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Review

Circulating tumor DNA as a new class of tumor biomarker

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Abstract

Accumulation of somatic mutations in genomic DNA is nearly exclusive to cancer cells. During proliferation, a fraction of cancer cells die and release genomic DNA fragments. These released genomic DNA fragments, termed circulating tumor DNA (ctDNA), can be found in blood. ctDNA has potential uses in cancer diagnostics, particularly since the emergence of "massively parallel" next generation sequencing (NGS). However, the detection limit of NGSs was recently shown to be far higher than the actual concentration of ctDNA. To quantify ctDNA as a tumor marker, digital PCR (dPCR) is required to detect very low (i.e., $<1\%$) concentrations of somatic mutations. We have designed a dPCR primer/probe library specific for cancer mutations that were selected from public databases containing 12.5 million entries. Our newly developed system using this library, the Off-The-Shelf (OTS)-Assay, can measure as little as 0.01% of ctDNA, which allows highly sensitive post-treatment cancer surveillance for cancer patients. The high sensitivity of the OTS-Assay may change approaches for measurement of residual cancer during and after treatment.

Key words : ctDNA, digital PCR, residual tumor, post-treatment

I. Introduction

Tumor burden is the ultimate indicator of the success of cancer therapy. To measure the tumor burden of a patient, serum tumor markers and imaging technologies have been used in clinical practice. Serum tumor markers have been widely built using the default practice "clinical pathway" in hospitals, but the main purpose of these measurements is not necessarily precise tracking of tumor burden. Instead, such measurements can define the baseline for each patient so as not to miss patients whose serum tumor marker level is extremely high. Imaging modalities, such as CT scanning, are perhaps the most essential tool to direct therapeutic strategies. Currently, diagnosis of post-treatment relapse of solid tumors can only be made after confirmation by imaging modalities. In daily practice, a lesion having 3-10 mm diameter at the relapse site is generally required for detection. In addition, frequent monitoring can be challenging since radiation exposure

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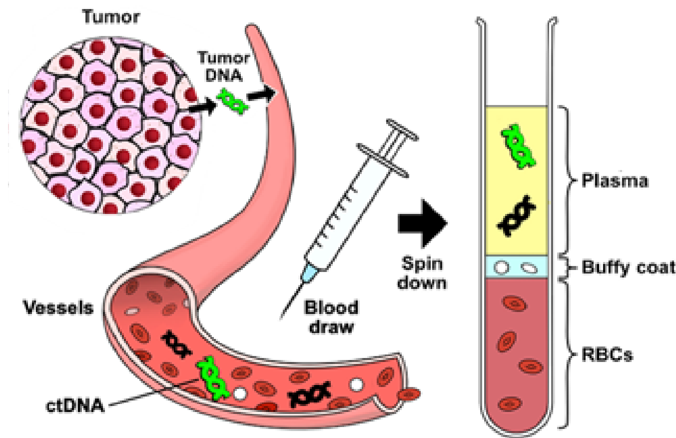

Fig. 1. Circulating tumor DNA. Cancer cells release short DNA fragments into the blood stream during turnover. The short DNA fragments are present in the plasma fraction. Most DNA fragments in plasma originated from normal cells. Hence, cancer cell-derived DNA fragments are distinguished from normal cells by the presence of somatic mutations.

accompanying imaging is unavoidable and the preparation for examination in terms of the administrative, economic, and physical burden, is much more demanding than that for a simple blood draw.

Although the significance of serum tumor markers and imaging modalities has long been accepted in practice, previous studies have reported that "early detection" using these modalities may not improve post-treatment prognosis for colorectal cancers [1, 2]. PSA has been used for broad prostate cancer screening, and is considered to be an exceptionally useful marker with respect to early detection in healthy individuals [3]. However, the actual clinical utility of PSA screening is still a matter of debate, particularly for younger individuals [4]. Many other cancer types do not have significant serum tumor markers. Thus, there is a demand for a new class of tumor marker, not necessarily to replace current modalities, but to complement current tools by providing precise and quantitative, yet affordable, monitoring for cancer treatment.

In this review we introduce our current approach to provide a new class of tumor markers and strategies for their clinical implementation.

