


# The Role Of Circulating Tumor DNA In Therapeutic Resistance

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**Abstract:** The application of precision medicine in cancer treatment has partly succeeded in reducing the side effects of unnecessary chemotherapeutics and in improving the survival rate of patients. However, with the long-term use of therapy, the dynamically changing intratumoral and intertumoral heterogeneity eventually gives rise to therapeutic resistance. In recent years, a novel testing technology (termed liquid biopsy) using circulating tumor DNAs (ctDNAs) extracted from peripheral blood samples from patients with cancer has brought about new expectations to the medical community. Using ctDNAs, clinicians can trace the heterogeneity pattern to duly adjust individual therapy and prolong overall survival for patients with cancer. Technological advances in detecting and characterizing ctDNAs (eg, development of next-generation sequencing) have provided clinicians with a valuable tool for genotyping tumors individually and identifying genetic and epigenetic alterations of the entire tumor to capture mutations associated with therapeutic resistance.

**Keywords:** cancer, heterogeneity, drug resistance, targeted therapies, sequencing technologies

## Introduction

Cancer is a leading cause of death worldwide in both developed and developing countries. Based on GLOBOCAN estimates, in 2012, approximately 14.1 million new cancer cases and 8.2 million cancer-induced deaths occurred globally.<sup>1</sup> Cancer is a genetic disease characterized by years of progressive accumulation of genomic aberrations that are occasionally augmented by predisposing germline mutations.<sup>2</sup> In 1976, Nowell proposed that this accumulation of mutations is caused by a process of diversification and selection of beneficial mutations promoting tumor cell proliferation and survival.<sup>3</sup> This process also occurs during long-term targeted therapy, causing changing mutations within tumor cells and eventually inducing intratumoral and intertumoral heterogeneity. This reduces the efficacy of therapeutic regimens, a phenomenon referred to as development of therapeutic resistance. The identification of selected genomic aberrations that target clinically relevant signaling pathways in patients, especially those giving rise to drug tolerance, remains a fundamental challenge.<sup>4</sup> Malignant tumors occasionally release fragments of nucleic acids into peripheral blood. Therefore, a noninvasive blood test detecting circulating tumor DNAs (ctDNAs) in the bloodstream (termed liquid biopsy) can inform clinicians regarding changes in the clonal composition of tumor tissues over time, thus allowing dynamic stratification of treatment.<sup>5</sup> Recent technological advances in isolating and analyzing ctDNAs bring about hope that liquid biopsies

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performed at different time points may be useful in tracing heterogeneous changes during treatment. Based on this concept, more precise therapeutic schedules can be formulated for patients with cancer of different stages.

This review focuses on the biological and technical aspects of ctDNAs and their valuable clinical applications in monitoring therapeutic resistance and improving outcomes in patients with cancer, as well as perspectives and challenges for future use.

## Underlying Reasons For Therapeutic Resistance: Intratumoral And Intertumoral Heterogeneity

Clonal mutations are detected in the majority of neoplastic cells in tumors. Beyond that, subclonal mutations resulting in heterogeneity within tumors also play an important role in tumor evolution, which must be considered when making individualized treatment decisions. The newly arisen concept of precision medicine aims to stratify patients into novel subgroups based on their individual information (eg, genetic, biomarker, phenotypic, or psychosocial characteristics) and offer personalized treatment strategies to each patient.<sup>6</sup> The application of targeted medicine offers an opportunity to improve patient care without the use of unnecessary chemotherapy or radiotherapy and reduce the side effects caused by these therapeutic modalities. An example which has shown remarkable clinical success to some extent is the administration of breakpoint cluster region-Abelson murine leukemia tyrosine kinase inhibitor (TKI) imatinib for the treatment of chronic myeloid leukemia.<sup>7</sup> However, tumor heterogeneity may give rise to differences in treatment responses and lead to the development of treatment resistance in patients with cancer. Tumor heterogeneity has two meanings: 1) intratumor heterogeneity, occurring within an individual tumor and 2) intertumor heterogeneity, appearing across several different tumors.<sup>8</sup> Explanations for the origin of intratumor heterogeneity fall into two categories. The cancer stem cell hypothesis proposes that a subset of cells with stem cell properties of indefinite self-renewal drive tumor initiation and progression. The differentiation of these cells may generate intratumor heterogeneity prior to or after treatment. In contrast, the clonal evolution model suggests that premalignant or malignant cells accumulate various hereditary changes over time, and those acquired advantageous genetic changes are selected according to natural selection. The accumulation of continuous mutations in

cancer cells ultimately contributes to the diversification of the tumor and emergence of further genetic and epigenetic alterations. These changes confer more aggressive, invasive, and drug-resistant phenotypes, thus resulting in intratumor heterogeneity.<sup>9,10</sup> Although surgical resection can remove the primary tumor along with a large proportion of the trunk mutations in them, a fraction of the residual branch heterogeneous mutation may cause tumor metastasis or relapse. In addition, the changing heterogeneity may reduce the initial efficacy of treatment. Mechanisms of drug resistance that have been identified thus far include the following: prevention of drug entry into the cells; extrusion of drugs out of cells; induction of enzymatic inactivation of drugs; prevention of drug activity by mutation or altered expression of the target and defects in apoptosis, senescence and repair mechanisms, etc.<sup>11</sup> Clinicians must consider this aspect in diagnosis and treatment planning to achieve optimal therapeutic efficacy. When selecting optimized therapy approaches, it is necessary to obtain an in-depth characterization of the clonal composition of each tumor.<sup>12</sup>

