib, ripretinib, sunitinib
KRAS	G12C	Non-small cell lung cancer	Sotorasib, adagrasib
MET	D1010, exon 14 deletion, exon 14 in-frame deletions, exon 14 splice mutations	Non-small cell lung cancer	Capmatinib, tepotinib
NF1	Oncogenic mutations	Neurofibroma	Selumetinib
NTRK1, NTRK2, NTRK3	Fusions	All solid tumors	Entrectinib, larotrectinib
PDGFB	COL1A1-PDGFB fusion	Dermatofibrosarcoma protuberans	Imatinib
PDGFRA	Exon 18 in-frame deletions, exon 18 in-frame insertions, exon 18 missense mutations	Gastrointestinal stromal tumor	Avapritinib
PIK3CA	C420R, E542K, E545A, E545D, E545G, E545K, H1047L, H1047R, H1047Y, Q546E, Q546R	Breast cancer	Alpelisib+fulvestrant
RET	Fusions	All solid tumors	Selpercatinib
		Non-small cell lung cancer	Pralsetinib, selpercatinib
		Thyroid cancer	Pralsetinib, selpercatinib
	Oncogenic mutations	Medullary thyroid cancer	Pralsetinib, selpercatinib
ROS1	Fusions	Non-small cell lung cancer	Crizotinib, entrectinib
SMARCB1	Deletion	Epithelioid sarcoma	Tazemetostat
TSC1, TSC2	Oncogenic mutations	Encapsulated glioma	Everolimus

The molecular barcoding strategy initially described by Kinde, *et al*. [48] as the Safe-Sequencing System employs single-strand UMIs. More recently, duplex UMIs tagging both strands of double-strand molecules, resulting in the generation of duplex consensus sequence reads, have been developed. This process eliminates asymmetric errors and consolidates SSCSs originating from complementary strands of the DNA molecule [49, 50].

Despite its advantages, molecular barcoding has certain limitations. One significant drawback is the necessity for redundant sequencing, leading to a low number of unique sequences and a high sequencing cost. This inefficiency becomes particularly pronounced in duplex UMI methods, where both DNA strands require redundant sequencing [51]. Furthermore, inaccuracies in quantifying unique molecules can arise because of errors within the UMI sequences [52].

In silico error correction

Recent advances in bioinformatics technologies have paved the way to integrating *in silico* error suppression methods. These techniques utilize bioinformatics algorithms to identify and remove artifacts, further enhancing the analytical sensitivity of NGS-based ctDNA assays. Pécuchet, *et al*. [53] recently presented a statistical approach that leverages base-position error rates to detect variants with VAFs as low as 0.003 for single-nucleotide variants and 0.001 for insertions/deletions. Newman, *et al*. [54] introduced an integrated digital error suppression–enhanced cancer personalized profiling by deep sequencing approach. This innovative method involves the *in silico* removal of artifacts identified in cfDNA sequencing data, resulting in highly sensitive tumor-derived cfDNA detection, with reported sensitivities of 0.00025–0.002%.

NGS-based ctDNA assays

The analytical performance of NGS-based ctDNA assays has undergone thorough evaluation in previous studies. Deveson, *et al*. [55] assessed five major NGS-based ctDNA assays, including the AVENIO ctDNA Expanded Kit from Roche Diagnostics (Indianapolis, IN), TruSight Tumor 170 from Illumina (San Diego, CA, USA), xGen Non-small Cell Lung Cancer from Integrated DNA Technologies (Coralville, IA, USA), Lung Plasma v4 from Burning Rock Biotech (Guangzhou, China), and Oncomine Lung cfDNA Assay from Thermo Fisher Scientific (USA). When using 25 ng input cfDNA, these assays demonstrated varying median unique fragment depths (Lung Plasma v4 and AVENIO ctDNA Expanded approximately 4,700×; TruSight Tumor 170 approximately 1,200×). This metric reflects the assays' capability to sequence unique DNA molecules in the input DNA sample, which significantly affects assay performance [56]. The amplicon-based Oncomine Lung cfDNA Assay exhibited a unique fragment depth akin to that of hybrid capture assays. Among the hybrid capture assays, variations were observed for variants with low VAFs ranging from 0.1% to 0.5% (analytical sensitivity, 0.39–0.83). The xGen Non-small Cell Lung Cancer and Lung Plasma v4 assays displayed the highest sensitivity, exceeding 0.90 for variants with VAFs ranging from 0.3% to 0.5%.

