

A digital PCR primer/probe library for physical tumor burden assessment through tumor-informed longitudinal ctDNA monitoring

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Abstract

Circulating tumor DNA (ctDNA) monitoring is informative for longitudinal physical tumor burden (PTB) assessment. However, NGS-based ctDNA monitoring is limited by sensitivity, cost, and turnaround time in clinical practice. We developed OTS-Probes, an off-the-shelf digital PCR (dPCR) primer/probe library prepared for more than 1,000 frequently registered somatic mutations. Oligonucleotide sequences with chemically modified bases facilitate to produce approximately 80 bp amplicons for fragmented plasma DNA. Based on the OTS-Select algorithm from an NGS report, 1–4 mutations are readily selected from OTS-Probes for ctDNA monitoring. OTS-Probes provide a sensitive, frequent, and readily PTB assessment for dPCR-based longitudinal ctDNA monitoring.

Background

Quantitative physical tumor burden (PTB) dynamics of a cancer patient is the ultimate indicator for treatment navigation and diagnostics. While serum tumor markers support tumor burden monitoring, their performance remains limited (1). Recent studies suggest circulating tumor DNA (ctDNA) as a biomarker for treatment response and prognostic evaluation using next generation sequencing (NGS)-driven qualitative data (2–4). However, this approach is limited for stratification, whereas quantitative, periodic, and longitudinal tumor burden monitoring is most demanded in daily practice. To date, no standard quantitative ctDNA method is established because the variant allele frequency (VAF) of ctDNA in blood is typically < 1%, which still reflects most clinical events (2, 3, 5). For practical use, such as minimal residual disease (MRD), a part of PTB for those received surgery with curative intent, detection, the ideal method should be sufficiently sensitive, manageable, and cost-effective in addition to having adequate quantitative function.

Digital PCR (dPCR) offers excellent sensitivity by separating PCR products into single reaction units, thus enabling binary counting at the single-nucleotide level (6, 7). The initial concept of dPCR was proposed by Vogelstein and Kinzler, whereas the detectable variants have fully depended on how variants were selected (8). Currently, despite several semi-automated dPCR platforms on the market (9, 10), a major limitation remains: the lack of ready-to-use primer/probe sets (P/P) for designated nucleotide changes. In oncology, ctDNA applications require a ready-to-use preparation for prompt turn-around-time (TAT). Moreover, since ctDNA in blood is fragmented, a short amplicon design is critical for PCR success (11). For prompt TAT, the P/P should be supplied with prior validation and optimization of dPCR conditions. Overcoming these requirements will help establish sensitive, affordable, and secure dPCR assay for ctDNA monitoring (12, 13). With broad, ready-to-use P/P coverage, ctDNA assays could be fully applicable for frequent and longitudinal tumor burden monitoring.

In this report, we propose a ready-to-use P/P library dedicated for dPCR, called Off-The-Shelf (OTS)-Probes, which targets more than 1000 frequently-found somatic mutations in ctDNA of human cancer. For PTB assessment by tumor-informed ctDNA monitoring, somatic mutations that are suitable for ctDNA monitoring need to be selected from the tumor sequencing data for each patient (14). To

facilitate the somatic mutation selection process, an originally developed algorithm is used for designated sequencing panel reports. In addition, efficient mutation assessment as well as potential clinical usefulness are demonstrated.

Materials and Methods

Mutation curation for OTS-1000ex

To provide essential probes for the rapid monitoring of ctDNA with the dPCR technique, OTS-Probes was designed to detect more than 1,000 mutations in human cancer (Dataset S1, Fig S1). The P/P sets were selected using an originally developed algorithm (Fig. S2) with the Catalogue Of Somatic Mutations In Cancer (COSMIC; <https://cancer.sanger.ac.uk/cosmic>) and The Cancer Genome Atlas (TCGA; <https://portal.gdc.cancer.gov/>). Tohoku Medical Megabank Organization (ToMMo) and the Genome Aggregation Database (gnomAD; <https://gnomad.broadinstitute.org>) were searched through the Japanese Multi Omics Reference Panel (jMorp; <https://jmorp.megabank.tohoku.ac.jp>) website, and the Database of Single Nucleotide Polymorphisms (dbSNP; <https://www.ncbi.nlm.nih.gov/snp/>) was also used to exclude germline mutations. Mutations whose translated DNA sequences were undefined or had less than 0.01 allele frequency in at least one database were removed.

Dataset

To evaluate the coverage of OTS-Probes in human cancer specimens, TCGA, the Center for Cancer Genomics and Advanced Therapeutics (C-CAT) and the Monitoring Recurrence of Individual tumor by serial Observation of Known gene Alterations (MORIOKA study) datasets were used (Fig. S3, Dataset S2). TCGA is an international landmark cancer genomic program in the U.S., and C-CAT is a Japanese cancer genome profiling database. The MORIOKA study is an observational study in which monitoring ctDNA dynamics in pan-cancer patients was conducted at the Iwate Medical University from 2019 to 2023. A list of OTS-1000ex were matched up with each database and the unique case numbers were counted.

