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REVIEW ARTICLE

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The Current State and Future of Plasma Cell-Free DNA Analysis in Urologic Malignancies

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Genomic medicine based on comprehensive genomic profiling (CGP) has revolutionized cancer treatment. However, there are certain limitations to CGP based on tissue analysis. Liquid biopsy, particularly plasma cell-free DNA (cfDNA), has emerged as a less invasive source of information to complement tissue-based analysis. To use cfDNA analysis effectively in the clinical setting, it is important to know the characteristics and specific limitations of cfDNA analysis. Moreover, the utility of cfDNA testing differs between cancer types, which is not widely recognized. Furthermore, in addition to its use in CGP, there are broader applications for cfDNA testing, including its use in detecting minimal residual disease or even epigenomic profiling. In this review, we first describe the detailed characteristics of cfDNA and the limitations of cfDNA analysis, and then focus on the utility of cfDNA analysis in urologic malignancies.

Key Words: Liquid biopsy, Cell-free nucleic acids, Prostatic neoplasms, Carcinoma, Renal cell, Urinary bladder neoplasms, Ureteral neoplasms

INTRODUCTION

In 2023, cancer genome analysis is clinically available in many parts of the world. Instead of developing and administering drugs for each type of cancer, as in the past, cancer genome medicine, which treats cancer across cancer types based on genomic abnormalities, is now being practiced. Undoubtedly, precision medicine based on the cancer genome, in addition to morphological pathological diagnosis, will be further promoted in the future.

It has been revealed that cancer genomes dynamically change with treatment stress [1]. Considering the dynamic nature of cancer genomes, the most ideal cancer genomic medicine for advanced cancer following multiple lines of treatment would be biopsy of a metastatic lesion and selection of drugs based on genomic information from the tumor tissue. However, biopsy of metastatic lesions may be difficult due to the invasiveness of the procedure in some organs; and bone metastases require ethylenediaminetetraacetic acid decalcification to collect DNA suitable for cancer genome analysis, which may not ensure sufficient quality and quantity of DNA. Furthermore, there is heterogeneity in cancer genomes between metastases in heavily treated patients, and genetic information from a particular metastasis site may not reflect the most important alteration in the overall clinical picture [1-3]. For example, if a patient with prostate cancer maintains lymph node shrinkage with hormonal therapy but only bone metastases worsen, analysis of the cancer genome of metastatic lymph nodes may not identify the genomic abnormality that drives tumor progression at that time. If a

tissue sample cannot be obtained at the time of progression, an alternative would be to perform cancer genome analysis using a tissue sample from the time of the initial diagnosis of cancer. However, there are 2 major concerns for this approach. First, it is possible that the cancer genome at the time of progression may have changed from the cancer genome at the time of diagnosis. In prostate cancer, *BRCA1* and *BRCA2* mutations, for which the poly(ADP-ribose) polymerase (PARP) inhibitor olaparib is effective, are reported to be already present at the time of diagnosis, and acquisition of new alterations during treatment is rare, except when treated with PARP inhibitors. On the contrary, hormonal therapy, including androgen receptor (AR) pathway inhibitors, significantly alters androgen receptor (*AR*) [1, 4, 5]. In addition, some genes important for prostate cancer progression, such as *TP53* and *RB1*, accumulate new genomic alterations with progression. In the future, as the number of drugs targeting specific genomic alterations increases, the discrepancy between the cancer genome of the biopsied tissue at diagnosis and that at progression may become a clinical problem. The second concern is the degradation of DNA in formalin-fixed paraffin-embedded (FFPE) specimens over time [6]. In particular, the success rate of genomic analysis using large gene panels drops below 50% when FFPE specimens are stored for more than 3 years. Due to these combined factors, the success rate for the analysis of tissue specimens from 4,047 patients analyzed in the Phase III PROfound trial to test the effectiveness of olaparib in metastatic castration-resistant prostate cancer (mCRPC) was unsatisfactory at 69% [7].

