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Circulating Tumour DNA as Resistance Biomarkers: Real-Time Monitoring of Therapeutic Response.

Sofya Shavikova[1], Margarita Mazurok[1], Sona Nadirova[2], Arzu Gumbatova[3], Salome Svanadze[1], Ilya Kravtsov[3], Alexander Lebedev[4], Anna Pavlova[5], Duan Shuqing[6]

[1] First Pavlov State Medical University of St. Petersburg, Saint Petersburg, Russian Federation

[2] Altai State Medical University, Barnaul, Russian Federation

[3] Ivanovo State Medical University, Ivanovo, Russian Federation

[4] Far Eastern Federal University, Vladivostok, Russian Federation

[5] Ural Federal University named after the First President of Russia B.N. Yeltsin, Yekaterinburg, Russian Federation

[6] Department of Pharmacology and Pathophysiology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, China.

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KEYWORDS

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ABSTRACT:
Circulating tumour DNA (ctDNA) is a promising minimally invasive biomarker that reflects the genetic makeup and dynamic changes of tumours, with utility in monitoring treatment response and the emergence of drug resistance in patients with cancer. “biomarker of resistance” and “real-time monitoring of treatment response”. We aimed to review and collate the current evidence regarding the use of ctDNA as resistance biomarkers and its potential for use in real-time monitoring of treatment response. We performed a systematic review following PRISMA protocol. We searched electronic databases, including PubMed, Scopus, and Web of Science and Cochrane Library for studies published from January 2014 to December 2025. We included studies that used ctDNA to measure resistance mutations, monitor treatment response, and use dosing or treatment choices in treatment. We screened 1,421 records and included 40 studies. The patient ctDNA analysis enables early detection of resistance-associated mutations in often preceding radiologic progression. High sensitivity of ctDNA in evaluating the volume of tumour on-monitor as well as in identifying minimal residual disease. Use of ctDNA profiling improves the ability to modify treatment algorithms in real time and improves patient stratification.

1. Introduction

Circulating tumour DNA (ctDNA) represents a paradigm-shifting biomarker in the realm of liquid biopsy, offering non-invasive access to the genetic landscape of tumours via analyses of fragments of circulating free DNA in blood. Unlike traditional tissue biopsy, which provides a temporal snapshot of limited site specific biology, ctDNA reflects the inherently widespread and dynamic nature of malignant disease, allowing genomic alterations from multiple sites to be captured simultaneously. The ability to monitor both tumour heterogeneity and clonal evolution in real time creates exceptional opportunities for the assessment of therapeutic response and detection of minimal residual disease and mechanisms of treatment resistance. The increasing assimilation of ctDNA into clinical oncology practice highlights the fundamental requirements for robust analytical platforms and standardised workflows. [1]

2. Biology of Circulating Tumour DNA

Origins and Release Mechanisms

Circulating tumour DNA (ctDNA) arises from a host of biological processes, both related to the turnover of the tumour cell itself, and to its interaction with the microenvironment that it is growing in. Of the sources of ctDNA, apoptosis is thought to be the largest, particularly in the case of treated tumours, leading to its highly fragmented DNA fragments, of ~180 base pairs, which correspond to the nucleosomal units. Necrosis, particularly in rapidly proliferating, poorly vascularised tumours, leads to larger, more heterogeneous fragments. Apart from passive release mechanisms, tumour cells can also actively release DNA, particularly via exosomes and microvesicles, concentrating the DNA, and likely to be

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involved in cell-cell communication. Additionally, phagocytic clearance of tumour cells by macrophages leads to secondary release of DNA into circulation.[2]

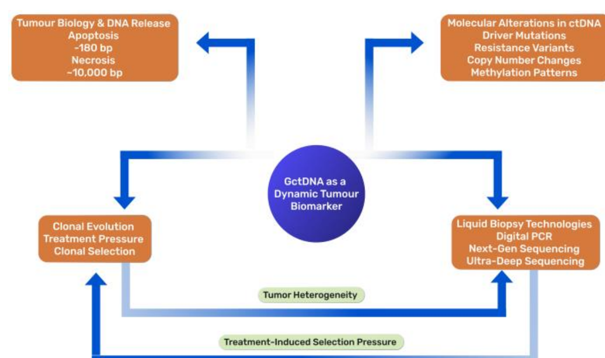

Figure 1. Biological Dynamics and Molecular Features of Circulating Tumour DNA in Therapy Resistance.