Digital-PCR

In the QX200 Droplet Digital PCR System (Bio-Rad Laboratories, Hercules, CA, USA), the DG8 Cartridge (#1864008), DG8 gasket (#1863009), and Droplet Generator oil for Probes (#1863005) were used for droplet generation. In the QuantStudio 3D Digital Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA), the QuantStudio 3D Digital PCR Master Mix v2 (A26358) and QuantStudio 3D Digital PCR 20K Chip Kit v2 (A26316) were used. In the Applied Biosystems QuantStudio Absolute Q Digital-PCR System (Thermo Fisher Scientific), the Absolute Q DNA Digital PCR Master Mix (5X) (#A52490) or QuantStudio 3D Digital PCR Master Mix v2 (#A26358), QuantStudio Absolute Q MAP16 Plate Kit (#A52732), and QuantStudio Absolute Q Isolation Buffer (#A52730) were used for sample preparation. In

the QIAcuity Digital PCR system (QIAGEN, Hilden, Germany), the Nanoplate 26K 24-well plate (#250031) and QIAcuity Probe PCR Kit (#250102) were used. In the Crystal Digital PCR Naica system (Stilla Technologies, Villejuif, France), the Sapphire Chip (#SU0004), Naica PCR MIX 10X (#SU0011), and Naica IQ/OQ Kit (#SU0012) were used. In the Digital LightCycler (Roche Diagnostics, Rotkreuz, Switzerland), the Digital LightCycler Universal Nanowell Plate (#518-401955) and Digital LightCycler 5 x DNA Master (#518-401924) were used. In all dPCR systems, 10X OTS-Probes were used. The OTS-Probes include either 900 or 1800 nM of forward and reverse primer sets. Probes were adjusted to 250 nM in the final PCR solution. The annealing temperature was optimized for 97.3% of the OTS-Probes at 60°C, whereas the range of annealing temperature for the rest of the 2.7% samples was 56°C to 64°C (Table S1). The synthesized P/P validation was performed by the arbitrary cutoff that divides the scatter plot into the following four quadrants: (i) double positive of wild-type (wt) and mutant-type (mt) signals; (ii) single positive of mt; (iii) double negative of wt and mt signals; and (iv) single positive of wt signal. For dPCR analysis, the double positive dots of mt and wt were excluded to reduce mt false positives. Thus, VAF% were simply calculated by the following formula in all dPCR systems:

$$VAF(\%) = \frac{mt}{mt + wt} \times 100$$

Plasma and PBMC collection and DNA extraction

For whole blood collection, Cell-free DNA Blood Collection Tubes (Streck, La Vista, NE, USA) were used. The collected whole blood was stored at room temperature for up to seven days. Approximately 8 mL of blood was centrifuged at 1800 x g for 20 min using a swing-out rotor (Kubota, Tokyo, Japan). Subsequently, 4–5 mL of the plasma layer was transferred to a new 15 mL tube and centrifuged under the same conditions. The resulting supernatant was then carefully transferred to a cryotube and stored at – 80°C until DNA extraction. The QIAamp Circulating Nucleic Acid Kit (QIAGEN) was used for cell-free DNA extraction. To isolate peripheral blood mononuclear cells (PBMCs), whole blood was transferred to BD Vacutainer CPT Cell Preparation Tubes (Streck, La Vista, NE, USA) and centrifuged at 1800 x g for 20 min. PBMCs were then collected from the buffy coat layer together with 1 mL of plasma. The solution, which included PBMCs, was transferred to a new 1.5 mL tube and centrifuged at 10,000 x g for 3 min. The plasma was then carefully removed and the PBMCs were stored at – 80°C until DNA extraction. To extract DNA from tissue, cell lines, and PBMCs, an ISOSPIN Tissue DNA kit (#316–08891, Nippon Gene Co., Ltd., Japan) was used. For the FFPE samples, the WaxFree Paraffin Sample DNA Extraction Kit (Trimgen Corp., Sparks Glencoe, MD) was used.

DNA concentration measurement

Either the Qbit dsDNA HS Assay Kit (#Q32854, Thermo Fisher Scientific) or Qbit dsDNA BR Assay Kit (#Q32850, Thermo Fisher Scientific) was used to measure the concentration of extracted DNA.

Panel sequencing by NGS and mutation detection by dPCR

Primary sample DNA (tumor or plasma) was sequenced by CancerSCAN (GENINUS Inc. Seoul, Republic of Korea), LiquidSCAN (GENINUS Inc. Seoul, Republic of Korea) with Illumina sequencer (Illumina, San Diego, CA, USA) at Geninus Inc. (Seoul, Republic of Korea)(11); Comprehensive Cancer Panel, Cancer Hotspot Panel version 2 (CHPv2), SCC Panel, *TP53*-Panel, Lung/Colon Cancer Panel, Stomach/Duodenum Panel (DU1), Pancreas Cancer Panel (Panc16), or Colon Cancer Panel TSA2 using Ion amplicon sequencing at the Research Institute for Frontier Medicine of Sapporo Medical University (Sapporo, Japan)(12, 15); Oncomine Precision Assay by Genexus (Thermo Fisher Scientific, Basel, Switzerland; OTS-Probes Sequencing Panel (DNA Chip Research, Inc., Tokyo, Japan); and reimbursement eligible panels, including FoundationOne CDx and FoundationOne Liquid CDx (Foundation Medicine, Inc., MA, USA). Two hotspot mutations in the *TERT* promoter, C228T and C250T, were detected by QuantStudio 3D using a TaqMan Probe for the *TERT* promoter at C228T and C250T (#A44177 Hs000000092_rm and Hs000000093_rm, Thermo Fisher Scientific) or QX200 (Bio-Rad Laboratories) with OTS-Probes (#OTS-0833 and #OTS-0834, Quantdetect, Inc., Tokyo, Japan).