Andor et al found a link between individuals with intermediate copy number variation burdens detected in their primary tumor prior to receiving treatment and their worst overall survival (OS) across several types of tumors.<sup>13</sup> However, the strength of this association varies depending on the different types of therapies the patients receive. Islam et al conducted a study involving patients with high-grade diffuse large B-cell lymphomas treated with the aurora kinase inhibitor alisertib. They found that the generation of drug-induced aneuploid/polyploid cells, which are thought to be responsible for treatment resistance owing to their capability of re-entering the cell cycle during off-therapy periods, are commonly induced by anti-diffuse large B-cell lymphoma therapies.<sup>14</sup> The factors underlying the contribution of drug-induced aneuploid/polyploid cells to therapeutic resistance are the following: amplifying receptor tyrosine kinase or T-cell receptor signaling, or MYC-mediated dysregulation of three spindle assembly checkpoints *Ran GTPase-activating protein 1 (RanGAP1)*, *TPX2*, and *karyopherin alpha 2 (KPNA2)*. These factors can be targeted in combination with the administration of aurora kinase inhibitors to reduce the occurrence of therapeutic resistance. Goodall et al recently reported an analysis of targeted and whole-exome sequencing of serial circulating-free DNA samples collected during a Phase II clinical trial (TOPARP-A) of the poly (ADP)-ribose polymerase inhibitor olaparib in metastatic

prostate cancer. As disease progressed following response to olaparib, they detected multiple sub-clonal aberrations reverting the reading frame of mutated homologous recombination genes in tumors harboring germline and somatic loss of DNA repair mutations (eg, BRCA2, PALB2). These aberrations emerged as mechanisms of therapeutic resistance.<sup>15</sup> Collectively, these discoveries suggest that drug resistance may be induced by the long-term use of a single chemotherapeutic drug. Hence, it is of great importance to monitor the alterations occurring in tumor cells during the course of treatment and duly adjust the therapeutic strategies.

## The Definition And Characterization Of ctDNA

The tumor molecular landscape is continuously altered during treatment with targeted drugs. Thus, the genomic profiles of patients with cancer ought to be repeatedly evaluated during the course of therapy.<sup>16</sup> The repeated use of tissue biopsies is hindered by the inherent risk of complications and limited availability of samples. As a result, this approach cannot reveal all subclone oncogenic mutations caused by tumor heterogeneity, which are concealed in primary or metastasized tumor tissue. In response to this limitation, a new technology for the analysis of ctDNAs (termed liquid biopsy) has emerged.

ctDNA is a small fraction of cell-free DNA, which refers to DNA fragments released from cells, presenting in body fluids (eg, plasma, cerebrospinal fluid, and urine). In plasma, most of the circulating-free DNA originates from leukocytes. A small proportion of this DNA, termed ctDNA, is derived from tumor cells.<sup>17</sup> However, the quantity of ctDNA ranges from 3% to 93% of all circulating DNA. The source of ctDNA in the peripheral blood of patients with cancer is disintegrating cancer cells due to apoptosis and/or necrosis, like other circulating DNA which is released from normal dying cells.<sup>18</sup> This brings difficulty in distinguishing ctDNAs from other cell-free DNAs through the detection of apoptotic or necrosis-related markers. Therefore, the development of accurate and specific technologies for isolating ctDNAs is warranted. The detection of ctDNA can identify somatic genomic alterations missed in biopsy studies on account of tumor heterogeneity or lesions in distant sites.

Apart from ctDNAs, liquid biopsies include other circulating molecular indices, such as circulating tumor cells (CTCs), exosomes, tumor-specific proteins, and several

kinds of non-coding RNAs. The clinical applications of these molecular indicators rely on advantages that distinguish them from other factors (Table 1). Among them, CTCs are most frequently investigated and compared with ctDNAs.<sup>19</sup> CTCs are a type of tumor cells derived from the multicellular groupings of a primary tumor, entering the hematogenous or lymphatic circulation to initiate metastasis.<sup>20</sup> The vast majority of CTCs floating in the bloodstream die during circulation. However, a small cluster of surviving CTCs are capable of migrating to a distant site and initiate the invasion of another organ. Among all molecules containing genetic information in the blood, CTCs are the first to be approved for use as a biomarker of metastatic cancer by the Food and Drug Administration (USA).<sup>21</sup> Owing to intratumor heterogeneity, CTCs offer whole tumor cell-derived materials to noninvasively provide a better representation of the invasive clones for serial analysis during treatment compared with traditional surgical biopsies.