Koessler, *et al*. [57] compared three NGS-based ctDNA assays, including the Oncomine Lung cfDNA assay, AVENIO ctDNA Expanded Kit, and QIAseq Human Lung Cancer Panel from Qiagen. The authors reported that reliable detection of variants with VAFs as low as 1% is achievable, whereas detecting variants with VAFs of 0.1% presents a considerable challenge. Notably, the QIAseq platform failed to detect two *EGFR* insertion/deletion variants with VAFs of 0.1% and underestimated the VAF in samples with a VAF of 0.5%.

In Korea, several NGS-based ctDNA assays are commercially available. Several clinical laboratories provide ctDNA NGS services using domestic or imported reagents, including DxLiquid Pan100 and DxLiquid TMB500 from Dxome, AlphaLiquid 100 from IMBDdx TruSight Oncology 500 ctDNA from Illumina, and Oncomine Pan-Cancer Cell-Free Assay from Thermo Fisher Scientific. Outsourcing-based ctDNA NGS assay services are available from foreign vendors, such as the Guardant360 NGS assay service provided by Guardant Health, Inc. and FoundationOne Liquid CDx by Foundation Medicine. Specimens are sent to Clinical Laboratory Improvement Amendment laboratories in the U. S., and reports are sent back to Korea. These services are not covered by Korean health insurance reimbursement.

It is essential to implement QC procedures to ensure proper execution of NGS library preparation, sequencing, and bioinformatic analysis. Valuable QC metrics for NGS-based ctDNA assay monitoring include the assessment of cfDNA quality and quantity, library qualification and quantification, base call quality scores, cluster density, the count of sequenced read pairs, GC bias, alignment rate, transition/transversion ratio, mapping quality, duplication rate, strand bias, sequencing depth, unique depth, on-target rate, and coverage uniformity [58].

ASSAY VALIDATION

Currently, most NGS-based ctDNA assays in clinical laboratories are laboratory-developed tests (LDTs), necessitating validation through established clinical validation processes and standards. Recently, the Blood Profiling Atlas in Cancer (BloodPAC) working group was established to tackle the challenges associated with the development, validation, and clinical utilization of liquid biopsy tests [59]. This group has formulated a set of recommended analytical validation protocols specifically for NGS-based ctDNA assays. The challenges posed by the minuscule quantities of cfDNA extracted from blood and the low concentrations of tumor-derived cfDNA have been carefully considered by the BloodPAC working group. These challenges encompass the need for highly sensitive assays to detect ctDNA, the potential for false-negative results because of the rarity of ctDNA molecules, and the necessity for contrived samples to achieve sufficient ctDNA quantities. The BloodPAC working group guidelines encompass determining the limit of detection (LOD) and limit of quantification (LOQ), analytical accuracy, linearity, precision, and interference. These protocols are readily accessible as supplementary materials in [60].

Specific assay validation guidelines for ctDNA testing are lacking in Korea. As an alternative, the Ministry of Food and Drug Safety in Korea provides detailed protocols for analytical and clinical performance evaluations that should be conducted in laboratories when implementing NGS assays. Analytical performance evaluation parameters include the LOD, measurement range, cut-off value, analytical specificity, and precision, including repeatability, reproducibility, robustness, accuracy, and cross-reactivity. Clinical performance evaluation covers clinical sensitivity and clinical specificity. For each of these aspects, the guidelines describe general explanations, materials, validation methods, and how to present the results [61]. In addition, the Laboratory Medicine Foundation of Korea and the Korean Institute of Genetic Testing Evaluation strive to maintain the qualitative reliability of clinical laboratories and offer a laboratory ac-

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creditation program. They provide standards for documented guidelines that define the verification process and QC methodology assessing LDT performance. The guidelines recommend that each laboratory should have a detailed manual specifying the method of LDT verification. LDT validation methods should cover analytical performance (accuracy or correlation with currently used tests, precision, linearity or reportable range, LOD, LOQ, stability, and interference) and clinical performance (sensitivity, specificity, and positive and negative predictive values) [62,63].