OTS-Select algorithm

OTS-Select is a program that runs in a Python environment. Based on a list of gene mutations obtained by NGS or other techniques, it ranks from S to E (i.e., S, A, B, C, D, and E) for each mutation, evaluating their suitability for ctDNA monitoring. At the beginning, the user must specify whether the mutation list originated from primary tumor tissue or plasma, and select the cancer type (i.e., the organ of origin) associated with mutation list. The selected cancer type determines the reference gene set, whereby the mutation list is matched against a precompiled list of the top 100 most frequently mutated genes for each organ (Dataset S3). The required input format for running the OTS-Select algorithm is provided in a template file (OTS-Select_input_format.csv), which is available in the GitHub repository. This is because the optimal VAF threshold for mutation selection differs between tumor and plasma samples. The algorithm processes mutations in descending order of VAF values. Factors that are unfavorable for ctDNA monitoring, such as low NGS coverage, known germline registration in ToMMo, gnomAD and dbSNP, low VAF, suspected germline VAF values, low representation in the COSMIC database, and C-terminal proximity of the gene product, will result in the assignment of specific negative flags (NFs). Conversely, variants supported by prior biological or clinical evidence are assigned positive flags (PFs), including those annotated as “Pathogenic” or “Likely pathogenic” in the ClinVar database, as well as variants occurring in genes that rank among the top 100 most frequently mutated genes in the corresponding cancer type according to the COSMIC database. Once the algorithm identifies four mutations without any NFs, the program ends without processing the remaining mutations. Alternatively, this stopping function can be disabled, allowing the software to assign ranks to all listed mutations. The OTS-Select pipeline generates two types of output files. The first is a comprehensive result file in which all input variants are assigned final ranks (S, A, B, C, D, or E), together with all associated positive and negative flags. The second is a filtered result file containing variants marked with `Selected_Flag = 1`,

representing candidate variants selected for ctDNA monitoring. For the purpose of ensuring computational reproducibility, we provide synthetic versions of the probe-validation files required by OTS-Select. A representative output of the OTS-Select algorithm, corresponding to the comprehensive result file that assigns final ranks and associated flags to all input variants, is shown in Dataset S4.

Statistical analysis

A two-sided unpaired t-test or Mann-Whitney test was used for two group comparisons. *P* values < 0.05 were considered statistically significant. Pearson and/or Spearman correlation coefficients were calculated between two independent variables. Analysis was performed in the R statistical environment version 4.4.0 or GraphPad Prism version 8 (GraphPad, Software, San Diego, CA, USA).

Results

Somatic mutation-specific primer/probe library for dPCR

We established a clinically applicable framework, OTS-Assay, which comprises a dPCR-based ctDNA monitoring system with its dedicated P/P library, OTS-Probes (Fig. 1A). The OTS-Probes were curated from high-frequency mutations to generate the OTS-1000ex set, designed to maximize coverage across cancer patients (Fig. 1B). Mutations not represented in OTS-1000ex were synthesized on demand, resulting in the OTS-1000ex alone covering more than half of cases, the on-demand approach extending coverage by an additional 20% (Fig. 1C). To build a P/P library for PTB monitoring, we mainly selected single nucleotide variants (SNVs) as P/P targets from registered mutations in the COSMIC. Selection was based on an originally developed algorithm using TCGA, NCBI ClinVar databases, jMorp provided by ToMMo, and gnomAD, as well as manually curated literature as “counter-references”.

TP53 was the most frequently mutated, mainly in the DNA binding domain coding region (DNABD-CR, Fig. 1D) (16, 17). A logarithmic relationship was observed between the number of *TP53* mutations and their cumulative frequencies, indicating that a small number of mutations account for most registries (Fig. 1D; left bottom). The concentrated mutations are more obvious when focusing on “hotspot” regions of the DNABD-CR. Out of 1,284 mutations in the DNABD-CR, only 10 mutations account for more than 30%, 100 mutations account for more than 65%, and 500 mutations account for more than 90% of the entire list of DNABD-CR mutations. However, even with 1,000 mutations, the total coverage remains incomplete (Fig. 1D; right bottom). Therefore, we investigated other frequently mutated genes. *KRAS* and *BRAF* mutations, with relatively limited variations, showed an even steeper logarithmic association than *TP53* in terms of the relationship between the number of distinct mutations and cumulative frequencies (Fig. 1E). Indeed, only 35 mutations of *KRAS* account for 97.7% of the entire *KRAS*-mutated cases. Similarly, only 12 mutations account for 88.9% of cases with *BRAF* mutations (Fig. S1). In contrast, genes without hotspot mutations, such as *KMT2D*, would require 4,447 P/Ps to cover 95% of cases (Fig. 1E). Overall, cumulative frequency plots by log mutation count showed three patterns: logarithmic (*BRAF*, *KRAS*, *NRAS*, *HRAS*, *PIK3CA*, *FGFR3*, and *EGFR*), linear (*TP53*), and exponential (*KMT2D*, *ARID1A*, and

FGFR2) (Fig. 1E). For OTS-Probes mutation selection, exponential-pattern genes (i.e., no hotspots) were excluded or had only a few mutations selected. The mutation frequency-based selection algorithm was repeated for over 600 genes in principle with manual curation (Fig. S2). Finally, the P/P library consists of 1,117 P/P sets from 106 genes, which was called “OTS-1000ex ver.1.0.0” (Dataset S1).