Liquid biopsy is expected to complement a diagnosis using tissue specimens. Liquid biopsy examines components such as DNA, RNA, and proteins derived from tumors (or stromal cells around tumors) released in body fluids instead of conventional tissue diagnosis by tumor biopsy. Although cell-free DNA (cfDNA) is the only currently available liquid biopsy clinically, research is ongoing to develop other forms of liquid biopsy, such as circulating tumor cells, circulating free RNA, exosomes, microRNAs, and proteins. In addition to blood, all body fluids, including urine, ascites fluid, spinal fluid, and pancreatic fluid, are also sources for liquid biopsy. Urine is a valuable source of information, particularly in the field of urology, and research on the development of liquid biopsies for urine is vigorously pursued [8]. The common features of all liquid biopsies are that they are less invasive than tissue biopsies, can be repeated in a timely manner, and that they provide information not only on one metastasis, but also on all cancers in the body. In this review, we focus on the utility of plasma cfDNA analysis in urological malignancies.

cfDNA AND ctDNA

cfDNA is fragmented DNA from cells undergoing apoptosis or necrosis, and is released into body fluids; each fragment consists of approximately 170 bases, which is roughly the number of nucleotides constituting a mononucleosome. However, it has been reported that there is variation in the size of cfDNA depending on the organ of origin and tumor or nontumor status, attempts are being made to use the size of cfDNA as a biomarker [9]. Since cfDNA retains the methylation status of cells of origin, in addition to genomic analysis, epigenomic analysis is also possible [10]. However, since cfDNA is fragmented, it does not provide information on transcripts or splice variants. Additionally, although structural variations such as gene fusion can be detected at the DNA level, sensitive identification using cfDNA is difficult because each fragment is very short. This limits the usefulness of cfDNA analysis in tumor types, where fusion genes are more important drivers than gene mutations, such as sarcoma.

A challenge common to all liquid biopsies, including cfDNA, is the discrimination between the information derived from normal cells and cancer cells. cfDNA is released from all cells in the body. Especially in plasma, most of the cfDNA is derived from leukocytes (Fig. 1). Tumor-derived cfDNA is called circulating tumor DNA (ctDNA), and the ctDNA fraction is the percentage of ctDNA among all cfDNA in the plasma. The ctDNA fraction can be as high as 30% or more, or less than 1% [11]. Even when the ctDNA fraction is less than 1%, the ctDNA information is clinically relevant [12], and a highly sensitive analysis system is required to accurately detect such low-frequency mutations. The analysis of cfDNA distinguishes ctDNA from cfDNA derived from normal cells mainly based on single nucleotide mutations. In other words, cfDNAs with mutations that are not found in the germline or human

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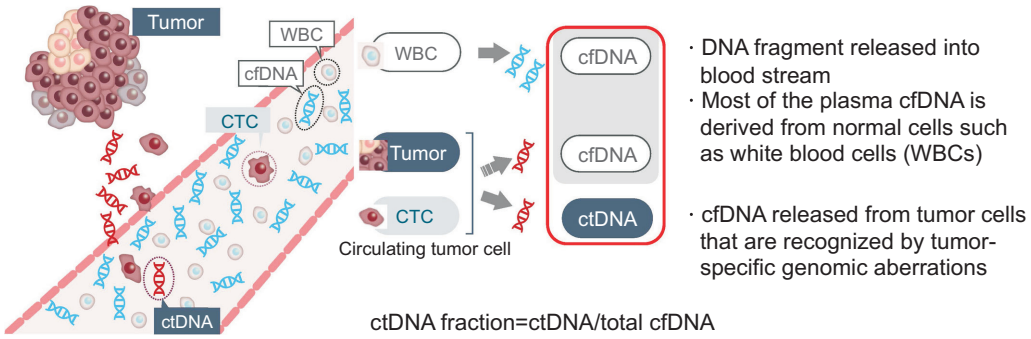

Fig. 1. Schema describing the differences between cell-free DNA (cfDNA) and circulating tumor DNA (ctDNA). CTC, circulating tumor cell.