Comprehensive schematic outlining the origin and signature of ctDNA in the context of therapy resistance focused on the node “ctDNA as a Dynamic Tumor Biomarker” (colored deep blue) that further branches out into three domains: “Tumor biology and Release of DNA (eg Apoptosis, necrosis, clonal evolution under treatment pressure)” “Molecular Signatures in ctDNA (Eg driver mutations, resistance-variation, copy number changes, methylation)”, and “the platform used (eg digital PCR, next-generation sequencing (NGS), ultra-deep-sequencing platform)”. Arrows denote tumor heterogeneity and treatment selection pressure leading to the dynamic nature of ctDNA and the possibility of being able to perform periodic sampling of tumor DNA for real time imaging of changing resistance. [3]

ctDNA Fraction and Tumor Burden

The ctDNA fraction, or proportion of DNA shed into the circulation attributable to tumour DNA relative to total circulating cell free DNA, varies substantially by tumour biology and disease burden. In early-stage disease or minimal residual disease, the ctDNA fraction may be as low as 0.01% of the total cfDNA in the circulation, while with advanced metastatic disease it may be more than 50%. This fraction is correlated with the tumour volume, spread of metastases and biological aggressiveness of the tumour and is a useful quantitative marker of disease burden. ctDNA clears rapidly, with a half-life of approximately 30-120 min, allowing monitoring in near real time. This rapid clearance of circulating cell free DNA enables immediate molecular readouts of tumour responsiveness after treatment, even prior to therapy-related clinical or radiographic events. [4]

Biological Advantages over Tissue Biopsy

Perhaps the greatest part of the power of ctDNA techniques is in their ability to collate some idea of tumour heterogeneity from those lesions in multiple sites of disease. Whereas tissue biopsy samples a single lesion in time and space, ctDNA incorporates material from both the primary and metastatic sites. The blood-sampling assay is also non-invasive, allowing serial samplings throughout the course of treatment, allowing for longitudinal analysis of evolutionary trends in the tumour, and probably foreshadowing radiographic progression by weeks to months through detecting resistance mutations. Thus, the clonal dynamics can be tracked and appropriate therapeutic responses initiated. [5]

Preanalytical Considerations

ctDNA analysis is susceptible to several preanalytical variables, and contamination and degradation must be avoided. Blood should be collected in special cell-free DNA stabilization tubes (seek to minimize genomic DNA being released from lysed blood cells), and plasma preferred to serum as cellular DNA can contaminate serum and dilute any signal present from ctDNA. Standardized centrifugation protocols (minimum double spin) are also required to pellet intact cells and cellular debris. Packaging and transport must be carefully optimised as ctDNA can remain stable in the appropriate tubes for several days at room temperature, but for long-term preservation requires storage at ultra cold temperatures. All of these preanalytical steps affect assay sensitivity and should be carefully standardized in both research as well as clinical labs. [6]

3. **Analytical Platforms for ctDNA Analysis**

Digital PCR (dPCR) and Droplet Digital PCR (ddPCR)

Among the various digital PCR technologies (for example, droplet digital PCR) are highly sensitive detection techniques of specific alterations within ctDNA. These systems define thousands of individual reactions from droplets of DNA, with absolute quantification based on Poisson statistics. With sensitivity of down to 0.01–0.1% mutant allele fraction, these are ideally suited to detecting mutations in known genes with high specificity. Their rapid, turnaround time, low cost and infrastructural demands make them attractive for clinical use; however, their chief limiting factor is the prior knowledge required before use, restricting their application to follow-up on detected mutations rather than exploratory or wide-ranging genomics.[7]

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Targeted Next-Generation Sequencing (NGS)

Targeted next-generation sequencing platforms allow a more comprehensive approach to sequencing by detecting multiple genomic alterations across multiple regions of interest. Amplicon-based platforms allow for extremely high sequencing depths to detect very low-frequency sequencing variants, whilst hybrid capture allows for broader coverage of the genome including for structural variants, gene fusions, and rearrangements. The incorporation of unique molecular identifiers allows for increased sensitivity and reduced sequencing error resulting in the detection of mutation at low allele frequencies. Targeted platforms are used widely in clinical practice as a means to balance sensitivity, breadth of coverage, and scale to allow monitoring of tumour genomics on an ongoing basis. [8]

Whole-Genome and Whole-Exome Sequencing

Whole-exome and whole-genome sequencing approaches provide the most comprehensive view of tumour genomics, capturing coding regions (exome sequencing) or the full genome (whole genome sequencing). WES/WGS can uncover novel mutations, structural rearrangements, and copy number alterations not present on the targeted panels, but have more modest clinical impact due to increased expense, longer turnaround time, and poorer sensitivity for low frequency variants (unless ultra-deep sequencing is adopted). Such technologies are more commonly used in research settings and in the occasional clinical scenario where deep extensive genomic profiling is required. [9]