Primer and probe sequence design for ctDNA monitoring

Through more than 4,000 ctDNA measurements from cancer patients, we found that the VAF of ctDNA is low, especially after treatment (Fig. 2A). Approximately 90% of overall VAFs were below 1%, whereas the range was significantly decreased in post-treatment (Fig. 2A), suggesting that VAF measurement technology should be sensitive enough to stably quantify very low VAFs. The ctDNA is known to show a highly fragmented peak at 167 bp in blood (18, 19), and therefore shorter PCR products are more suitable for efficient amplification (20). To cover a wide mutation spectrum, we aimed to design the P/P for product lengths of approximately 70 bp. Due to the short amplicon length, maintaining specificity is challenging. Hence, we employed the Hypercool Primer and Probe™ technology (HPPT, Nihon Gene Research Laboratories, Inc, NGRL), which uses modified bases, including 2-amino-dA (2aA) and 5-methyl-dC (5mC), into P/P sequences. While Lebedev et al. demonstrated the benefit of modified bases for PCR, NGRL has developed an enhanced approach by using short primers with elevated melting temperatures, which provides a robust solution for high-sensitivity applications (21). While OTS-Probes targets more than 1,000 somatic mutations, we prioritized mutations based on real-world mutation emerging frequencies. As a result, we have currently 500 designed P/Ps, of which 366 P/Ps have been synthesized. In addition to these frequently found mutations, 236 P/Ps were synthesized for patients who did not have mutations targeted in the OTS-1000ex (Dataset S1). The amplicon size of the designed and validated OTS-Probes had a median length of 82 bp ($n = 736$) (Fig. 2B), which are optimized to increase the PCR success rate with highly fragmented DNA such as ctDNA (18). PCR success was defined as finding both wild-type and mutant signals across quadrants as a component of a 2D scatter plot. Of 626 initial attempts, 602 P/Ps were successfully obtained, yielding a first-attempt PCR success rate of 96.2% (Fig. 2C). The result suggests that the binding affinity of modified bases, 2aA and 5mC, allows one to design: (i) specific but short P/Ps; (ii) blocked DNA with secondary structure; and (iii) P/P within a palindromic sequence (22, 23). Importantly, 97.3% of the successful P/P sets functioned under the same thermal conditions allowing a parallel duplex (i.e., mutant and wild type) dPCR (Fig. 2D). Finally, only 4.2% (25/602) of the OTS-Probes required adjustment to primer concentration, additives, and/or annealing temperature (Table S1).

When monitoring ctDNA with genes that have pseudogenes or are homologous, off-target amplification may increase the wild-type copy number and underestimate VAF. Statistically, long amplicons reduce the off-target PCR product whereas short amplicons give a great chance to obtain PCR product. As expected, probes with short amplicons demonstrated greater detectability with both fresh plasma DNA (Fig. 2E, 2F) and seven-year old formalin-fixed paraffin-embedded (FFPE) templates (Fig. 2G, 2H) than long amplicons. Overall, the results suggest that the short amplicons yielded significantly more analyzable copies than long amplicon, particularly in old FFPE DNA template (Fig. 2I). The high PCR

success of OTS-Probes enables stable tumor-informed ctDNA monitoring, OTS-Monitor, in daily practice. It also allows prompt personalized application for patients requiring ctDNA monitoring with a short TAT. The overall median TAT of OTS-Monitor is 12 business days.

Statistical evaluation of OTS-Probes

On completion of mutation selection for the OTS-Probes, we evaluated the “cover rate”, which represents the capacity of immediate ctDNA monitoring based on individual somatic mutations. We first investigated public mutation databases, including TCGA, C-CAT, and our pan-cancer study, MORIOKA study (Fig. S3, Table S2, Dataset S2). The OTS-Probes covered most cases at a rate of 59.4% for TCGA (Fig. 3A), 69.0% for C-CAT (Fig. 3B), and 78.4% for MORIOKA study datasets (Fig. 3C). The cover rate analysis by organs of origin revealed that *TP53* mutations were well covered in almost all organs in three databases, whereas *KRAS*, *PIK3CA*, and *TERT* mutations had a high cover rate in specific organs of origin (Fig. 3D-3F).

Currently, the OTS-Probes library includes 1,117 P/Ps that are listed at high frequency in public databases (i.e., OTS-1000ex) and 236 P/Ps from our previous studies (11, 15, 24–28). According to the COSMIC database, 108 (10.8%) and 611 (54.7%) out of 1,117 P/Ps targeting mutations occurred at less than 0.1% and 1% in population frequencies, respectively. In practice, we managed 469 patients with 366 OTS-Probes from statistically selected 1,117 P/Ps (11, 15, 24–28). The 366 OTS-Probes are a great set for evaluating statistical validity regarding mutation emergence probability in real-world settings. As expected, the 366 sets demonstrated the coverage of 50.7% of TCGA (Fig. 3G), 62.8% of C-CAT (Fig. 3H), and 69.4% of MORIOKA study (Fig. 3I). These results suggest that producing the remaining 751 OTS-Probes would contribute to only 5–10% additional coverage (Fig. 3A-3C), although completing the OTS-1000ex still remains valuable.