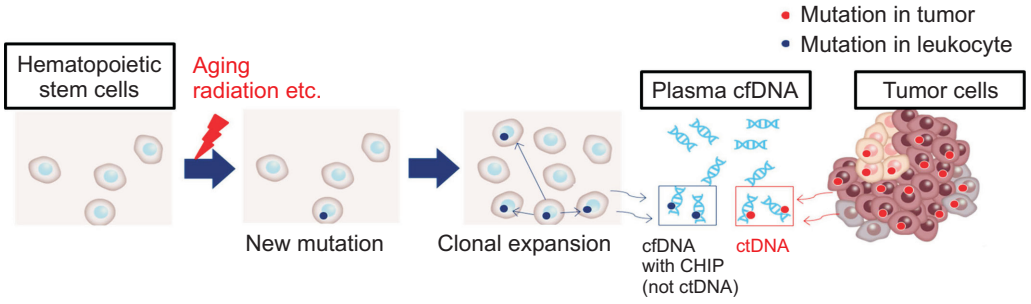

Fig. 2. Schema describing clonal hematopoiesis of indeterminate potential (CHIP). cfDNA, cell-free DNA; ctDNA, circulating tumor DNA.

reference genome are considered ctDNAs. However, it has recently become clear that there is a major pitfall in this discrimination. Traditionally, the germline gene sequence was thought to be inherited by all normal somatic cells and remains unchanged as long as the cells remain normal. In other words, except in patients with hematologic cancers, the leukocyte DNA sequence was considered equivalent to the germline gene sequence. Recently, it was found that normal hematopoietic cells also accumulate gene mutations due to external stimuli, such as aging and radiation, and clonally proliferate (clonal hematopoiesis of indeterminate potential, CHIP) [13]. Therefore, in the plasma of patients with cancer, there is a mixture of cfDNA derived from perfectly normal leukocytes reflecting germline gene sequences, leukocyte-derived DNA with CHIP mutations, and ctDNA (Fig. 2). Thus, the assumption that "mutated DNA"="DNA derived from cancer cells" is no longer valid. To distinguish between CHIP and cancer cell-derived DNA, both cfDNA and leukocyte DNA should be analyzed in the same patient. However, currently commercialized FoundationOne Liquid CDx (Foundation Medicine, Cambridge, MA, USA) and Guardant360 (Guardant Health, Redwood City, CA, USA) both identify ctDNA based on the reference genome. There are many cases where the identified gene mutation is not actually derived from cancer cells, but is CHIP. The frequency of CHIP increases with age [14], and genes such as *ASXL1*, *ATM*, *CBL*, *CHEK2*, *DNMT3A*, *JAK2*, *KMT2D*, *MPL*, *MYD88*, *SF3B1*, *TET2*, *TP53*, and *U2AF1* are known to be susceptible to CHIP. Since CHIP is known to be particularly prevalent among low-frequency mutations [15, 16], CHIP should be strongly suspected, especially when mutations in the above genes are detected at allele frequencies of 1% or less. Among the genes associated with homologous recombination repair that are relevant for the use of PARP inhibitors, in addition to *ATM* and *CHEK2*, CHIP has been reported to be also present in *BRCA1* and *BRCA2*. Therefore, failure to correctly identify CHIP can lead to inappropriate use of PARP inhibitors [12]. The development of commercial gene panels that analyze both cfDNA and leukocyte DNA from the same patient is awaited.

Copy number variations, such as gene amplification, can also be detected by cfDNA analysis. For example, *AR*, the

driver gene for prostate cancer, undergoes amplification at the DNA level in many cases where castration resistance is acquired, often resulting in a 10-fold or higher copy number. Since *AR* is on the X chromosome and there is only one copy per cell, if the ctDNA fraction is 10% and the *AR* copy number is 10-fold, the copy number in the cfDNA analysis is calculated as 1 copy×0.9+10 copies×0.1=1.9, indicating that the cfDNA from the amplified *AR* region has approximately doubled (Fig. 3). However, the detection of copy number loss is difficult unless the ctDNA fraction is high (at least 20%) [17]. For example, in normal cells, there are 2 copies of *PTEN*, a gene often lost in prostate cancer. Assuming diploid status, if the ctDNA fraction is 10% and *PTEN* is lost in both alleles, the copy number in the cfDNA analysis is calculated as 2 copies×0.9+0 copy×0.1=1.8, which means a small decrease from 2 to 1.8 needs to be sensitively detected, which is not possible using current standard methods. Therefore, although FoundationOne Liquid CDx reports abnormalities in gene copy number, in Japan, copy number reports of FoundationOne Liquid CDx have not been approved for use in companion diagnostics. Furthermore, the consistency of blood tumor mutation burden (TMB) with tissue TMB has not been verified, and TMB in FoundationOne Liquid CDx has not been approved in Japan as a companion diagnostic. Microsatellite instability (MSI) can be analyzed using cfDNA, depending on the design method of the gene panel [18]. However, since FoundationOne Liquid CDx was not designed for this purpose, MSI detected with FoundationOne Liquid CDx is also unapproved as a companion diagnostic tool in Japan. In tumors where TMB, MSI, gene copy number alterations, or gene fusions are clinically more frequent and relevant for drug selection, comprehensive genomic profiling (CGP) using tumor tissue is preferable to CGP using cfDNA.