Emerging Technologies

New innovations are now beginning to widen the applications of ctDNA analysis beyond traditional sequencing methods. CRISPR-based detection systems leverage the trans-cleavage activity of Cas enzymes for highly sensitive and amplification-free detection of specific sequences that have promising applications in rapid point-of-care diagnostics. Electrochemical-based biosensors that use nanomaterials allow for real-time detection of ctDNA with high sensitivity and minimal processing requirements, while mass spectrometry-based techniques use multiplexed genotyping of known mutations for ctDNA analysis. Nanopore sequencing has the potential to allow real-time, long-read sequencing capable of detecting structural variants and epigenetic changes. These technologies represent some of the most recent changes to the field of ctDNA diagnostics. [10]

4. ctDNA as a Biomarker of Primary Resistance

Baseline ctDNA Detection and Prognosis

The identification of circulating tumour DNA at baseline is prognostically important across multiple cancer types and disease stages. In metastatic disease ctDNA can be detected in the majority of patients, often at rates greater than 80%, and in early stage disease at lower rates due to lower tumour burden and less shedding of DNA. Detectable ctDNA at baseline is associated with poor progression free survival and overall survival, consistent with worse biology and greater burden of disease at a systemic level. At the quantitative level higher mutant allele fractions (>1-5% in general) independently predict for worse outcome. CtDNA measures also add prognostic information to classic clinical staging systems. [11]

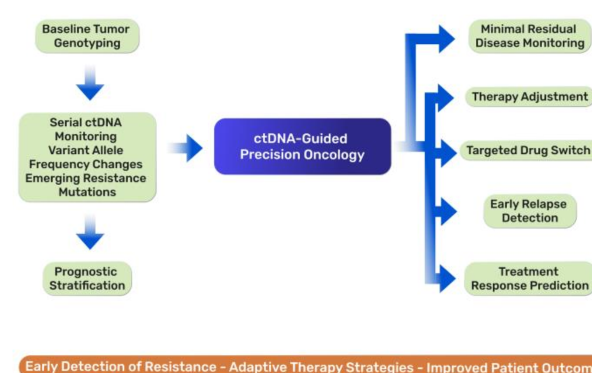

Figure 2. Clinical Integration of ctDNA for Real-Time Monitoring and Adaptive Cancer Therapy.

Schematic summarizing potential broad applications of ctDNA toward monitoring therapeutic response and clinical decision-making seen through “ctDNA-Guided Precision Oncology” lens (highlighted in dark blue). Offshoots off of baseline tumor genotyping through serial ctDNA monitoring during therapy (changes in variant allele frequencies, detection of resistance mutations) and clinical decision making (changes to therapy or targeted drug switching, early detection of relapse); modules demonstrating a few potential clinical applications (monitoring minimal residual disease, prognosis assessment, treatment stratification and prediction of response). Arrows highlight continuous monitoring of ctDNA replicates, early awareness of resistance, and improved outcomes in patients through intervention to therapeutic regimens. [12]

Genomic Predictors of Primary Resistance

ctDNA analysis reveals alterations present from the outset that drive intrinsic therapy resistance-discovery of specific driver mutations predictive of primary resistance to focal targeted agents(such as KRAS in lung cancer, BRAF in colorectal cancer etc.) which confer limited

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sensitivity to pathway directed agents, while additional concurrent mutations -of co-occurring genomic alterations(eg mutations in a tumour suppressor gene of TP53, STK11, KEAP1 etc.) further erodes therapeutic efficacy by activating any number of alternative survival pathways. Measures of tumour mutational burden(TMB) from ctDNA sequencing correlates with immunotherapy response - where lower mutational burdens = less responsive etc. Copy number alterations at both large(whole gene amplifications of ERBB2, MET, MYC etc. etc.) or small(whole exon or intronic amplifications of genes) scales may cause oncogenic signalling redundancy and are thus further mechanisms of baseline resistance detectable by ctDNA profiling [13].

Baseline Tumour Heterogeneity

An important aspect of ctDNA is that it embodies the heterogeneity of the tumour that is important for dictating its responsiveness to therapy. ctDNA is often seen to be derived from patients' tumours that contain multiple subclonally divergent populations, each having different mutations that may be involved in conferring resistance to specific agents; such polyclonality is not present in monoclonal tumours which are managed more often effectively with targeted therapies. High genomic complexity and clonal diversity correlates with rapid emergence of resistance to treatment. By using phylogenetics on ctDNA data, one can distinguish between truncal mutations (which are present in many or most of the tumour cells) and which may be sequentially or simultaneously acquired with certain evolutionary consistency, as opposed to branch mutations (confined only to certain subclones) and able to help track down where therapy directed ebbs and flow. This information has clinical application, providing a roadmap for agents that might be beneficial together for targeting multiple resistance routes simultaneously in order to hold the disease at bay for longer. [14]