Analytical assessment of OTS-Probes on multiple dPCR systems

Next, we examined whether all synthesized P/P of the OTS-Probes produced the expected PCR product with human DNA or synthetic DNA on the following dPCR systems: QuantStudio 3D, Absolute Q (Thermo Fisher Scientific); QX200 (Bio-Rad Laboratories); QIAcuity (QIAGEN), Naica (Stilla Technologies); and Digital LightCycler (Roche Diagnostics) (see Methods). Cross-platform correlation coefficient (r) exceeded 0.98 among possible combinations (Fig. 4A; Table S3). Once PCR conditions are established with available P/Ps, the detection limit of dPCR can be determined solely by the number of analyzable “reaction units” (i.e., droplet or microwell), which depends on the DNA input copy number. Theoretically, one ng of genomic DNA is approximately equivalent to 333 genomic copies (29). We investigated the number of analyzable reaction units yielded from FFPE DNA and plasma DNA of cancer patients. The median value was 131.0 dots/ng for FFPE ($n = 327$) and 191.2 dots/ng for plasma cell-free DNA ($n = 2,403$) (Fig. 4B). Although there was a significant difference in DNA recovery rate between FFPE and plasma, in general, the recovery rates in microwell format systems were stable at high range. The trend was consistent in both FFPE (Fig. 4C) and plasma samples (Fig. 4D).

We then investigated whether probe sequences affect non-specific PCR amplification. A previous study reported that dPCR P/Ps targeting transition mutations (i.e., base changes within purines or within pyrimidines) had high false-positive rates caused by probe mis-annealing (30). The risk of mis-annealing can be examined using excess template DNA with probes targeting transition mutations. However, OTS-Probes targeting transition mutations showed no false-positives under excess DNA input conditions, even with 150 ng DNA input across 16 P/Ps (Fig. 4E, Fig. S4) (31). Moreover, the use of modified bases from the HPPT allows for the production of highly-specific PCR products, even when using template DNA with high G/C content (Fig. S5). Of note, amplification of the GC-rich regions can be optimized by incorporating additives such as 7-deaza-2'-deoxyguanosine 5'-triphosphate (7-deaza-dGTP), dimethyl sulfoxide (DMSO), or Q-Solution (QIAGEN). ethylenediaminetetraacetic acid (EDTA), 7-deaza-dGTP, and DMSO are essential for PCR with GC-rich templates (32, 33). Finally, the effect of pseudogenes was assessed in the case of *KRAS* for *KRAS* (34). Since the *KRAS* codon 12/13 bears greater than 95% homologous nucleotide sequences (35), the P/P sequence design was highly restricted even when using the HPPT. Hence, we optimized the cross-reactivity conditions for six *KRAS* codon 12/13 mutations using EDTA (Fig. S6). As the final evaluation with fully optimized P/P sets, the concordance of 604 VAF pairs between NGS and dPCR using the OTS -Probes showed an excellent correlation of $r = 0.92$ (Fig. 4F). However, when NGS VAFs were less than 1%, the r dropped to -0.26 (Fig. 4G), consistent with previous reports (36, 37).

Somatic mutation selection algorithm for tumor-informed ctDNA monitoring

Technically, dPCR offers 10-100-fold higher sensitivity than NGS (7), but requires pre-validated P/P for specific targets. In the context of tumor mutational heterogeneity, truncal mutations are considered constantly present at invasion front or multiple metastatic lesions (38). Therefore, truncal mutations are suitable for ctDNA monitoring via dPCR (26, 28).

The algorithm we developed, “OTS-Select”, requires only an essential list: gene name, VAF, coding DNA sequence (CDS) change, and amino acid change in an Excel format reported from an NGS panel (see Online Methods). The OTS-Select includes 30 principal steps referring to COSMIC, ClinVar, ToMMo and gnomAD via jMorp, the Database of Single Nucleotide Polymorphisms (dbSNP), and our OTS-Probes validation records (Fig. 5A). The OTS-Select algorithm prioritizes mutations that are a high frequency in the target cancer type, driver genes, cancer-related genes, and those included in the OTS-1000ex list. OTS-Select algorithm can also rule out germline mutations or single nucleotide polymorphisms (SNPs) according to VAF and validity from dbSNP, gnomAD and ToMMo; and otherwise NGS error. We consider these changes excluded if the VAF exceeds 40%, except for genes such as *TP53*, as this gene region frequently exhibits loss of heterozygosity (16). The OTS-Select algorithm loops until four high-ranking mutations are selected.

To examine the feasibility of the OTS-Select algorithm, 612 mutations ranked by OTS-Select were evaluated using ctDNA monitoring up to two years in the MORIOKA study and OTS-Assay Observational

(OTS-AO) study protocols. The clinical concordance was defined as either the case of detection of ctDNA with at least one mutation-positive time point or consistently negative ctDNA results with no tumor detected by CT imaging during the observation. Strikingly, 88.8% (318/358) of rank S mutations showed clinical concordance, followed by 83.3% (60/72) of rank A mutations, 71.7% (86/120) of rank B mutations, 55.9% (19/34) of rank C mutations, and 29.4% (5/17) of rank D mutations. Rank E mutations showed 0% (0/11) concordance (Fig. 5B, Table S4).

OTS-Probes markedly improves prompt diagnosis during cancer surveillance

During cancer surveillance in our clinical studies, nearly 5,000 timepoints have been analyzed over 1,200 OTS-Monitor events as a component of the OTS-Assay. For those who did not have a mutation immediately available for OTS-1000ex, we designed and synthesized custom P/Ps within weeks of mutation identification.