In addition to its use in CGP, ctDNA can also be quantified and used as a surrogate of disease volume. ctDNA fraction or the variant allele frequecy of ctDNA harboring a specific mutation may be used to monitor treatment response during therapy, or to detect minimally residual disease (MRD). Recently, it has also been reported that ctDNA fraction can be a good prognostic marker in 4 major types of cancer with metastasis (prostate cancer, breast cancer, non-small-cell lung cancer, and colorectal cancer) [18]. Even in cases where the shedding of ctDNA is low, methylation status of cfDNA derived from both cancer cells and surrounding stromal cells may be informative as a biomarker, and is actively being explored in research [19]. The role of cfDNA analysis in the clinical setting is expected to further expand beyond mutation detection in the future [20].

UTILITY AND LIMITATIONS OF cfDNA ANALYSIS IN PROSTATE CANCER

Prostate cancer is the most studied urological cancer in terms of liquid biopsy analysis. This is because *AR* is the driver of progression and drug resistance in almost all cases, and because *AR* is a single copy gene, the analysis can be performed without considering the alternate allele. Another advantage is that hotspots of mutations associated with drug resistance are already known. Furthermore, many cases of metastatic prostate cancer have only bone metastases at the time of progression, and biopsy of metastases is technically difficult and highly invasive. Therefore, liquid biopsy is highly anticipated. Prostate cancer has a relatively high ctDNA fraction among urological cancers. It does not have as many passenger mutations as urothelial carcinoma, making it easier to target the gene mutations identified for treatment.

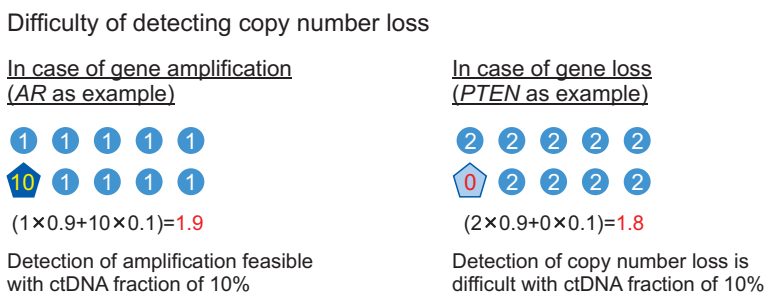

Fig. 3. Schema describing the difficulty of detecting copy number loss using cell-free DNA analysis. ctDNA, circulating tumor DNA.

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The reliability of cfDNA analysis has been demonstrated, for example, in the *post hoc* analysis of the PROfound study. In the PROfound study, the positive concordance rate for *BRCA1*, *BRCA2*, and *ATM* mutations was 81%, and the negative concordance rate was 92% when the results of tissue-based analysis using FoundationOne CDx and cfDNA based analysis by FoundationOne Liquid CDx results were compared [7]. In particular, the concordance rate for single nucleotide aberrations was 93% for nonsense variants, 87% for splice site variants, and 86% for frameshift variants, while the detection sensitivity of ctDNA was 63% for structural variants and 27% for copy number loss. In another *post hoc* analysis of the same study, among the cohort enrolled in the study with *BRCA1*, *BRCA2*, or *ATM* mutations, when the analysis was limited to patients who were also mutation positive using FoundationOne Liquid CDx, there were no differences in the hazard ratio for progression-free survival compared to the overall study group [21].