RESULT INTERPRETATION

Variant interpretation

Variant interpretation involves two distinct processes: biological interpretation and clinical interpretation. Biological interpretation focuses on assessing the oncogenic potential of a variant. The 2015 guidelines from the American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) are widely accepted as the standard for the biological interpretation of germline variants [64]. These guidelines classify variants into five categories: pathogenic, likely pathogenic, variant of uncertain significance, likely benign, and benign, considering various factors, such as population data, computational and prediction data, functional data, segregation data, *de novo* data, and allelic data.

The Sequence Variant Interpretation working group, established by the Clinical Genome Resource (ClinGen), has released gene- and disease-specific guidelines to ensure consistent and harmonized interpretation [65]. It is important to note that these guidelines are primarily suited for assessing the pathogenicity of germline variants.

In May 2022, guidelines for determining the oncogenicity of somatic variants were published collaboratively by ClinGen, the Cancer Genomics Consortium (CGC), and the Variant Interpretation for Cancer Consortium (VICC) [66]. The ClinGen/CGC/VICC guidelines classify variants into the same five categories as the ACMG/AMP guidelines (oncogenic, likely oncogenic, variant of uncertain significance, likely benign, and benign), considering population data, functional data, predictive data, cancer hotspots, and computational evidence.

Clinical interpretation involves determining the actionability of a variant. For the clinical interpretation of somatic variants, the 2017 AMP/American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines are widely adopted [67]. These guidelines utilize a system of four evidence levels (A, B, C, D) to assess the clinical impact of a variant. Based on this evidence, variants are categorized into four tiers (I, II, III, IV) according to their relevance to cancer diagnosis, prognosis, and treatment. Tier I comprises variants of strong clinical significance (level A and B evidence), Tier II includes potential clinical significance (level C and D evidence), Tier III encompasses variants of unknown clinical significance, and Tier IV consists of benign or likely benign variants.

In addition to the AMP/ASCO/CAP guidelines, the European Society for Medical Oncology Scale of Clinical Actionability for Molecular Targets (ESCAT) guidelines provide another framework for variant classification. ESCAT categorizes variants into six tiers (I, II, III, IV, V, and X) based on their implications for patient management [68].

The Precision Oncology Knowledge Base, or OncoKB, offers annotations for the biological and clinical interpretation of somatic variants [40]. OncoKB employs a levels-of-evidence system, classifying variants into six levels encompassing sensitivity (levels 1–4) and resistance (levels R1 and R2). The OncoKB levels align with the 2017 AMP/ASCO/CAP guidelines tiers as follows: OncoKB levels 1, 2, and R1 correspond to Tier 1A; OncoKB level 3A corresponds to Tier 1B; OncoKB level 3B corresponds to Tier IIB, and OncoKB levels 4 and R2 correspond to Tier 2D. Variants are further categorized as oncogenic, likely oncogenic, likely neutral, or inconclusive based on their oncogenic effects. Notably, the degree of concordance with the ClinGen/CGC/VICC guidelines has not yet been established.

These annotations are publicly accessible via the OncoKB website (http://oncokb.org) and cBioPortal for Cancer Genomics (http://www.cbioportal.org). In addition to OncoKB, several other knowledgebases are available for variant interpretation. Notably, the VICC database is a meta-knowledgebase that integrates six different knowledgebases [69], including the Cancer Genome Interpreter Cancer Biomarkers Database, Clinical Interpretation of Variants in Cancer, Jackson Laboratory Clinical Knowledgebase, MolecularMatch, OncoKB, and the Precision Medicine Knowledgebase.