Nevertheless, a few limitations hinder the investigation of CTCs. In recent years, advances in detection technologies and devices have optimized the methods of CTC enrichment and isolation, rendering the analysis of this measurement index achievable. Both ctDNAs and CTCs show great value in temporally and spatially tracing mutation profiles. However, ctDNAs have the preponderance of superior sensitivity to CTCs, larger quantities in the bloodstream, and greater dynamic range that are in accord with changes in tumor burden.<sup>22</sup> Hence, ctDNA is the preferred source for molecular diagnosis, especially in patients with early stage cancer.

## Current Sequencing Techniques Applied To The Analysis Of ctDNA In Clinical Cancer Research

Recent advances in sequencing techniques provide new opportunities for analyzing ctDNAs (Table 2). These developments provide clinicians with a more valuable tool for monitoring tumor burden and treatment response based on molecular profiles. Here, we present a portion of these newly developed techniques.

### Digital Droplet Polymerase Chain Reaction (ddPCR) And Next-Generation Sequencing

Genotyping of ctDNA provides a chance to probe into heterogeneity during the evolution of cancer, monitor

**Table 1** Comparison Of Strengths And Limitations Between ctDNAs And Other Circulating Biomarkers

	Strengths	Limitations	References
Similarities	<ul style="list-style-type: none"> <li>-Noninvasive</li> <li>-High specificity for multiple types of cancer</li> <li>-Vital function in the diagnosis, progression monitoring, and prognosis of cancer</li> </ul>	<ul style="list-style-type: none"> <li>-Lack of standardization in isolation, enrichment, or detection techniques</li> <li>-Combined application is necessary for a more comprehensive insight into cancer specificity</li> </ul>	19,68,69
ctDNAs	<ul style="list-style-type: none"> <li>-Allowing for early detection and high sensitivity</li> <li>-Relatively higher detection rates</li> <li>-Tracing tumor heterogeneity temporally and spatially</li> </ul>	<ul style="list-style-type: none"> <li>-Analysis limited to segments of DNA</li> <li>-Functional genomic testing cannot be performed</li> <li>-No co-localization of signal</li> </ul>	70–73
CTCs	<ul style="list-style-type: none"> <li>-Fully showing the molecular profile of RNAs and proteins</li> <li>-Achievable structural evaluation of intact tumor cells</li> <li>-Show alterations in the whole genome</li> <li>-Co-localization of signal</li> <li>-Available for ex-vivo functional studies</li> </ul>	<ul style="list-style-type: none"> <li>-Influence of heterogeneity on selection methods</li> <li>-Low signal-to-noise rate, especially in early stage cancer</li> <li>-Detection limited to metastasis period</li> </ul>	74–76
Exosomes	<ul style="list-style-type: none"> <li>-Extractable from many biological fluids</li> <li>-Allowing for early detection and stable</li> <li>-mRNA and miRNAs enriched from exosomes, as well as lipids and metabolites, can serve as reliable biomarkers</li> </ul>	<ul style="list-style-type: none"> <li>-Lack of large scale in vivo studies</li> <li>-Function in immune response and interplay with the tumor environment require further research into the mechanism</li> </ul>	77–80
miRNAs	<ul style="list-style-type: none"> <li>-Exist in different biological fluids and benefit the early detection of cancer</li> <li>-More stable and resistant to storage and handling</li> <li>-Detectable in low quantity of sample with high specificity</li> </ul>	<ul style="list-style-type: none"> <li>-Hemolysis causing specific miRNAs mixed with ruptured erythrocytes containing miRNAs</li> <li>-Difficulty in accurately measuring the expression level</li> </ul>	81–83
Proteins	<ul style="list-style-type: none"> <li>-Easy detection and quantification</li> <li>-Proved efficiency in the prediction of recurrence and monitoring of therapeutic efficacy</li> </ul>	<ul style="list-style-type: none"> <li>-Low accuracy for early clinical diagnosis</li> </ul>	68
lncRNAs	<ul style="list-style-type: none"> <li>-Stable and levels indicative of severity</li> <li>-Wide range of expression patterns in various cancers</li> </ul>	<ul style="list-style-type: none"> <li>-Low quantity and quality resulting in low signal-to-noise ratios</li> <li>-Development of efficient detection technologies is required</li> <li>-Lack of clinical trials</li> </ul>	84–86

**Abbreviations:** CTC, circulating tumor cell; ctDNA, circulating tumor DNA; lncRNA, long non-coding RNA; miRNA, microRNA.

cancer development, and potentially detect the emergence of therapeutic resistance. However, the development of new technologies, such as ddPCR and next-generation sequencing (NGS), is required for the identification of valuable variants to be targeted when making clinical decisions. Plasma ddPCR is capable of rapidly detecting mutations, such as epidermal growth factor receptor (EGFR) and Kirsten rat sarcoma viral oncogene homolog (KRAS) mutations, with a low rate of false-positive test results and the required robustness for real-world testing. Sacher AG et al conducted a plasma ddPCR assay on 180 patients with non-small cell lung cancer (NSCLC) and showed a sensitivity of 82% for *EGFR* 19 del, 77% for