ctDNA as Companion Diagnostic

Clinical utility of ctDNA as a companion diagnostic has gained regulatory approval through the development of assays that guide targeted therapy selection. An example of this would be the Guardant360 CDx and FoundationOne Liquid CDx platforms. The assays on these platforms detect actionable mutations in plasma samples obtained from patients, enabling selection of effective targeted therapy for that patient. These "liquid biopsy" assays are approved for detection of alterations in plasma for both EGFR mutations (lung), KRAS mutations (colorectal), PIK3CA mutations (breast), and BRCA1/2 alterations (ovarian). In most cases concordance with tissue testing exceeds 95% for these driver mutations; however, ctDNA may reveal additional alterations not detected by single-site tissue biopsy. Limitations exist with ctDNA in non-shedding tumours and those of low tumour burden where false-negative results have been found, warranting tissue analysis when indicated. [15]

4. ctDNA for Monitoring Response to Targeted Therapy

Early Molecular Response

Dynamic changes in ctDNA levels early during treatment can provide an early indicator of treatment response, as effective targeted therapy is associated with a decline in the ctDNA fraction arising within days to weeks after treatment initiation, and a reduction of greater than 50% within weeks two to four predicts radiographic response and improved outcomes. Complete clearance of ctDNA detected at early time points is prognostic, with a high proportion of patients remaining progression-free at one year, whereas persistent or minimally declining ctDNA levels in early treatment phases suggest primary resistance and worse outcomes, often seen prior to radiographic evidence of treatment failure. [16]

Molecular Relapse Detection

ctDNA analysis makes it possible to detect molecular relapse earlier than imaging techniques. The reappearance/increase in circulating tumor DNA levels following initial response is usually several months before imaging is able to detect disease progression, a timeline that varies from two to twelve months (depending on histology and assay sensitivity). In the postoperative or post-curative setting the presence of circulating tumor DNA implies impending disease recurrence, with sensitivity and specificity dependent on assay design. Close surveillance in a defined interval of ctDNA detection gives rise to early indication of relapse and hence provides targeted treatment opportunities, enabling initiation of a second-line approach or clinical trial [17].

Depth of Response and Residual Disease

In potentially curable settings ctDNA can be valuable in concept of molecular residual disease. Here if ctDNA is detected following surgery or chemoradiation, this implies the presence of microscopic residual disease and patients should have more intense follow-up due to a higher risk of recurrence. Patients with undetectable ctDNA are in comparison at much lower risk for failure. Serial quantitative ctDNA analysis may also give some insight into depth of response, and perhaps guide the need for adjuvant therapy. With ctDNA as a guide, trials are ongoing to evaluate using molecular residual disease status to guide modulation of 'intensity' of therapy. [18]

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Patient-Specific Assays for MRD Monitoring
Highly sensitive patient-specific assays are emerging to further refine detection of minimal residual disease. Using tumour-informed approaches, genomic data from the primary tumour are used to develop personalised assays that target several patient-specific mutations, achieving sensitively for detection of low-frequency variants. In contrast to these tumour-informed approaches, one can simply use mutation panels that are predefined and based on common mutations, offering broader application but less sensitivity. Tumour informed assays have been shown to detect ctDNA at very low allele fractions that allow for detection of residual disease months prior to clinical progression. There are many examples of how these technologies are beginning to be integrated into workflows of increasingly personalised disease monitoring and risk stratification. [19].

5. **ctDNA for Detection of Acquired Resistance**
On-Target Resistance Mutations
ctDNA analysis plays a key role in uncovering on-target resistance mechanisms that occur during the course of therapy as a response to being targeted. These mutations occur within the target itself and impair drug binding or activity. In lung cancer, for example, EGFR inhibitors fail in the setting of secondary mutations in the target such as T790M or C797S granting resistance to first- and third-generation inhibitors, respectively. The same pattern continues in other targeted therapies such as ALK inhibitors and BRAF inhibitors where specific mutations in the target protein confer inadequate drug coverage unearthing resistance. Tumors treated with PARP inhibitors develop reversion mutations within BRCA thereby regaining DNA repair function and treatment resistance. ctDNA can detect these alterations leading to timely" reassessment and reworking of therapy. [20]

Off-Target Resistance Mechanisms
Aside from target modification, tumours can also learn new tricks and activate different pathways altogether to drive growth. ctDNA analysis can find mutations in MAPK, PI3K/AKT/mTOR etc., which do not affect the originally targeted protein, and instead promote continued proliferation. Amplification or upregulation of receptor tyrosine kinases whose target lies off the pathway are frequent, with MET, HER2 or FGFR the usual suspects appearing. [21]

Histologic Transformation
In a different flavour of the resistance phenomenon is histologic transformation whereby the tumour obtains a new histological phenotype at the behest of a therapeutic pressure - lung adenocarcinomas with alterations to the EGFR coding region may become transformed into small cell lung cancer with different genetic and clinical features. ctDNA may bear clues to such a transformation e. g. in a concomitant appearance of RB1 and TP53 mutation [22].