The greatest advantage of OTS-Probes is that the P/Ps have been validated for dPCR with human tumor samples and/or synthesized DNA. In practice, dPCR allows for highly sensitive and frequent monitoring, defining “clinical validity” in terms of: (i) early relapse prediction (Fig. 6A), (ii) treatment efficacy evaluation (Fig. 6B), and (iii) no relapse corroboration (Fig. 6C) (15, 39–41). Under our longitudinal ctDNA monitoring, the longest monitoring duration is now 10 years, in which OTS-Monitor contributed to providing on the clinical validities. To include more patients, we are expanding our analyzed mutations, including those that are not feasible to design all possible point mutation-specific P/P sets in a “hotspot” region. For example, we will include *EGFR* exon 20 insertions by designing a universal “drop-off” P/P set that quantifies VAF with any sequence changes due to exon 20 insertions (Fig. S7).

Another potential advantage of OTS-Monitor in clinical diagnostics is that a sustained duration of ctDNA VAF below a limit of detection may indicate drug withdrawal based on our sensitive detection of ctDNA (Fig. 6D). In contrast to stratification of drug administration by 1–3 time points (2–4), OTS-Monitor may offer reassurance by identifying optimal timing to resume treatment. Recently, we also confirmed that dPCR of a somatic mutation identified from the primary esophageal cancer for a lung metastatic site could identify the tumor origin (Fig. 6E). When the same mutation is identified at multiple metastatic sites, they likely share a common origin.

Discussion

OTS-Probes represent the dPCR P/P library dedicated to tumor-informed ctDNA monitoring, which has the potential to markedly improve cancer patient surveillance. Although currently available serum tumor markers, such as carcinoembryonic antigen (CEA), offer clinically useful information, they are not fully deterministic (42). In contrast, OTS-Probes offer quantitative ctDNA VAF, which reflects either PTB or tumor proliferation velocity via patient-specific somatic mutations (11, 15, 26, 28). Designed for immediate application in patients with identified somatic mutations, OTS-Assay offers a broad range of validated P/Ps. For example, OTS-Probes include P/Ps against 232 somatic mutations of *TP53*. Notably,

the top 10 mutations account for approximately 30% of missense mutations, and the top 50 missense mutations are located in the exon 5–8 hotspot (i.e., coding region of the DNA binding domain) (43). In addition, the 232 target mutations cover 80% in this region. Even an expansion to 300 *TP53* mutations would only increase the coverage by 4.5%. Therefore, we believe that the probability of encountering less than 0.1% of “virtual” mutation frequencies from public databases would be very rare in daily practice. Moreover, *KRAS*, *BRAF*, *PIK3CA*, and *EGFR* mutations are well represented by a selected number of OTS-Probes for the majority of their registered mutations, including pancreatic cancer, which bears *KRAS* mutations in more than 90% of cases (44, 45). In contrast, *FGFR4* mutations are disperse; for instance, c.407C > T (p.P136L) accounts for 9.3% of all *FGFR4* mutations, while other ‘major’ *FGFR4* mutations including c.1162G > A (p.G388R), c.28G > A (p.V10I), and c.1648G > C (p.V550L) represent 6.3%, 3.6%, and 1.7%, respectively, suggesting that the *FGFR4* mutations were not located in concentrated hotspots. OTS-Probes is currently comprised of 1,117 P/Ps designed to target mutations across 106 genes, which are prioritized based on population-level “virtual” frequencies. Although this is not currently exhaustive, it has already allowed for immediate ctDNA monitoring in 60–80% of cancer patients with somatic mutations.

Some cancer types seem quite effective even if tumor-agnostic ctDNA monitoring is performed. For example, the cover rate of the OTS-Probes in our previous study of bladder cancer was 86.7% (26/30 cases), in which 76.7% (23/30) of cases were covered by either one of the two C228T or C250T mutations in the *TERT* promoter (*TERTp*) (11). Since *TERTp* mutations were occasionally found in normal or atypical epithelium located in close proximity to tumors (46), the false positives during ctDNA monitoring were of concern. However, with the OTS-Assay, 94.4% (17/18) of cases from our cohort exhibited VAFs of *TERTp* mutations, which accurately reflected the degree of tumor progression (11). In the MORIOKA study, *TERTp* mutations were *de novo* screened in liver, bladder, and upper tract urothelial carcinoma (UTUC) as well as glioblastoma cases by dPCR. The screening identified *TERTp* mutations that were not detected by NGS, leading to a high cover rate in the MORIOKA study. The cases covered included 76% in liver, 80% in bladder, and 89% in UTUC as well as 75% of glioblastoma. TCGA includes a population of rare cancers that have been collected for the project rather than actual incidence rates (47–49), which may explain the lower coverage by OTS-Probes, especially in cancers with fewer hotspot mutations, such as paraganglioma, pheochromocytoma, and acute myeloid leukemia. Notably, the frequency of *TP53* mutations in the Japanese population was higher than that of the Caucasian population (50). In our analysis across cancer types, the frequency of *TP53* mutation was 39.4% (4,003/10,152 cases) in TCGA, 59.9% (31,662/52,886 cases) in C-CAT, and 59.1% (178/301 cases) in the MORIOKA study. In contrast, the respective coverage by available OTS-Probes was 61.4%, 61.9%, and 80.9%. Therefore, the 232 P/P sets for *TP53* mutations in OTS-Probes would be a powerful driving force for prompt and frequent tumor-informed ctDNA monitoring, particularly in the Japanese population.

For mutational status requiring information of only its presence or absence of a given alteration, such as deletion in the targeted region, we demonstrated P/P sets for *EGFR* exon 20 mutations. Theoretically only two P/P sets can cover 101 reported mutations in the *EGFR* exon 20. While mutations in these regions indicate the molecular targeting agent as companion diagnostics (CDx) (51, 52), these

mutations may also be used for ctDNA monitoring. In the OTS-Assay workflow, we incorporated the sequencing results of CDx may be used for tumor ctDNA monitoring for the PTB monitoring in response to targeting therapy.