T790M, 74% for L858R, and 64% for *KRAS*. It also exhibited a positive predictive value of 100% for *EGFR* 19 del, L858R, and *KRAS*; of note, this value was lower for T790M (79%).<sup>23</sup> This evidence indicated the ability of ddPCR to rapidly and sensitively detect EGFR and KRAS mutations. This approach offers the advantage of absolute quantification and sample differentiation with tiny concentration distinction, highlighting its potential for application in the clinical setting. Nevertheless, ddPCR is limited by its inability to detect unknown genotypes or rearrangements and difficulty in multiplexing to determine the amounts of genetic variants. The breakthrough concept for the detection of circulating genomic subclones through

**Table 2** Sequencing Technologies For ctDNA Analysis

Categories Of Sequencing Technologies	Type Of Alterations	Cancer Types	References	Advantages	Limitations
<b>PCR-based</b>					
Nested real-time PCR	Genomic rearrangements	Breast cancer, osteosarcoma	87	-Ease of use -Lowest cost	-Lower sensitivity -Only detect limited genomic loci
ARMS-Scorpion PCR	PIK3CA mutation	Breast cancer	88		
PAP-A amplification	TP53 mutation	Breast cancer	89		
	EGFR deletion	Non-small cell lung cancer			
<b>Digital PCR-based</b>					
BEAMing	NRAS, KRAS mutation	Colorectal cancer	90	-Ease of use	-Only detect limited genomic loci or single nucleotide variants
	KRAS mutation	Pancreatic cancer	91		
	EGFR mutation	Non-small cell lung cancer	92		
Droplet-based digital PCR	NPY methylation	Colorectal cancer	93	-High sensitivity	
Microfluidic digital PCR	TP53 and PTEN mutation	Ovarian, pancreatic cancer	94	-Clinically validated	
<b>Targeted deep sequencing</b>					
Safe-SeqS	KIT mutation	Gastrointestinal stromal tumor	95	-High sensitivity -Relatively inexpensive	For selected alterations across targeted regions
TAm-Seq	TP53 mutation	Ovarian cancer	96		
	EGFR mutation	Non-small cell lung cancer			
CAPP-Seq	KRAS, APC, and TP53 mutation	Ovarian cancer	97		
Ion-AmpliSeq	TP53, KRAS, and EGFR mutation	Non-small cell lung cancer	98,99		
Whole-genome sequencing (with NGS)	Copy number aberrations	Non-small cell lung cancer	100	-Broad application	-Expensive -Low sensitivity
	Alterations in DNA repair deficiency genes and biallelic inactivation	Prostate cancer	101		
	KRAS and BRAF mutation	Colorectal cancer	102		
Whole-exome sequencing (with NGS)	SNVs and copy number aberrations	Wilms' tumor, neuroblastoma	103,104	-Broad application	-Expensive -Low sensitivity -Lack of standardization
	EGFR mutation	Non-small cell lung cancer	105		

**Abbreviations:** ARMS, amplification refractory mutation system; BEAMing, beads, emulsion, amplification, magnetics; CAPP-seq, cancer personalized profiling by deep sequencing; NGS, next generation sequencing; PAP-A, pyrophosphorolysis-activated polymerization allele-specific-amplification; PCR, polymerase chain reaction; Safe-SeqS, safe-sequencing system; SNV: single-nucleotide variants; TAm-Seq, tagged amplicon deep sequencing.

ctDNA-NGS has recently been investigated as a formidable platform to develop noninvasive blood tests. The purpose of this approach is to complement and further enhance the currently available screening and diagnostic strategies. With similar sensitivity to ddPCR, plasma NGS can compensate for the disadvantage of ddPCR by detecting a wide range of driver and resistance mutations in NSCLC, such as anaplastic lymphoma kinase (ALK), ROS1, and RET rearrangements, mesenchymal–epithelial transition (MET) amplification, and human epidermal growth factor receptor 2 (HER2) insertions, with 100% specificity.<sup>24</sup> NGS mainly consists of three specific techniques, namely whole-genome sequencing (WGS), whole-exome sequencing, and targeted region sequencing. Single ctDNA samples are inadequate to reveal subclonal populations or their responses to therapy. Therefore, the combined application of whole-exome sequencing and targeted longitudinal monitoring of the ctDNA can reveal both the presence of individual subclones with their genetic constitution and distinct responses to therapy.<sup>25</sup> Compared with WGS, exomic analyses exhibit markedly higher sequence coverage, with generally more than 100-fold versus just approximately 30-fold of WGS. Nevertheless, whole-genome plasma DNA sequencing enables the identification of novel mutant clones and potentially facilitates early adjustments of clinical therapies that may delay or prevent the progression of cancer.<sup>26</sup>