Somatic variant interpretation has predominantly relied on clinical assessment. Based on a survey conducted among 152 organizations involved in NGS proficiency testing for solid tumors [70], a significant majority, 84.9% (129/152), utilized the 2017 AMP/ASCO/CAP guidelines for somatic variant interpretation. Of these, 68.2% (88/129) exclusively relied on the 2017 AMP/ASCO/CAP guidelines. The remaining 31.8% (41/129) combined the 2017 AMP/ASCO/CAP guidelines with other reference guidelines, with the 2015 ACMG/AMP guidelines being the

most frequently used.

A comprehensive understanding of somatic variants requires consideration of their biological and clinical aspects. Therefore, assessing somatic variants using a combination of clinical and biological interpretation approaches is recommended.

In pursuing precision medicine, engaging in collaborative discussions with multidisciplinary experts is paramount to exploring potential therapeutic options based on genetic findings. Such consultations play a pivotal role in the clinical decision-making process for patient management. Institutions can establish Molecular Tumor Boards (MTBs) comprising a diverse team of experts in the fields of genetics, oncology, pathology, genetic counseling, and bioinformatics. The MTB conducts a comprehensive evaluation of each patient case, aiming to optimize the utilization of targeted therapies. Therefore, patients can access the most suitable treatment options available or become candidates for participation in clinical trials exploring novel therapies. A systematic review conducted by Larson, *et al*. [71] indicated that patients who received therapy recommended by an MTB had better clinical outcomes than those treated using conventional approaches.

Considerations for interpretation

Low VAF

Interpreting variants with low VAFs can be challenging because it can be difficult to differentiate a genuine genetic alteration from an artifact. In ctDNA testing, cfDNA originating from non-cancerous cells can dilute ctDNA, resulting in a low VAF. This is particularly challenging in scenarios where the ctDNA quantity is limited, such as cases with a low tumor burden, subclonal variants, or brain metastasis. Although the clinical significance of low-VAF variants remains uncertain, some studies have indicated that variants located in driver genes can respond effectively to targeted therapies, even when the VAF is low [72, 73]. Therefore, accurately distinguishing true genetic alterations from artifacts is crucial. Variants falling below the LOD should not be reported unless additional verification steps have been undertaken. Increasing the depth of sequencing can be advantageous in identifying genuine alterations and reducing the likelihood of false positives.

Clonal hematopoiesis of indeterminate potential (CHIP)

CHIP is a natural aging process characterized by the accumulation of somatic variants in hematopoietic stem cells, resulting in their clonal expansion. This phenomenon is observed in approximately 10% of individuals aged >65 yrs [74]. As cfDNA primarily originates from hematopoietic stem cells, CHIP is a significant confounder when interpreting ctDNA results. This challenge is particularly pronounced when dealing with low-VAF variants detected in genes commonly associated with CHIP, such as *DNMT3A*, *TET2*, and *ASXL1* [74]. Furthermore, CHIP has been reported in genes typically associated with solid cancers, including *KRAS*, *GNAS*, *NRAS*, and *PIK3CA* [75], further complicating the interpretation.

To distinguish ctDNA-derived variants from those related to CHIP, paired sequencing of peripheral blood mononuclear cells (PBMCs) can be employed. Additionally, as ctDNA fragments tend to be shorter than non-tumor-derived cfDNA fragments, employing bioinformatics techniques that consider fragment size can be a valuable and effective alternative to PBMC sequencing [76].

Incidental germline variants

ctDNA testing can identify both germline and somatic variants. Particularly, the likelihood of detecting germline variants increases with an increasing number of analyzed genes. Acknowledging that germline variants may incidentally appear in ctDNA results is imperative, and patients should be made aware of this possibility. Common criteria used to suggest somatic variants encompass a VAF <50%, hotspot variants known to have clinical significance in cancer, and variants not frequently observed in population databases [1]. Conversely, VAFs of approximately 50% and 100% typically indicate heterozygous and homozygous germline variants, respectively. However, caution must be exercised, as VAFs of germline variants do not always align with these expected values [77].