From a clinical viewpoint, the value of OTS-Probes includes assay sensitivity, personalization, and the use of affordable biomarkers for informing PTB. As previous studies have shown, the clinical validity of ctDNA monitoring did not require pathogenicity of targeted mutations (15, 28). A critical aspect for selecting the targeted mutation for ctDNA monitoring is the identification of at least one truncal mutation in individual tumors (28). The Darwinian model for cancer genetic clonal evolution considers that driver genes that contribute to cancer cell survival are likely to be truncal mutations (53). In fact, our previous multiregional sequencing together with the statistical simulation for genetic evolution revealed that ctDNA was detected in 62.1% of branch (i.e., non-truncal) mutations whereas 75% of truncal mutations (28). OTS-Select primarily avoids SNPs, CHiPs, and germline mutations by searching public databases. In practice, we perform validation using tumor DNA that has been used for NGS and genomic DNA from peripheral blood mononuclear cells by dPCR. This approach helps to rule out potential technical errors.

In line with the selection of truncal mutations, there has always been a question of whether limited number of mutations for ctDNA monitoring is sufficient to capture tumor genetic heterogeneity (54). This question may have arisen from the premise that genetic analysis should be generated from comprehensive methods, such as NGS (55). Although longitudinal ctDNA monitoring requires technologies that are sufficiently sensitive and frequently usable, several approaches have suggested that monitoring PTB with NGS is also effective, such as CAPP-Seq (56), Safe-SeqS (57, 58), liquid biopsy by exome followed by target sequencing (54), and even whole genome sequencing (59). These techniques allow longitudinal monitoring in terms of the “sensitive” region, though the practical detection limits of NGS is above 1%; however, the “frequent” need of testing is not a practical solution in terms of cost. Therefore, it is more reasonable to monitor the VAF of ctDNA in the context of cancer patient surveillance. In our practice of more than 200 patients who enrolled in the OTS-AO study, the OTS-Probes used only 1–2 mutations for ctDNA monitoring. The OTS-Assay is designed for longitudinal PTB monitoring rather than comprehensive genomic profiling; however, its sensitivity and cost-effective testing frequency enables capture of ctDNA dynamics that are not accessible through conventional modalities for cancer surveillance. The OTS-Probes is therefore the core resource technology for implementing the OTS-Assay in daily practice.

Conclusions

In this study, we developed OTS-Probes, an off-the-shelf primer/probe library dedicated to dPCR-based longitudinal ctDNA monitoring. We also demonstrated that appropriate primer/probe sets for ctDNA monitoring by dPCR can be selected by the OTS-Select algorithm from OTS-Probes. Subsequent longitudinal tumor-informed ctDNA monitoring by the selected OTS-Probes using dPCR, OTS-Monitor, provides clinically relevant PTB information such as early relapse prediction, treatment efficacy

evaluation, and no relapse corroboration. These findings support the OTS-Assay system as a practical strategy to bridge the gap between genomic profiling and longitudinal monitoring, thereby enabling precision ctDNA surveillance in clinical settings.

Abbreviations

ctDNA

Circulating Tumor DNA

PTB

Physical Tumor Burden

dPCR

Digital PCR

VAF

Variant Allele Frequency

PBMC

Peripheral Blood Mononuclear Cells

FFPE

Formalin-Fixed Paraffin-Embedded

NGS

Next Generation Sequencing

CDx

Companion Diagnostics

SNPs

Single Nucleotide Polymorphisms

UTUC

Upper Tract Urothelial Carcinoma

7-deaza-dGTP

7-deaza-2'-deoxyguanosine 5'-triphosphate

2aA

2-amino-2'-deoxyadenosine

5mC

5-methyl-2'-deoxycytidine

DMSO

dimethyl sulfoxide

EDTA

ethylenediaminetetraacetic acid

DNABD-CR

DNA Binding Domain Coding Region

CDS

Coding DNA Sequence

COSMIC

Catalogue Of Somatic Mutations In Cancer

dbSNP

Database of Single Nucleotide Polymorphisms

TCGA

The Cancer Genome Atlas

C-CAT

Center for Cancer Genomics and Advanced Therapeutics

ToMMo

Tohoku Medical Megabank Organization

gnomAD

Genome Aggregation Database

jMorp

Japanese Multi Omics Reference Panel

TAT

Turn-Around-Time

P/P

Primer and Probe

OTS-Probes

off-the-shelf digital PCR primer/probe library

HPPT

Hypercool Primer and Probe™ technology

MORIOKA study

Monitoring Recurrence of Individual tumor by serial Observation of Known gene Alterations

OTS-AO study

OTS-Assay Observational study

Declarations

Approval and consent to participate

This study was approved by the Institutional Review Board of Iwate Medical University as OTS-155 study (Approval No. HG2020-027). Biological specimens and clinical data included tumor tissue, plasma, and PBMCs. These materials comprised archival samples from previously approved studies at Iwate Medical University, samples provided by collaborating institutions, and datasets derived from the MORIOKA study (HG2019-003) and the ongoing OTS-AO study (HG2022-001), and other IRB-approved studies (HGH28-16, HGH27-16, HGH28-15, HG2019-030, and HG2019-001) (11, 15, 24–28). A portion of the tumor samples was purchased from Kyoto Bridge for Breakthrough Medicine (KBBM), Inc. (Kyoto, Japan) for P/P validation. The clinical information was obtained from the corresponding IRB approval studies listed above. All procedures were conducted in accordance with institutional guidelines and the Declaration of Helsinki.