## Deep Sequencing Of ctDNA

Diehn et al developed a new strategy based on NGS, termed CAncer Personalized Profiling by deep Sequencing (CAPP-seq), for the analysis of ctDNA. This strategy involves the design of a “selector” consisting of labeled DNA oligonucleotides that target recurrently mutated regions in ctDNAs of interest for their quantification. This methodology is the first to achieve ctDNA analysis with an ultralow detection limit and broad patient coverage (ie, early or advanced stages of human malignancy with available recurrent mutation data) at a reasonable cost.<sup>27</sup> Integrated digital error suppression (iDES) was developed to further improve the performance of CAPP-seq by eliminating most artifacts observed in ctDNA sequencing data and simultaneously maximizing the recovery of ctDNA molecules. As a result, the iDES-enhanced CAPP-seq can sensitively and precisely facilitate the noninvasive detection of variants across hundreds of kilobases. For example, when applied to ctDNAs obtained from a cohort of patients with NSCLC, this approach enabled the profiling of mutations in the *EGFR* kinase domain. At the variant level,

the sensitivity and specificity were 92% and nearly 100%, respectively. At the patient level, these values were almost 90% and 96%, respectively.<sup>28</sup>

## Safe-Sequencing System (Safe-SeqS) And Simple, Multiplexed, PCR-Based Barcoding Of DNA

Compared with conventional digital PCR methods, massively parallel sequencing can sequentially and easily analyze hundreds of millions of template molecules and multiple bases in an automated fashion. However, this sequencing technique cannot be used for the detection of rare variants (ie, variant alleles with fractions  $\leq 0.1\%$  in ctDNA), owing to the high error rate associated with the sequencing process. Vogelstein described that Safe-SeqS can substantially increase the sensitivity of massively parallel sequencing by assigning a unique identifier (UID) to each amplified template molecule to create UID families, followed by implementation of redundant sequencing of the amplification products. In this method, the presence of an identical mutation in  $\geq 95\%$  of the PCR fragments with the same UID indicates mutation (“supermutants”).<sup>29</sup> Although Safe-SeqS has considerably improved massively parallel sequencing, this technology has not been widely used probably due to its complex protocol and the requirement of a gel purification step. In a laboratory in Boston, Tony E. Godfrey et al developed a method based on Safe-SeqS termed Simple, Multiplexed, PCR-based barcoding of DNA for Sensitive mutation detection using Sequencing (SiMSen-Seq). This approach employed a molecular hairpin serving as the UID to protect the barcode sequences from polymerase-induced errors for a more efficient identification of true mutations during the preparation of a PCR-based NGS library. Moreover, it involves a standard multiplex preamplification approach using lower primer concentrations as well as an elongated PCR extension time.<sup>30</sup> The strengths of the SiMSen-Seq methodology are as follows: 1) facilitates the detection of sequence variants at an allele frequency  $\leq 0.1\%$ ; 2) requires  $< 50$  ng of DNA input; and 3) can be applied to interrogate multiple genome loci covering  $> 1000$  kb of a target sequence.

## Assessment Of Epigenetic Heterogeneity Using DREAMing (Discrimination Of Rare EpiAlleles By Melt)

The ever-growing number of genes that show epigenetic alterations in cell biology and tissue physiology in the

course of human disease emphasizes the crucial role of these epigenetic alterations, especially DNA methylation, in future diagnosis, prognosis, and prediction of response to therapies. Epigenetic drugs which target mutated modifying enzymes, such as DNA methyltransferase, improve to some extent the outcome of various patients with cancer whose methylation profiles had been analyzed. Thus, comprehensive DNA methylation profiling presents a valid tool for the analysis of regions offering a possibility of therapeutic intervention.<sup>31</sup> High-resolution data can serve as a source of cancer subtype-specific profiles at various stages of disease. This is achieved by performing analyses of alterations in methylation profiles using convenient and noninvasive methods with powerful technologies, such as ctDNAs. Wang at Johns Hopkins University (Baltimore, MD, USA) discussed a method termed DREAMing for ultrasensitive assessment of locus-specific epigenetic heterogeneity in specimens obtained from liquid biopsies. This approach applies semi-limiting dilution and precise melt curve analysis to discriminate and enumerate individual copies of epiallelic variants at single-CpG-site resolution, with fractions as low as 0.005%, while using only a single 96-well microtiter plate.<sup>32</sup> This technique is particularly suitable for the ultrasensitive assessment of epigenetic heterogeneity in specimens with low abundance of epialleles and may be utilized for the evaluation of methylation dynamics in ctDNAs during progression of cancer.

## Clinical Implication Of ctDNAs As A Monitoring Tool Of Drug Resistance

According to the time of development, drug resistance can be divided as follows: present at the time of initial treatment (ie, primary resistance) or arising during the course of treatment (ie, acquired resistance).<sup>33</sup> Thus far, four main strategies employed by cancer cells to induce drug resistance have been found: 1) direct reactivation of small-molecule targets; 2) activation of signaling nodes upstream or downstream of oncogenes; 3) engagement of parallel oncogenic signaling pathways; and 4) adaptive survival mechanisms.<sup>34</sup> An important commonality of all four strategies is the persistent activation of the drug target itself or its critical signaling pathway.<sup>35</sup> ctDNAs can reveal the tremendous heterogeneity and genetic composition of primary or metastatic tumors; thus, they can also reflect mutations associated with changes in the activation state.<sup>36</sup> Based on this, the isolation, purification, and analysis of ctDNAs can expound the reasons for the generation and

development of primary or acquired resistance after chemotherapy and guide the administration of drugs in the clinical setting.<sup>37</sup> Thus far, numerous mutations resulting in resistance to different kinds of targeted medicines have been found in ctDNAs. Here, we discuss some of these mutations according to the classification of cancer types and targeted medicine.