When germline pathogenic variants are suspected in ctDNA results, considering confirmatory germline testing using normal tissue and offering genetic counseling to the patient is advisable. Furthermore, each laboratory should have well-established policies for interpreting and reporting germline variants. Incidental germline variants should be interpreted according to the 2015 ACMG/AMP guidelines and reported following the ACMG recommendations for reporting secondary findings [78]. In cases where confirmatory germline testing is not performed, patients should be informed that the differentiation between germline and somatic variants may not be feasible. Laboratories that already conduct normal tissue testing with PBMCs to filter CHIP-related variants can also identify germline variants. However, germline variants may be filtered out when the laboratory's testing strategy involves matched normal tissue testing with germ-

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line variant subtraction [79]. In such cases, it should be clearly stated that germline variants were subtracted during the analytical process.

Discordance with the results of tissue analysis

The results obtained from ctDNA testing may diverge from those derived from tissue analysis. When variants are not detected in ctDNA, it may represent a true-negative result, but the possibility of a false negative cannot be entirely ruled out. A false-negative result might occur because of a low concentration of tumor DNA in the plasma that is insufficient for detection. This is particularly relevant in cases involving central nervous system cancer or brain metastasis, where the blood–brain barrier constrains the release of tumor DNA. In such scenarios, ctDNA testing using cerebrospinal fluid can yield informative results. It is crucial to communicate these limitations to the patient, and terms such as "not detected," "undetected," or "uninformative" are preferred over "negative" [1].

Conversely, there may be instances where variants are exclusively detected in ctDNA and not in tissue samples [80]. This phenomenon can be attributed to tumor heterogeneity, which may not be accurately reflected in tissue-based testing. Consequently, ctDNA analysis can open up additional therapeutic options when the therapeutic target cannot be identified in tissue samples.

REPORTING RESULTS

The ctDNA report should encompass details crucial for clinical decision-making and ideally should be kept concise (no more than two pages). In essence, it should comprise patient and sample information. Patient information should include name, sex, age, tumor type, and histology. Sample information should include the sample identifier, sample type, and collection date.

Variants should be meticulously described following the Human Genome Variation Society nomenclature, which can be accessed at http://varnomen.hgvs.org/, at the coding DNA and protein levels. To ensure clarity and accuracy, it is essential to employ the approved gene symbol per the HUGO Gene Nomenclature Committee guidelines, available at https://www.genenames.org/. For reference sequences, please refer to the Matched Annotation from NCBI and EMBL-EBI (MANE) Select, accessible at https://www.ncbi.nlm.nih.gov/refseq/MANE.

In addition to the standard nomenclature, colloquial nomenclature may be included to facilitate clear communication and enhance understanding of the variant [67].

Studies have indicated a correlation between tumor size and the VAF in ctDNA [81, 82]. Consequently, VAF serves as a valuable tool for estimating tumor burden. Furthermore, a comparative analysis of VAF for variants within the same sample can unveil subclonal variants, shedding light on tumor heterogeneity [83]. It is worth noting that variations in the quantity of leukocyte DNA between samples may arise from pre-analytical factors. Therefore, VAF should be interpreted cautiously, and in such cases, reporting the mutation burden in copies per milliliter of plasma can provide valuable insights [84, 85].

The clinical interpretation of variants is a crucial component of the report, as it plays a pivotal role in selecting an appropriate treatment strategy. According to the 2017 AMP/ASCO/CAP guidelines, variables should be categorized into a four-tiered system. Specifically, Tiers I to III should be reported, whereas Tier IV should not be reported because it includes variants of known insignificance that are benign or likely benign [67]. If available, information on oncogenicity can also be incorporated in the report.