II. Circulating tumor DNA

Recent studies have established that short DNA fragments, termed circulating tumor DNA (ctDNA), with somatic mutations are released from cancer cells and circulate in the blood stream (Fig. 1). The existence of DNA fragments in blood was first reported in 1948 [5]. Although few studies concerning these fragments appeared in the ensuing 20 years, the association between the concentration of DNA fragments circulating in the blood stream and tumor burden in the body was noted in the 1970s [6]. Interestingly, there was no DNA amplification process involved in the detection of such DNA, which could be quantified at nanogram levels in serum from cancer patients using a radioimmunoassay. These assays demonstrated that circulating DNA levels decreased after radiation therapy.

Table 1. DNA measuring methods

Method	Device	Gene throughput	Limit of detection (VAF*)	TAT**	Frequent monitoring ctDNA
Sanger sequence	Capillary sequencer	Low	> 10%	2-3 days	Yes
Exome sequence	NGS	High	5%	> 1 week	No
Target sequence	NGS	Medium	1%	>1 week	No
Quantitative PCR	Dedicated system	Low	1%	2-3 days	Yes
BEAMing	FCM***	Medium	0.01%	> 1 week	No
Digital PCR	Dedicated system	Low	0.01%	2-3 days	Yes

*, variant allele frequency; **, turn around time; ***, flowcytometer.

One of the most recent advances in measurement of ctDNA involves a next generation sequencer (NGS) that enables quantitative and genome-level profiling through "massively parallel" sequencing [7, 8]. The potential of ctDNA to act as a surrogate for tissue genome profiling, companion diagnostics, assessment of tumor genetic heterogeneity, and possibly minimum residual disease detection has been demonstrated. However, the clinical significance of extremely low levels of ctDNA (i.e., <1% of variant allele frequency (VAF)), most of which are below the detection limit of ordinary methods to measure DNA, including NGS, is unclear (Table 1).

III. Clinical significance of ctDNA

Over the last several years, ctDNA levels were reported to be surrogates for serum tumor markers or imaging with a "lead-time" of clinical findings such as relapse [9, 10]. In our previous research, we also reported that ctDNA levels can be useful for evaluating factors associated with clinical validity including: (i) early relapse prediction; (ii) treatment efficacy evaluation; and (iii) no relapse corroboration [11, 12]. One notable example was for a patient with esophageal squamous cell carcinoma, for whom relapse was noted through elevation of ctDNA levels around 6 months in advance of radiographic confirmation. Upon treatment of this patient, the ctDNA levels immediately responded (Fig. 2). In addition, the dynamics of ctDNA level assessed in pre- and post-treatment blood samples revealed that patients whose ctDNA level decreased showed significantly better survival, which supports the clinical utility of measuring ctDNA levels [13].

To use ctDNA as a tumor marker, effects on tumor genetic mutational heterogeneity should be clearly defined [14-17]. Our previous phylogenetic analysis in gastric and colorectal cancers using multiregional sequencing revealed that both types of tumors developed through cell proliferation when genetic alterations accumulated to produce multiple clones in a single tumor [12, 18]. Here, the founder mutations can be defined as those that are commonly present in the "trunk" of the cancer evolutionary tree [19]. Using multiregional sequencing, founder mutations can be identified in the majority of specimens (Fig. 3). Importantly, we found that the founder mutations tend to have high VAFs