## Resistance To EGFR-Targeted Therapies Anti-EGFR Therapy With The Monoclonal Antibodies In Colorectal Cancer (CRC)

Overexpression of EGFR has been associated with more aggressive clinical progression and acts as a factor of poor prognosis in a wide variety of cancers. In CRC, the EGFR gene is frequently amplified and overexpressed at the RNA and protein levels.<sup>38</sup> Thus, EGFR-targeted therapies may improve the OS and progression-free survival (PFS) of patients with CRC. Cetuximab and panitumumab are monoclonal antibodies binding to EGFR, which are routinely used in the treatment of metastatic CRC (mCRC). However, these drugs show clinical benefits in only a portion of patients with mCRC owing to molecular alterations in EGFR pathway effectors.<sup>39</sup> The EGFR effector KRAS is frequently mutated in CRC, resulting in activation of the downstream RAS-RAF-mitogen-activated protein kinase (RAS-RAF-MAPK) signaling pathway, regardless of EGFR activation or blockade. Therefore, KRAS mutations may predict the development of resistance to anti-EGFR antibodies and are consistently correlated with reduced OS and PFS.<sup>40</sup>

RAS testing has become mandatory for patients with mCRC to identify the specific tumor genotype for treatment decision. Diaz et al traced KRAS mutations in ctDNAs obtained from patients with CRC who received monotherapy with anti-EGFR drugs.<sup>41</sup> They found that 38% of the patients had a detectable conversion from KRAS wild type to KRAS mutant type in their ctDNAs under EGFR blockade. According to the KRAS type tested through allele-specific real-time PCR, clinicians decide to further analyze all RAS through Sanger sequencing (if KRAS ex2 wild-type is mutated) or treat patients with standard chemotherapy (if KRAS ex2 is mutated). Standard chemotherapy is required for patients with identified RAS mutations. Only all RAS wild-type patients receive targeted treatment with an anti-EGFR monoclonal antibody.<sup>42</sup>

Apart from RAS mutations, amplification of the MET protooncogene is also associated with acquired resistance in patients treated with anti-EGFR therapy. Amplification

of the MET locus can be present in ctDNA before relapse becomes clinically evident, and timely administration of MET kinase inhibitors can overcome the induced resistance to EGFR blockade.<sup>43</sup> Low-abundance somatic mutations, such as phosphoinositide-3-kinase, catalytic, alpha polypeptide (PIK3CA), can also be detected through a targeted amplicon ultra-deep sequencing method in ctDNAs collected from blood of patients with mCRC.<sup>44</sup> As the PIK3CA mutation may result in acquired resistance to cetuximab in patients with mCRC, ctDNA sequencing can provide a reference for adjusting the clinical therapeutic approach.

### EGFR-TKI In NSCLC

EGFR-TKI have been introduced into the first-line treatment of patients with NSCLC harboring an EGFR mutation, improving PFS and OS to various degrees.<sup>45</sup> Nevertheless, almost all patients with EGFR-mutant NSCLC ultimately develop clinically progressive disease, as a result of resistance to treatment with an EGFR-TKI (ie, gefitinib or erlotinib). Thus far, two main mechanisms underlying acquired resistance to TKI have been identified. Secondary EGFR T790M mutation is a major mechanism, accounting for half of all reported resistance cases. Using the ddPCR method, T790M ctDNA can be detected in plasma as a poor prognostic factor and an indicator for modifying therapeutic strategies.<sup>46</sup> MET amplification is another cause of resistance to gefitinib, which can be detected in approximately 20% of the patients. Amplification of MET can drive ERBB3 (HER3) sustained phosphorylation in the presence of gefitinib. In addition, upregulation of the ERBB3/PI3K/Akt signaling pathway impedes the induction of apoptosis by gefitinib in EGFR-mutant cells.<sup>47</sup> In a CAPP-Seq ctDNA analysis, researchers found that MET amplification was the most frequent mechanism of resistance in patients with NSCLC receiving the third-generation EGFR inhibitor rociletinib.<sup>48</sup> Those results indicated that ctDNA analysis is capable of noninvasively presenting resistance mechanisms and defining patterns of resistance to targeted therapies.

According to the T790M-mutation status shown in ctDNA analysis through multiplexed deep sequencing, 52% of the patients with NSCLC who were T790M-mutation positive and received treatment with osimertinib ultimately achieved a 62.5% response rate and a 12-month PFS.<sup>49</sup> Another trial showed that even in patients with NSCLC with unknown tumor mutation status, but merely harboring T790M mutation detected through ctDNA

analysis, treatment with osimertinib resulted in favorable efficacy.<sup>50</sup> Similarly, crizotinib is a common targeted medicine used to counter MET amplification, and ctDNA can reveal a number of genetic mutations which contribute to resistance to crizotinib and progression of cancer (eg, multiple secondary MET mutations, increase of fibroblast growth factor receptor 2 gene relative copy number, etc).<sup>51</sup> Thus, ctDNA analysis may illustrate the complex and heterogeneous molecular mechanisms of drug resistance, and allow personalized selection of therapies in response to resistance.