Temporal Patterns of Resistance Emergence
The development of resistance is a dynamic phenotype characterized by clonal evolution driven by therapy pressure. ctDNA allows this evolution to be tracked over time distinguishing between the expansion of an existing mutant subclone and the appearance of novel mutations during treatment. Resistance can be polyclonal, with multiple distinct mechanisms co-existing in a patient. Serial ctDNA analysis at intervals during therapy provides early insight into progressive change prior to recognising clinical progression. This knowledge then drives real-time tumour evolution to it adapting the treatment to oncogenesis to block resistance or outsmart it. [23]

6. **ctDNA in Immuno-Oncology**
Baseline Predictors of Immunotherapy Response
Circulating tumour DNA has arisen as a powerful non-invasive biomarker predictive for response to immune checkpoint inhibitors. Among these, one of the most clinically relevant is blood-based tumour mutational burden (bTMB), which correlates with and is reflective of a tissue-derived TMB, as well as a surrogate of tumour neoantigen load. Increased bTMB is associated with improved response to PD-1/PD-L1 blockade, as improved exposure to "non-self" neoantigen increases recognition by the immune system. Likewise, detection of ctDNA-based microsatellite instability (bMSI) allows identification of immunotherapy-eligible patients, in most cases MS-high tumours who display exquisite sensitivity to agents such as pembrolizumab. Inversely, high burden of copy number alterations has been associated with "cold" tumours and poor prognosis. Finally, ctDNA fraction at baseline is also prognostic, with lower measures correlating with improved outcomes, although this may reflect lower tumour burden rather than inherent immunogenicity. [24]

On-Treatment ctDNA Dynamics with Immunotherapy
Longitudinal measurement of ctDNA during immunotherapy can provide unique information regarding patterns of response that imaging may miss. Rapid and sustained declines or even eradications in ctDNA is highly associated with long term clinical benefit and survival. Alternatively, atypical patterns of response like pseudoprogression (increase in tumour size attributable to immune infiltration) can be deconvoluted

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by ctDNA: if ctDNA transiently increases before decreasing, the immune system may be controlling the disease rather than the only true progression of disease. In contrast, "hyperprogression" of the tumour in immune therapy (the paradox wherein the cancer suddenly grows much faster because of failure of therapy) is marked by a rapid rise and sustained elevation in DNA levels, associated with poor outcomes. CTDNA suppression within the first one or two months of therapy (greater than 50% in 4-6 weeks) should be considered early evidence of efficacy of therapy and conversely persistent ctDNA burden beyond 8-12 weeks should trigger reconsideration of the therapeutic strategy. [25]

ctDNA in Combination Immunotherapy

Within combination immunotherapy paradigms, ctDNA represents a multidimensional biomarker platform. For example, more complex immune responses stemmed from dual checkpoint blockade (CTLA-4 + PD-1 inhibition), and ctDNA dynamics can facilitate earlier stratification of responders vs. non-responders. When ctDNA assessment is combined with T cell receptor sequencing, it enables simultaneous assessment of tumour burden and immune clonal expansion, informing on the adaptive immune response. ctDNA methylation profiling indicates indirect information of the tumour microenvironment including immune cell infiltration and inflammatory signalling. Composite biomarker panels taking account of bTMB, bMSI and dynamics of ctDNA change outperformed single biomarkers in predictive performance, exemplifying rationale for an integrated approach to patient stratification. [26]

Immune-Related Adverse Events (irAEs)

ctDNA also plays a role in the management of immune-related adverse events, which are a signature complication of immunotherapy. In patients suffering high-grade toxicities, dynamics of ctDNA could assist in determining whether there is genuine anti-tumour immune activation as opposed to non-specific autoimmunity. Persistent/rising ctDNA levels in the setting of severe irAE suggests inadequate control of the malignancy, while declining ctDNA suggests ongoing benefit from treatment. In patients with ‘difficult’ imaging features, e.g., pneumonitis, colitis within the context of immunotherapy, ctDNA provides an objective means to determine true disease progression and inflammation. ctDNA monitoring can influence decisions regarding reinitiating immunotherapy following resolution of toxicity, to try to ensure the best balance of efficacy and safety in complex patients. [27]