In cfDNA analysis, tumor volume can affect the detection of ctDNA, since the amount of ctDNA released in the bloodstream depends on tumor volume [22]. If the tumor volume is small, it may result in less ctDNA being released into the bloodstream, causing false-negative results. In fact, in the PROfound study, ctDNA was detected in 81% of all cases, and in approximately 20% of cases, ctDNA was undetectable. Furthermore, cfDNA has a short half-life in the blood, and the ctDNA fraction decreases rapidly after effective treatment. For example, in prostate cancer, the ctDNA fraction significantly decreases within 2 weeks after the initiation of hormonal therapy for untreated metastatic prostate cancer [23]. If cfDNA analysis is conducted in response to current therapy, or immediately after a drug change, the likelihood of false-negative results increases. However, ctDNA has been reported to be detected in 88% of cases of castration-resistant prostate cancer if cfDNA is collected immediately before a change of treatment, even in a cohort that included many cases before the first-line AR pathway inhibitor treatment for mCRPC [12], emphasizing the importance of analysis timing. Generally, CGP testing using tissue samples takes a relatively long time from test submission until the analysis results are reported. In some cases, test results cannot be reported because of problems with DNA quality or quantity. However, CGP testing using cfDNA shortens the time from test submission to reporting of analysis results, and there are few cases of test failure due to poor specimen quality [24]. It is therefore recommended to only perform the cfDNA test after confirming resistance to current therapy.

In Japan, regarding whether a tumor tissue-based test or a cfDNA test should be submitted as a CGP test, the Joint Task Force for the Promotion of Genomic Medicine of the Japanese Society of Clinical Oncology, the Japanese Cancer Association and the Japanese Society of Medical Oncology issued a "Policy Recommendation on the Proper Use of Cancer Genome Profiling Tests Using Circulating Tumor DNA in Blood" [25]. Among all solid tumors, prostate cancer is one of the tumor types for which cfDNA testing is the most clinically useful because (1) there are many cases with only bone metastasis progression; (2) the course of treatment is relatively long, and in many cases, more than 3 years have passed since the initial diagnostic biopsy when a CGP test is performed; (3) currently, fusion genes, TMB, and MSI are relatively less important in prostate cancer for the determination of drug use; (4) the detection rate of ctDNA in large-scale clinical trials is high, and the concordance rate with tumor tissue tests is also high [7, 26]. Alternatively, as mentioned above, cfDNA analysis cannot detect biallelic loss of *BRCA2* due to the low sensitivity of copy number analysis. Therefore, CGP using tumor tissue should be prioritized in cases where good quality DNA can be extracted from FFPE within 3 years of the initial biopsy, or in cases where metastatic sites can be biopsied relatively easily, such as liver or lymph node metastases.

In addition to CGP, cfDNA analysis has been applied for the early diagnosis and detection of MRD in other types of cancer [27]. However, the barrier to clinical application in these areas is high in prostate cancer, because prostate-specific antigen (PSA), a remarkably sensitive and inexpensive biomarker, is already available. There is also growing interest in the use of cfDNA epigenomic markers to diagnose neuroendocrine prostate cancer (NEPC), since a large shift in the epigenome occurs upon transdifferentiation from adenocarcinoma to NEPC [28, 29]. If the feasibility of this approach is confirmed, it would allow the early diagnosis of NEPC without performing a metastatic biopsy.