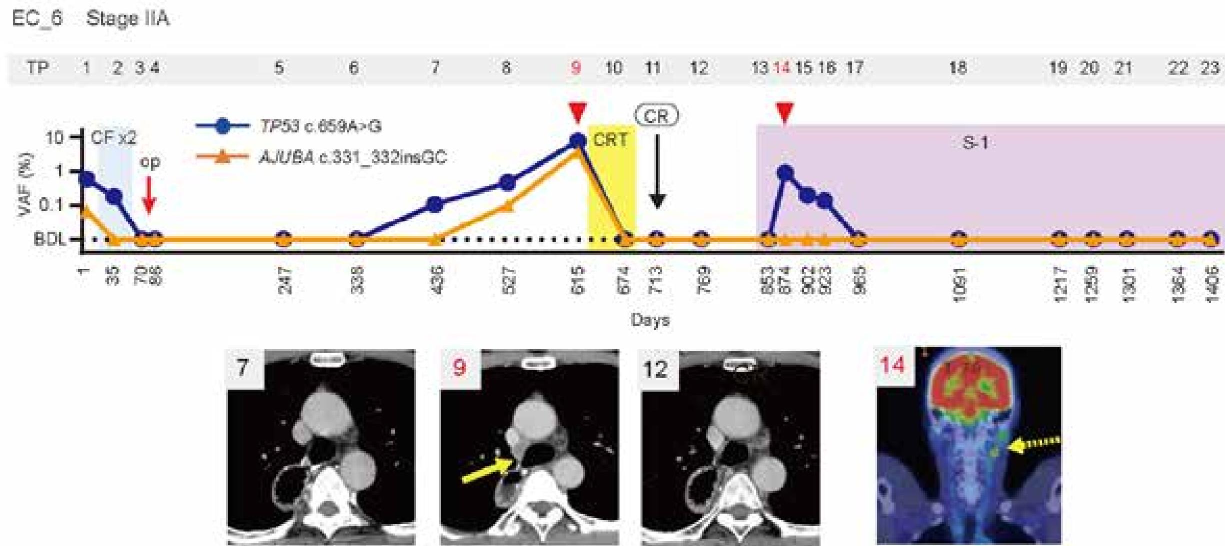

Fig. 2. Tumor burden monitoring using ctDNA for an esophageal cancer patient. Two somatic mutations that were identified from the primary lesion were quantified from plasma DNA. Pre-treatment VAF of ctDNA was approximately 1% for the TP53 mutation. This fraction dropped immediately after surgery, and began increasing nearly 10 months before diagnosis of radiographic relapse (red arrowhead). TP, time point; BDL, below detection limit; CF, cisplatin and 5-fluorouracil; CRT, chemoradiotherapy; CR, complete response. Red numbers indicate the timing of relapse diagnosis by CT scan. Yellow arrows indicate lesions considered to be relapse. Permission to reproduce the abovementioned display items was obtained from Elsevier [11].

among mutations identified in the tumor [12, 18]. In addition, a relatively high VAF mutation in a tumor tissue tended to increase the likelihood of detection in blood compared to samples that had lower VAF mutations. Therefore, for clinical application of ctDNA monitoring as a tumor marker, mutations with high VAF are the most practical candidate for ctDNA monitoring, even from a single biopsy, with the exception of hypermutated tumors [12].

IV. Digital PCR

To quantify minute amounts of DNA in complex DNA mixtures, digital PCR (dPCR) technologies were developed in the late 1990s and early 2000s that count PCR products in an absolute manner and require no reference sample [20]. Although dPCR was a very sophisticated technology, it was not frequently used because many users did not intuitively recognize the intrinsic difference between such digitized (i.e., binary) technologies and ordinary PCR technologies. dPCR can be defined as PCR with an output that has binary units associated with identifying the presence of PCR products as single reaction units with either droplets or microwells [20-24]. In general, the number of reaction units ranges from a few to several thousand and is roughly equal to the genome copy number of the default amount of DNA template. For example, 1 ng genomic DNA has a genome copy number of around 333. Thus, around 3 ng genomic DNA is required to quantify mutations with 0.1% VAF. We have attempted to use dPCR for tumor burden monitoring from periodic blood