7. ctDNA in Combination Therapy and Novel Modalities

Antibody-Drug Conjugates (ADCs)

ctDNA analysis is increasingly being used to monitor response and resistance in patients undergoing immunoconjugate therapy. Resistance mechanisms may include: loss of target antigen expression, activation of drug efflux pathways, and mutations that affect payload sensitivity. For example, under HER2-directed ADC therapy, ctDNA can show loss of ERBB2 amplification, signalling weaker target engagement. Similarly mutations in topoisomerase I have been implicated in resistance to topoisomerase inhibitor-based immunoconjugates. Early evidence of ctDNA clearance (usually assessed longitudinally) has been associated with favourable response to therapy, whereas rising ctDNA levels may precede clinical progression and therefore allow earlier change in therapeutic strategy. [28]

Bispecific T-Cell Engagers (BiTEs)

In therapies based around bispecific T-cell engagers, ctDNA is able to monitor tumor burden and mechanism of resistance. Loss of target antigens (eg, CD19 in B-cell malignancies) can be observed in ctDNA prior to clinical relapse. Coupling this with T cell receptor sequencing can also provide insights into T cell clonal expansion and exhaustion, both of which are correlates of therapeutic efficacy. An early decrease in ctDNA represents effective engagement of immune targets, with subsequent increase alerting to the risk of evolving resistant / immune escape [29].

CAR-T Cell Therapy

ctDNA is also a dynamic biomarker of response to treatment and disease evolution in the context of CAR-T cell therapy. The baseline level of ctDNA is prognostic, with a higher burden of tumour correlating with lower response rates. Rapid decreases or extinction of ctDNA correlates with an effective kill and CAR-T cell expansion. ctDNA is particularly useful in distinguishing true progression from immune-mediated effects, such as cytokine release syndrome. This long-term monitoring also allows relapse because of loss of antigen or clonal evolution to be detected early allowing intervention. [30]

Targeted Radionuclide Therapy

For theranostic and targeted radionuclide therapies, ctDNA serves along with imaging in order to assess treatment response. For example, in prostate cancer loss of target expression (such as PSMA) is usually assessed by imaging; but this may be evaluated through ctDNA also. This may explain the mechanism of resistance behind some cases of radioligand therapy failure. Early decline or change in ctDNA levels is correlated with treatment response, while increases are also seen to occur ahead of radiologic progression. The combination of

ctDNA with molecular imaging together leads to improvement in how assessment is conducted, leading to more precise tools for which to perform personalized theragnostics. [31]

8. ctDNA in Early-Stage and Adjuvant Settings

Molecular Residual Disease (MRD) Detection

Molecular residual disease detection by ctDNA is one of the most impactful uses of liquid biopsy in early-stage cancer. Following curative-intent treatment, ctDNA detection indicates residual microscopic disease not detectable by imaging. MRD-positive patients carry a dramatically higher risk of recurrence (greater than tenfold compared to those who are ctDNA-negative) and ctDNA may detect recurrence several months in advance of clinical or radiographic evidence. [32]

MRD-Guided Adjuvant Therapy

ctDNA-guided adjuvant therapy approaches aim to individualize the intensity of adjuvant therapy according to the risk of recurrence. Patients found to have detectable ctDNA post-surgery can potentially benefit from more intensified adjuvant therapy, whereas those patients found not to have detectable ctDNA can be safely spared treatment and toxicity. Multiple randomized trials are ongoing across multiple tumour types, of note preliminary results show improved outcomes in high-risk patients whilst reducing overtreatment in low-risk patients representing a move towards precision oncology in the adjuvant space. [33]

ctDNA in Neoadjuvant Setting

In the neoadjuvant setting, ctDNA represents a real-time measure of treatment response. Baseline ctDNA is detectable in a significant proportion of patients, and clearance of ctDNA during therapy strongly predicts pathological complete response. Persistent ctDNA post-neoadjuvant therapy identifies patients at high risk of recurrence and may guide escalation of the intensity of adjuvant therapies. Moreover, dynamic ctDNA measurements facilitate response-adapted treatment strategies with the early switch of ineffective regimens. [34]

Surveillance After Curative-Intent Treatment

Post-treatment monitoring with ctDNA is a highly sensitive and specific strategy for early detection of recurrence. Serial assessment at defined intervals can pick up disease relapse several months before it can be identified by an abnormality on imaging. This enables timely intervention, often with resection of oligometastatic disease and/or initiation of systemic therapy. Preliminary data suggests ctDNA-based surveillance may be cost-effective relative to standard imaging approaches in a number of high-risk populations. [35]