UTILITY AND LIMITATIONS OF cfDNA ANALYSIS IN UROTHELIAL CARCINOMA

Urothelial carcinoma is one of the most frequently mutated solid tumors [30, 31], and the concordance between tissue-based mutation analysis and cfDNA analysis is relatively high [32]. However, most of them are passenger mutations, and *FGFR3* is the only driver gene that can be targeted. Additionally, some upper urinary tract urothelial cancers are associated with Lynch syndrome and are MSI-high. However, since immune checkpoint inhibitors are approved for advanced urothelial cancers regardless of genomic abnormalities, the likelihood that CGP testing will lead to new treatments based on genomic abnormalities is much lower than for prostate cancer. In contrast, urothelial carcinoma does not have a sensitive biomarker like PSA, making the early diagnosis of recurrence and disease follow-up difficult. Tissue samples are easily obtained during transurethral surgery, cystectomy, or nephroureterectomy for urothelial carcinoma. If the genetic mutations of individual patients can be listed in advance from the sequencing of the tissue samples, and ctDNA can be detected using them as indicators, it will be possible to detect MRD and diagnose recurrence at an early stage. Christensen et al. [33] first extracted patient-specific genetic mutations by whole exon sequencing of tumor tissue in 68 patients with nonmetastatic muscle invasive bladder carcinoma, and then identified 16 patient-specific mutations for each patient. The usefulness of MRD detection using cfDNA was examined by constructing individualized gene panels consisting of 16 mutations per patient and analyzing them by ultradeep sequencing (105,000 × : mutations with allele frequencies of >0.01% can be detected) before and after preoperative adjuvant chemotherapy, before surgery, and periodically after surgery [33]. The results showed that patients who were ctDNA positive before preoperative chemotherapy, before total cystectomy, and after total cystectomy had significantly shorter progression-free survival and overall survival than patients who were ctDNA-negative. In particular, the presence or absence of postoperative ctDNA detection was the strongest predictor of recurrence-free survival in multivariate analysis. Additionally, 85% of patients who were ctDNA positive before chemotherapy but no longer had detectable ctDNA after chemotherapy showed pathological downstaging, while none of the patients in whom ctDNA was still detectable after chemotherapy showed downstaging. Furthermore, postoperative temporal analysis of ctDNA could identify tumor recurrence with 100% sensitivity and 98% specificity, and could detect recurrence approximately 3 months earlier than tumor recurrence identified by imaging studies. In muscle invasive bladder cancer, a 3-month delay in treatment can lead to disease progression and affect the outcome. Therefore, the detection of MRD by ctDNA may improve the outcome of muscle invasive bladder cancer treatment. IMvigor010, a phase III trial of adjuvant atezolizumab in muscle invasive urothelial carcinoma, did not show an improvement in disease-free survival in the general population [32]. However, in this study, a postoperative analysis of cfDNA was conducted using a 16-gene mini-panel generated for each patient based on tumor tissue sequencing data. When stratified by MRD status based on ctDNA, postoperative adjuvant atezolizumab therapy extended progression-free survival in patients who were MRD positive, while no differences were observed in patients who were MRD negative [33]. A new phase III trial (IMvigor011: NCT04660344) is currently ongoing to assess the benefit of postoperative adjuvant atezolizumab in patients who are MRD positive for ctDNA. If the results of the study are positive, it could be a game-changer for treating muscle invasive bladder cancer.

UTILITY AND LIMITATIONS OF cfDNA ANALYSIS IN RENAL CARCINOMA

In contrast to urothelial carcinoma, renal cell carcinoma (RCC) is one of the cancer types that releases the least amount of ctDNA among all cancer types [30]. It is still unclear whether this is due to low ctDNA release or if ctDNA is diluted by cfDNA from the rich stromal components of RCC. Similarly to *AR* in prostate cancer, *VHL* is the main driver of clear cell renal carcinoma, and more than half of cases have *VHL* aberrations. Therefore, detection of ctDNA based on *VHL* mutations could be a useful biomarker. However, a study has shown that even though *VHL* mutations were detected in 71.8% of tumor tissues, the same mutation was detected in cfDNA in only 25% of

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cases in the same patient using an assay system that can accurately detect allele frequencies down to 0.1%, indicating that the agreement between tumor tissues and the results of the cfDNA analysis was disappointingly low [34]. Several reports by various authors have discussed the usefulness of cfDNA analysis in renal cancer. For example, in cases where ctDNA was detectable, ctDNA amount (the allele frequency of ctDNA with specific mutation) was informative to track treatment response to tyrosine kinase inhibitors [34] or immune checkpoint inhibitors [35, 36]. However, in most studies, the concordance rate with tumor tissue for *VHL* mutations was as low as 30% [37-39], suggesting that the usefulness of cfDNA analysis for RCC using genetic mutations as markers is limited, at least with the current detection sensitivity. Further improvement in ctDNA detection method is necessary for clinical implementation. Alternatively, as mentioned previously, cfDNA also allows epigenomic analysis. Nuzzo et al. [19] developed a method to specifically immunoprecipitate methylated cfNA for comprehensive analysis (cfMeDIP-seq). Using this method, the group identified methylation patterns specific to RCC. The methylation information in this case included not only cancer cells, but also cfDNA derived from the surrounding stromal cells. With the model based on the methylation pattern, the authors could diagnose RCC with a high accuracy of area under the curve (AUC) 0.99 for blood cfDNA and AUC 0.85 for urine cfDNA. Although further validation is required, if the clinical application of this detection system is realized, it will become the first highly accurate diagnostic marker for RCC.

SUMMARY

Although liquid biopsy using cfDNA has been clinically implemented as a CGP test, its utility is not limited to CGP. The clinical utility of cfDNA analysis is expected to increase with the development of more sensitive analytical methods and advances in epigenomic analysis.

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