### Resistance To anti-HER2 Therapies In Breast Cancer

Amplification of the HER2/neu oncogene occurs relatively frequently in breast cancer and correlates with disease relapse and OS.<sup>52</sup> With the development of HER2-targeted agents (eg, trastuzumab, pertuzumab, and other small molecule TKI of HER2), addition of these drugs to chemotherapy can prolong the time to disease progression and lengthen patient survival.<sup>53</sup> Although the efficacy of anti-HER2 therapy is obvious, therapeutic resistance prevents patients from achieving sustained clinical remission with this treatment strategy. The ctDNA assay can reveal markers of resistance to HER2 blockade, such as mutations in TP53 and genes implicated in the PI3K/mTOR pathway.<sup>54</sup> In a longitudinal follow-up study analyzing ctDNAs collected from 42 patients with metastatic breast cancer, it was demonstrated that ctDNA can show more dynamic changes to mutations and gene amplification than cancer antigen 15-3. Thus, ctDNA carries the potential of monitoring response to treatment, analyzing dynamic tumor heterogeneity, and stratifying targeted therapies.<sup>55</sup>

### Resistance To BRAF And MEK Inhibitor Therapies In Melanoma And CRC

BRAF inhibitors (eg, dabrafenib and vemurafenib) in combination with a MEK inhibitor (eg, trametinib) can improve the OS of patients with previously untreated BRAF V600-mutant metastatic melanoma.<sup>56</sup> However, drug resistance may cause progression of disease within a year, which usually involves MAPK reactivation, commonly via BRAF amplification and mutations affecting NRAS or MEK2.<sup>57</sup> Gray et al analyzed the ctDNAs extracted from melanoma patients with progressive disease who initially responded to treatment with dabrafenib/trametinib.<sup>58</sup> They found that NRAS mutations, such as NRASQ61K and NRASQ61R, can be detected in ctDNAs.



In addition, the number of these mutations was consistently lower than that of the BRAF mutation, possibly owing to their origin from a subset of tumor cells. Daniele et al reported a case of CRC relapse after combined treatment with BRAF and MEK inhibitors, with a KRAS G12C mutation and an amplification of mutant BRAF V600E shown through ctDNA analysis. They systematically assessed the efficacy of several candidate therapies across a panel of resistant cell lines. The results revealed that most resistant cells remain sensitive to suppression of the MAPK pathway after combination of extracellularly regulated kinase, BRAF, and EGFR inhibitors.<sup>59</sup>

## Resistance To Other Therapies In Various Cancers

Patients with castration-resistant prostate cancer demonstrated improved clinical efficacy and OS following the application of abiraterone acetate and enzalutamide; however, the inevitable occurrence of resistance impedes long-term therapeutic benefit. Genomic profiling of ctDNAs collected from 65 patients with metastatic castration-resistant prostate cancer receiving enzalutamide showed that aberrations associated with primary resistance include androgen receptor (AR) amplification, multiple AR mutations, and RB1 loss. Acquired resistance can also be identified in ctDNAs, such as AR-L702H, AR-T878A, and catenin beta 1 mutations, and PI3K pathway alterations.<sup>60</sup> ctDNAs can also be used as an identifier for patients with breast cancer who have become resistant to endocrine therapies. The detection of estrogen receptor 1 mutations in ctDNAs correlates with resistance to aromatase inhibitors (AIs) and may point to the application of more efficient treatments in such patients.<sup>61</sup> Through utilization of a targeted ctDNA NGS test, mechanisms of resistance to ALK inhibitors (eg, kinase domain mutations, alternative oncogenic mutations, and amplifications) have been found in patients with NSCLC.<sup>62</sup> ctDNAs were detected in all patients with metastatic gastrointestinal stromal tumors, and their levels were associated with tumor size. The mutations resulting in resistance to treatment with imatinib clustered in the region of the adenosine triphosphate-binding pocket or the activation loop; both could be detected in ctDNAs.<sup>63</sup> Furthermore, a study of four patients who underwent resection of imatinib-resistant gastrointestinal stromal tumor identified secondary C-KIT exon 13 or 18 mutations in ctDNAs with a mutation fraction range of 0.010–9.385%.<sup>64</sup> Consequently, the application of liquid biopsy

is a promising option to comprehensively determine the mutational profiles of various heterogeneous tumors and guide therapeutic decisions in drug-resistant cancers.