9. ctDNA Methylation and Epigenetic Resistance

ctDNA Methylation as a Biomarker

Circulating tumour DNA methylation is also a powerful epigenetic marker that can be used as a supplementary layer of information to more conventional genomic ctDNA analysis, capturing likely regulatory changes in gene expression that do not manifest as sequence-level mutations. Compared to genomic alterations, DNA methylation patterns are more stable in circulation because they are less prone to fragmentation and degradation whilst retaining tissue-specific signatures that allow correct identification of tumour origin. Platforms such as bisulfite sequencing, methylation-specific PCR and targeted methylation panels are used for high definition mapping of methylation landscapes across multiple tumour types, whilst quantitative assessment of methylation burden, often expressed as methylation fraction, is useful for correlation with tumour burden and disease activity and has diagnostic and monitoring utility especially in cancers with low mutational burden, or instances where genetic alterations are insufficient to fully characterise tumour biology. [36]

Epigenetic Drivers of Resistance

Epigenetic alterations have been implicated in mediating both de novo and acquired resistance to anticancer therapies. Aberrant promoter hypermethylation is a hallmark of silencing of tumour suppressor pathways related to therapy resistance, including MLH1 hypermethylation associated with resistance to prior chemotherapy courses and MGMT promoter methylation, which alters the response of tumours to alkylating agents (e.g. temozolomide). Epigenetically mediated hijacking of repair pathways may result in, for example, methylation of BRCA1 resulting in homologous recombination deficiency which sensitises tumours to PARP inhibition, but when reversed restores DNA repair function and resistance. Epigenetic reprogramming may also mediate lineage plasticity that causes tumour cells to return to earlier phenotypic states, i.e. neuroendocrine transformation in prostate cancer leading to treatment resistance. Epigenetic heterogeneity resulting from diverse patterns of methylation across tumour subclones may also promote emergence of resistance since they provide a plethora of relatively stable states. [37]

Monitoring Epigenetic Resistance

Flexible ctDNA methylation tracking enables in vivo tracking of epigenetic changes in response to therapy, giving biologists a ‘real time’ insight into the war of

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attrition between cancer and therapy. If new signalling resistant drives appear to be arising under pressure or if a signature appears where signalling is being 'switched off' by hypermethylation this could be suggestive of adaptive silencing of oncogenic drives. Conversely a globally hypomethylated pattern could potentially reflect an aggressive behaviour or destabilization of the genome. Methods such as cell-free methylated DNA immunoprecipitation (cfMeDIP) may allow low-cost and high-throughput profiling of methylation without requiring the use of bisulfite conversion, thus making this technology globally scalable. Incremental benefits might be derived by combining methylation profiling with mutation data and looking for overlaps in signalling pathways at play. Such an approach might reveal incipient resistance, for instance if resistance occurs through non-mutational signalling changes that can sometimes go undetected otherwise. [38]

Epigenetic Therapies and ctDNA

The integration of epigenetic therapies with ctDNA provenance and monitoring is an exciting potential avenue for localization within precision oncology. Hypomethylating agents e.g. azacitidine and decitabine reverse aberrant DNA methylation, reactivating silenced tumour suppressor genes and restoring sensitivity to other therapies whose benefits were previously buffered against. ctDNA analysis may provide real-time assessment of the dynamics of demethylation and response to therapy. Other histone deacetylate inhibitors, e.g. vorinostat or romidepsin, further facilitate modification of chromatin structure and gene expression and are often combined with other agents. The basic notion of epigenetic priming, where epigenetic therapies a) amplify the immunogenicity of tumours and in this way sensitize them to chemotherapy or immunotherapy can find relatable monitoring within ctDNA methylation profiles. However, it appears probable that epigenetic therapy could facilitate evolution towards a resistant model through mutation in master regulators, e.g. FET2 or DNMT3A - ctDNA serves as a good vantagehip at this evolutionary change. [39]

10. **Challenges and Limitations**

Biological Limitations

Despite its promise, ctDNA assays must contend with limiting biological factors. Some tumours, "non-shedding," release little or no detectable ctDNA into the circulation (reducing assay sensitivity). Other shedding tumours may provide ctDNA fractions that fall below the detection threshold for existing assays, notably in early-stage disease or minimal residual disease settings. Clonal haematopoiesis of indeterminate potential, which introduces age-related somatic mutations to some haematopoietic cells, also modifies ctDNA interpretation as these mutations may potentially yield a false-positive result if not recognised. Shedding of tumour-specific ctDNA also varies by metastatic site (more ctDNA released from liver metastases versus bone or brain), further complicating interpretation. [40]

Technical Challenges

Technically, the need for accurate detection of low-frequency variants presents a quandary in ctDNA analysis. Detection of low-frequency variants requires ultra-deep sequencing and sophisticated algorithms/rounds of error-correction yields more sensitive but higher complexity and higher cost sequencing. Discrepant reporting rules and pre-analytical variability limit reproducibility (both in terms of study to study and test to test). Turn around time of comprehensive next-generation sequencing (typically several days to 2 weeks) may limit additional application in urgent cases. Serial testing is also costly [41]