It is imperative to conduct clinical interpretation within the context of the patient's tumor type, as the clinical implications of the same variants can significantly vary depending on the specific tumor type. It is important to note that drug recommendations based on genetic information should not be overly specific, as the efficacy of therapy depends on numerous factors beyond genetic information. Therefore, drug recommendations should be general, and it is advisable to elucidate the overall association between the variant and potential therapeutic options [1]. For instance, it is appropriate to refrain from endorsing specific medications and elucidate how the variant may impact the medication's efficacy. This information should be pertinent to the patient's tumor type and substantiated with evidence, including proper citation of references [67].

Laboratories should regularly update the latest information to ensure that patients receive the most up-to-date and appropriate therapeutic guidance.

Microsatellite instability (MSI) and tumor mutational burden (TMB) are tumor-agnostic biomarkers for immune checkpoint inhibitors. Several studies have reported that blood MSI and blood TMB (bMSI and bTMB) estimated from ctDNA showed strong correlations with tissue MSI and tissue TMB (tMSI and tTMB) [86-89]. However, to date, only tMSI and tTMB determined using FoundationOne CDx developed by Foundation Medicine have been approved by the FDA as companion diagnostics for pembrolizumab [69, 90]. Further studies on the clinical efficacy and optimal cutoffs for bMSI and bTMB are required.

Table 5. Recommendations for result interpretation and reporting

Recommendation	Grade of recommendation	Level of evidence
Result interpretation		
It may be considered to assess somatic variants based on clinical and biological interpretation.	B	I
It is recommended to conduct clinical interpretation in the context of the tumor type of the patient.	A	I
It is recommended to consider the possibility of false positives and clonal hematopoiesis of indeterminate potential when interpreting variants.	A	I
If germline pathogenic variants are suspected, it may be considered to sequence normal, matched samples as a confirmation test and provide genetic counseling to the patient.	B	I
Result reporting		
It is recommended that the report includes essential information for clinical decision-making and is concise.	A	I
It is recommended to report general associations of variants and therapeutic options rather than specific recommendations.	A	I
It is recommended to acknowledge the possibility of false negatives as a limitation when a variant is not detected.	A	I
It may be considered to mention negative findings in actionable genes in a tumor-specific manner.	B	I

The report should encompass methodological details to provide a comprehensive understanding. These details should include the testing method employed, the specific genes and regions targeted, the reference genome used, assay performance parameters, critical quality metrics, and test limitations.

In cases where only specific portions of a gene were targeted, such as exons or hotspots, this information should be indicated. Regions that failed to meet the minimum depth of sequencing coverage, whether because of biological or technical factors, should also be explicitly mentioned.

Assay performance metrics, such as the LOD or minimum depth of sequencing coverage, along with critical quality metrics, such as the amount of input DNA or sequencing depth, can be provided to assess the test's overall success.

It is crucial to acknowledge the potential for false negatives, particularly when a variant is not detected, which may be attributed to limited test sensitivity. In instances where Tier I variants are not identified in cancer-specific actionable genes, such as *EGFR* in non-small cell lung cancer, it may be advisable to report these negative findings and recommend follow-up testing with tumor tissue [67, 91]. Therefore, each institution should compile a list of actionable genes tailored to the specific tumor type.

For easy reference, the recommendations for interpreting and reporting results are concisely summarized in Table 5.

CONCLUSION

ctDNA has emerged as a promising and minimally invasive tool with a wide range of clinical applications in precision medicine. This review provides practical recommendations encompassing various facets of the ctDNA assay, including pre-analytical procedures, analytical considerations, and result interpretation/reporting.

SUPPLEMENTARY MATERIALS

Supplementary materials can be found via https://doi.org/10.3343/alm.2023.0389

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AUTHOR CONTRIBUTIONS

Hong J, Kim Y, Jang JH, Kim YG, and Kim B reviewed the evidence-based recommendations and performed the literature search. Lee JS, Cho EH, and Shin S wrote the manuscript. Lee ST, Kong SY, and Lee W revised the article. Song EY organized the Clinical Practice Guidelines Committee and contributed to the conception and design.

CONFLICTS OF INTEREST

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