## Clinical Trials Demonstrating The Role Of ctDNAs In Treatment Monitoring

Based on the great potential value of ctDNAs in demonstrating mutations underlying primary or acquired resistance, researchers worldwide have conducted clinical trials to prove the practicability of this approach. The phase I/II multi-cohort eXalt2 trial (NCT01625234) employed hybrid-capture NGS to analyze ctDNAs in plasma collected from 76 patients with ALK+ NSCLC. The results showed serial changes in ALK mutation allelic frequencies while receiving treatment with ensartinib, demonstrating the clinical utility of ctDNAs in serially tracking genetic determinants of resistance.<sup>65</sup> A prospective, multicancer, biomarker trial applied Safe-SeqS to analyze plasma ctDNAs in 42 patients with early stage operable pancreatic adenocarcinoma (Australia New Zealand Clinical Trials registry number ACTRN12612000763842). The analysis revealed a KRAS mutation in 23 patient samples pre-operatively. Although 18 patients received standard therapy, 19 of the 23 patients relapsed at a median of 38.4 months, probably due to resistance. The researchers suggested to explore the use of neoadjuvant therapy strategies in these patients rather than immediately proceeding to surgery.<sup>66</sup> Using plasma samples collected from 83 patients with advanced breast cancer, a prospective plasma DNA AI study (CCR3297, London-Bromley Research Ethics Committee, REC 10/H0808/50) also demonstrated the potential value of ctDNA analysis in describing the mutant genetics of drug-resistant disease. ddPCR and enhanced tagged-amplicon sequencing of ctDNA samples showed high levels of genetic heterogeneity, such as estrogen receptor 1 mutations and sub-clonal KRAS mutations, with cancers progressing after first-line treatment with AI. The genetic diversity of AI-resistant cancers shown by ctDNAs may limit the effectiveness of subsequent targeted therapy approaches which target only one of the clones.<sup>67</sup> Thus, ctDNA analysis is essential for establishing clinical therapeutic strategies, as well as tracing their levels and changes in genetic heterogeneity during treatment to adjust or combine medications.

## Conclusions

Surgical techniques and targeted drugs are rapidly developing. However, the prognosis of a proportion of patients

with cancer remains poor owing to the development of resistance to chemotherapeutic and targeted agents during long-term use. The primary or mutant selection-induced intratumoral and intertumoral heterogeneity renders the adjustment of the therapeutic schedule essential for improving the outcomes of patients and effectively prolonging their survival. Liquid biopsy, such as ctDNAs analysis, is likely to play a complementary role as a cancer biomarker to show the mutation status in tumor cells and guide clinical practice. As plasma is easy to collect and there is no need to enrich or isolate a rare cluster of cells, ctDNA analysis is more appealing for clinical application. However, it can only provide information regarding genomes and has a limitation in detecting mutations occurring in the RNA or protein levels. Thus, CTC analysis is also useful, as it can compensate for the shortcomings of ctDNAs. Improvements in sequencing techniques, such as NGS and other sequencing techniques, have promoted the study of ctDNAs and CTCs, and offer the possibility of clinical application in the future. Nonetheless, obstacles continue to hinder the extensive use of liquid biopsies. For example, the sensitivity and specificity of ctDNA or CTC detection warrant further verification. It is currently unclear whether all alterations in tumor cells contribute equally to changes in the composition of CTCs or ctDNAs in different stages of cancer. In addition, the concentration of ctDNAs and CTCs in plasma is low. Hence, the development of more specific techniques for their isolation and detection is necessary. Another problem that impedes widespread clinical application of this method is the relatively high cost of sequencing. A number of prospective clinical trials have been conducted to detect genetic heterogeneity in several common cancers. However, whether ctDNAs can detect mutations in rare cancers warrants further clinical investigation. In spite of these difficulties, we firmly believe that liquid biopsies hold great promise for future genetic studies concerned with monitoring mutations in tumor cells. This method has the capacity to conveniently and noninvasively offer a wealth of information over the course of treatment, which is not possible with traditional tissue biopsies. Future advances in technologies will make ctDNA and CTC analysis essential in precision medicine.

## Abbreviations

ADP, adenosine diphosphate; AF, allelic frequency; AI, aromatase inhibitor; ARMS, amplification refractory mutation system; CAPP-seq: Cancer Personalized Profiling by

deep Sequencing; CRC, colorectal cancer; CRPC, castration-resistant prostate cancer; CTC, circulating tumor cell; ctDNA, circulating tumor DNA; DREAMing, Discrimination of Rare EpiAlleles by Melt; ddPCR, digital droplet PCR; EGFR, epidermal growth factor receptor; HER2, human epidermal receptor 2; iDES, integrated digital error suppression; mCRC, metastatic colorectal cancer; MET, mesenchymal–epithelial transition; NGS, next-generation sequencing; NSCLC, non-small cell lung cancer; OS, overall survival; PAP-A, pyrophosphorolysis-activated polymerization allele-specific-amplification; PCR, polymerase chain reaction; PFS, progression-free survival; Safe-SeqS, Safe-Sequencing System; SiMSen-Seq, Simple, Multiplexed, PCR-based barcoding of DNA for Sensitive mutation detection using Sequencing; SNV, single-nucleotide variants; TAM-Seq, tagged amplicon deep sequencing; TKI, tyrosine kinase inhibitors; UID, unique identifier; WGS, whole-genome sequencing.

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## Disclosure

The authors have no conflicts of interest to declare.

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