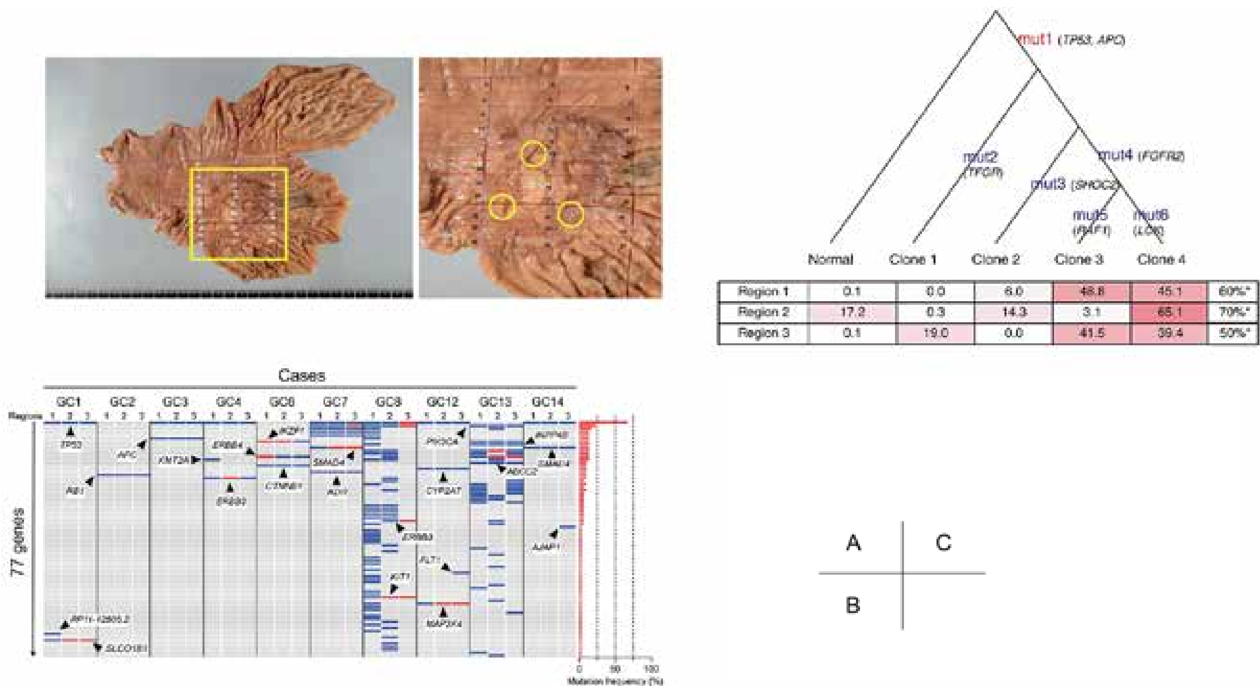

Fig. 3. Multiregional sequencing for phylogenetic analysis. (A) Samples for panel sequencing were acquired from multiple arbitrary regions of a gastric cancer tumor. (B) Multiregional sequencing results displayed in a colormap. Each tumor (GC) at the top has three sequencing results from three samples within the tumor. Blue and red indicate mutations identified by NGS and dPCR, respectively. (C) Phylogenetic simulation of a colorectal cancer. Samples from three regions are considered with several different clones that originated from one normal cell and contain gene mutations that accumulated during tumor development. Here, TP53 and APC mutations are considered to be "truncal" whereas others are "non-truncal". Display items in this figure were used under a CC-BY license [12, 18].

samples. Although the cost per dPCR run was affordable, it was nonetheless unrealistic for immediate individual quantitative measurements because each assay requires an individually-designed probe for specific tumor mutations.

The key component of successful dPCR is the "probe" , a simple oligonucleotide that has a base sequence that carries the mutation of interest. Probes can be synthesized within several weeks, followed by a quality validation process. Moreover, since there are very few commercially available probe "libraries", it is not convenient for clinical practices treating cancers that have a broad range of individual tumor mutations. We have designed and synthesized a knowledge-based dPCR probe library called Off-The-Shelf (OTS)-Probes, which is extracted from sequences in the publicly available database COSMIC that contains 12.5 million mutations (https://cancer.sanger.ac.uk/cosmic). This library allows immediate ctDNA monitoring once mutations are identified in primary tissue or pretreatment blood. We have evaluated the quality of OTS-Probes using publicly-available cancer mutation databases as well as in our ongoing clinical study, the MORIOKA study

(Hiraki et al., unpublished).

From previous studies we demonstrated that the VAF of majority ctDNA is quantified at levels that are too low to allow quantitation by NGS [11, 12, 18, 25]. However, ctDNA VAF dynamics have been demonstrated to be associated with clinical outcomes during treatment [26-29]. Although some variations have been introduced, dPCR could, in principle, be designed to detect a small fraction of DNA in a mixed population of DNAs [20-24]. Thus, ctDNA could be a new class of tumor biomarker for use in daily practice if appropriate dPCR probes are readily available in a library.

V. Limit of detection for clinical significance

One challenge for ctDNA monitoring is the quantification of ctDNA. Many instruments for genomic analysis are designed to measure DNA (Table 1). In principle, sensitivity for a variation that comprises 50% of a given DNA sample would be sufficient for germline genotyping. However, the fraction of somatic mutations from cancer cells often does not exceed 50%. For ctDNA monitoring, screening somatic mutations from cancer tissue or pre-treatment plasma is needed. The screening method is not necessarily whole genome sequencing, but should be, at least to some extent, exploratory (i.e., target sequencing). For most cases, the ctDNA VAF is less than 1%. Since the intrinsic detection limit of typical NGS is 1-5%, other methods are needed for ctDNA monitoring. We have confirmed in > 1,000 plasma samples that the majority have ctDNA VAF below 1%, even in patients with advanced cancer (Hiraki et al, unpublished data).

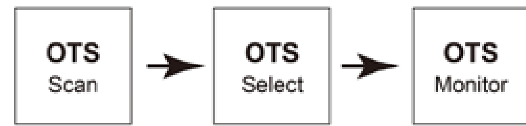

Fig. 4. The OTS-Assay.
OTS-Scan is for mutation screening for tumor tissues or pre-treatment plasma DNA. OTS-Select is for mutation selection based on the mutation profile determined from OTS-Scan. OTS-Monitor is for mutation monitoring as ctDNA using dPCR. dPCR probes were picked from OTS-Probes.