Clinical Implementation Barriers

Clinical integration of ctDNA testing is further challenging by practical issues such as reimbursement variability and lack of familiarity in assay interpretation among clinicians. Clinically relevant incorporation would require a mix of molecular diagnostics and integration with imaging and clinical assessments, but the best way to do this is still not well defined. In addition, some changes detected have no therapeutically actionable options yet, requiring that the patient enter a clinical trial to find a means of intervention. [42]

Regulatory and Ethical Considerations

Governance for ctDNA-based diagnostics is being developed, and many ctDNA assays are now approved as companion diagnostics used in conjunction with targeted therapies, whereas wider applications such as MRD detection and resistance to treatment are still being validated. ctDNA analysis may rarely reveal incidental findings, while germline mutations require genetic counselling. Data privacy is a major concern, owing to the sensitivity of genomic and epigenomic data. Equitable access for ctDNA technologies across populations and healthcare systems is essential to avoid a further widening of disparities in cancer care. [43]

11. **Future Directions**

Multi-Analyte Liquid Biopsy

The future will be greater for liquid biopsy when a variety of other analytes, including ctDNA, circulating tumour cells, exosomes, cfDNA methylation, etc, can be combined together to describe the tumour biology. Single-cell genomic analysis of circulating tumour cells

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can describe mechanisms of resistance in more detail whilst exosomal RNA profiling can provide information on transcriptional programs driving tumour progression/immune evasion etc and ctDNA combined with protein biomarkers improves sensitivity and specificity in early-stage disease. [44]

Artificial Intelligence and Machine Learning

Artificial intelligence and machine learning may allow ctDNA analysis to move toward more complex, high-dimensional signals. Predictive models that incorporate ctDNA kinetics together with imaging findings and clinical features can lead to better risk stratification and further inform patient-specific treatment algorithms. Advanced learning approaches can identify subtle signatures of resistance emergence that are less likely to be gleaned via traditional means, while generative learning has the potential to create and validate new assays using simulated data from ctDNA sequences. [45]

Point-of-Care and Home-Based Monitoring

New technologies are creating platforms for rapid, decentralized testing of ctDNA, with CRISPR-based detection systems showing promise for highly sensitive amplification-free assays via fast abscission compatible with point-of-care application. Coupled with microsampling and wearable technologies, ctDNA levels may be monitored frequently and non-invasively with patient focus and remote monitoring via smartphone applications. [46]

Therapeutic Integration

New technologies are building the foundations for rapid decentralized testing with ctDNA, CRISPR-based detection systems are promising even ultra-sensitive amplification-free assays using fast abscission compatible with point-of-care application. Combined with microsampling and wearable technology, ctDNA may be assessed several times a day minimally invasively with patient focus with use of smartphone applications for remote monitoring. [46-51]

12. **Conclusions**

Summary of Key Evidence

Circulating tumour DNA (ctDNA) has emerged as a cornerstone of biomarker discovery in modern oncology, providing real-time insight into tumour biology, heterogeneity, evolution, and microevolution. High-sensitivity analytical platforms can detect genomic and epigenetic alterations at low variant allele fractions, enabling a wide array of applications including prediction of primary resistance, monitoring for response, and early detection of acquired resistance. ctDNA has proven especially useful in the fields of immuno-oncology, MRD detection, and epigenetic profiling, and those epigenetic approaches relying on methylation markers appear to have greater stability and tissue specificity.

Clinical Implications

ctDNA allows for earlier detection of treatment resistance than by imaging in the clinical state, permitting the earlier reactive therapeutic intervention. As a companion diagnostic its use is well known for some forms of targeted therapy, and ongoing trials are evaluating its ability to guide adjuvant treatment. ctDNA acts as a supplement, not substitute, to tissue biopsy, and helps therapeutic decision making; and so still has the need for histopathological diagnosis.

Research Priorities

Future studies could focus on ctDNA methodology standardisation, prospective validation of clinical applications and the expansion of cognisable targets detected through ctDNA examination. Economics studies are possible, concordant to economic evaluation of surveillance strategies there may be interest in the cost effectiveness of routine ctDNA monitoring.

The Road Ahead

Improvements in analytics, integration of next-generation sequencing with patient data, and evolving therapies are now at critical mass to see ctDNA as the foundation of precision oncology. Multi-analyte liquid biopsy, artificial intelligence and real-time monitoring will make truly personalized cancer care a reality with ctDNA as the dynamic biomarker supported in every step of the patient journey.

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