Currently-available high market-share NGS technologies, including the semiconductor type and bridge-PCR type provided by Thermo Fisher Scientific and Illumina, respectively, are designed for "massively parallel" sequencing to yield sequencing results optimally from "many" genes [7, 8]. In contrast, to quantify rare mutations from ctDNA, a sequencing depth of over 3,000 is required (i.e., 0.03% VAF). As of December 2021, little substantial progress has been made in terms of the intrinsic detection limit of NGS. Overall, ctDNA measurement is a challenging assay in which the majority of target materials have <1% VAFs, which can be detected using the OTS-Assay. The OTS-Assay is comprised of the following three modules: OTS-Scan for somatic mutation screening using an NGS panel, OTS-Select for assigning appropriate mutations for ctDNA monitoring, and OTS-Monitor for quantifying ctDNA VAF using dPCR over time course (Fig. 4).

VI. Clinical validity and clinical utility

Regardless of marker type, clinical assays that examine genetic variants in ctDNA should assess clinical validity and utility.

The definitions of clinical validity and utility are slightly confusing since these terms can be used interchangeably. A review by the American Society of Clinical Oncology and the College of American Pathologists in 2018 that focused on ctDNA analysis in patients with cancer stated that clinical validity is defined as the "ability of an assay to divide, with statistical significance, one population into two or more groups on the basis of outcomes, such as presence of cancer or treatment response" whereas clinical utility is defined as the "ability to demonstrate, with statistical significance, improvement in the diagnosis, treatment, management, or prevention of cancer, with the use of the assay compared with not using the assay" [30]. In short, markers that have clinical validity would allow classification of patients into reasonable categories, whereas markers that have clinical utility must be able to predict better/worse survival. These definitions would seem to apply for a one-time ctDNA assay using NGS or quantitative PCR for target variant identification. Meanwhile, the OTS-Assay provides quantified dynamics of ctDNA that can reflect tumor burden in response to treatment.

In a previous study in which the originally developed OTS-Assay system was used for the first time, we found that the clinical validity for longitudinal measurement of ctDNA was 91% and 83% for esophageal and colorectal cancer, respectively [11, 12, 18]. As shown in Fig. 4, this assay flow does not intend to identify "actionable" mutations. Instead, we focused exclusively on patients who received treatment for relatively advanced stage cancers or for tumor burden monitoring during and after treatment.

VII. Implementation for daily clinical practice

One of the ultimate goals of tumor biomarker development is to reduce the number of cancer deaths. To achieve this goal, academic research activity should set a goal for real-world implementation. At the beginning, the assay technology should be patented and then licensed to commercial entities. Although there have been very few commercially successful bioventures in Japan, commercialization is a meaningful goal even for academic researchers. Commercialization may be particularly important when the research institution at which an assay was developed has limited intramural resources to support high quality research activities. Furthermore, commercialization would prompt patients to access new modalities, although the responsible researchers/organizations should prepare towards the final approval by authorities of the technology.

VIII. Conclusions

We demonstrated that our newly-developed cancer tumor marker assay system, OTS-Assay, can contribute to the ultimate goal of reducing cancer deaths through implementation and management of technology.

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All studies were conducted according to Helsinki Declaration principles.

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新しい腫瘍マーカーとしての腫瘍由来循環 DNA

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要旨

体細胞変異の蓄積は，ほぼがん細胞に特有の現象である．がん細胞増殖中の細胞死により細胞外に放出されたゲノム DNA は腫瘍由来循環 DNA（ctDNA）として知られている．「大量並列」処理を行う次世代シークエンサー（NGS）の登場以来、ctDNA の利便性ががん診断において示されている．しかしながら，ctDNA の血中濃度は NGS の検出限界（1-5%）よりも低いことが明らかになってきた．したがって，ctDNA 定量には超低濃度（1% 以下）の体細胞変異検出用のデジタル PCR（dPCR）が必要となる．我々は，公共データベースの 1250 万以上の登録変異から，がん特有の変異に対する dPCR プローブライブラリーを開発した．このライブラリーを用いる Off-The-Shelf (OTS)-アッセイでは，0.01% までの ctDNA を計測し再発に関する緻密な情報を提供できる．OTS- アッセイにより治療中再発の計測における既成概念が変化する可能性がある．