Consent for publication

Written informed consent for publication of clinical data was obtained from the patient.

Competing Interests

The authors declare the following competing interests: H.H. reports consulting for Quantdetect, Inc; lecture honoraria, travel support/research funding from Quantdetect, Inc; research funding from LSI Medience Co; reagents from Nippon Gene Co., Ltd; patent-related compensation (PCT/JP2022/43189) covering the dPCR primer/probe library (OTS-1000ex); and is an inventor on a pending Japanese patent application related to the probes for EGFR exon 20 mutations in this study (JP Application No. 2025-060775). A.Y.-A. reports consulting for Quantdetect, Inc., LSI Medience Co., Kotobiken Medical Laboratories, Inc; and lecture honoraria from AbbVie Inc., Chugai Pharmaceutical Co., Ltd., and Nippon Shinyaku Co., Ltd. M.A. reports research support from Nippon Kayaku Co., Ltd. and Quantdetect, Inc. M.J. is an employee of Quantdetect, Inc.; and reports research support from Quantdetect, Inc. W.-Y.P. is the CEO of Geninus Inc., and GxD Inc.; and a stockholder of Geninus Inc., and MacroGen Inc. F.E. is a stockholder of Quantdetect, Inc. M.Y. reports consulting for Johnson & Johnson K.K. Medical Company; and reports lecture honoraria from Johnson & Johnson K.K. Medical Company, Covidien Japan, Inc., Takeda Pharmaceutical Co., Ltd., Merck Biopharma Co., Ltd., Eli Lilly Japan K.K., Terumo Corporation, Kaken Pharmaceutical Co., Ltd., Daiichi Sankyo Company, Limited, and Taiho Pharmaceutical Co., Ltd. D.T. reports research funding from Nippon Kayaku; and research support from Quantdetect, Inc. P.M.J. is a director and employee of Thermo Fisher Scientific; and a consultant for University Hospital Basel. L.Q. is an employee; and stockholder of Thermo Fisher Scientific. S.T. reports honoraria from Quantdetect, Inc. for serving as a chairperson at a scientific meeting. M.M. reports lecture honoraria from Quantdetect, Inc.; and research support from Kyoto Bridge for Breakthrough Medicine. H.I. is a stockholder of Quantdetect, Inc. W.O. is a stockholder of Quantdetect, Inc. T.I. reports consulting for Quantdetect, Inc.; is a stockholder of Quantdetect, Inc.; reports lecture honoraria, travel fees, and research support from Quantdetect, Inc.; and patent-related compensation (JP Patent #6544783) covering the dPCR primer/probe library (OTS-Probes). S.S.N. is the CEO and a stockholder of Quantdetect, Inc.; reports consulting for Hitachi High-Tech Co.; lecture honoraria from Thermo Fisher Scientific, MSD Co., Ltd., Finggal Link Co., Ltd., Nippon Kayaku Co., Ltd., Chugai Pharmaceutical Co., Ltd., and Iwate Prefecture; research funding from Taiho Pharmaceutical Co., Ltd., Boehringer Ingelheim Japan, LSI Medience Co., QIAGEN, and Roche Diagnostics K.K.; research support from Geninus, Array Jet, Thermo Fisher Scientific, Nippon Gene Co., Ltd.; patent-related compensation (JP Patent #6544783, PCT/JP2022/43189) concerning the dPCR primer and probe library (OTS-Probes, OTS-1000ex); and is an inventor on a pending patent application related to the probes for EGFR exon 20 mutations in this study (JP Application No. 2025-060775). All other authors declare no competing interests.

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Author Contribution

H.H. contributed to conceptualization, methodology, data analysis, data curation, writing of the original draft, writing—review and editing, visualization, validation and funding. A.Y.-A. contributed to conceptualization, review and funding. N.S., Y.K., T.S., S.T., W.-Y.P., T.S., M.Y., H.N., R.F., Y.S., K.K. and S.M. contributed to data curation, sample acquisition and review. M.A. contributed to conceptualization, data curation, review and funding. M.J., M.I., Y.S., T.T., P.M.J., L.Q., H.N., T.Y., N.Y., R.S., E.M. and A.T. contributed to data curation and review. F.E. contributed to methodology, sample acquisition, data curation and review. H.S., M.K., T.B., D.T., K.T., Y.D., H.K., T.I., H.K., H.S., Y.S., Y.M., A.A.-M., M.B., R.K., T.N., Y.K., M.K., Y.T., T.Y., S.T. and M.M. contributed to sample acquisition and review. F.T. contributed to supervision and review. S.I., H.I., M.M. and W.O. contributed to conceptualization, sample acquisition and review. T.I. contributed to conceptualization, methodology, sample curation, data curation, funding and review. S.S.N. contributed to conceptualization, methodology, writing of the original draft, writing—review and editing, funding and supervision.

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Data Availability

Data availability The data supporting the main findings of this study are provided within the article and its Supporting Information files. Variant information used for our probe validation is provided in Dataset S1. Public cancer genomic datasets were obtained from TCGA, C-CAT and other publicly accessible repositories as described in the Methods. The primer and probe sequences of the OTS-Probes are proprietary to Quantdetect, Inc. (<https://www.quantdetect.com>) and therefore not publicly available. To enable reproducibility of the OTS-Select computational workflow, synthetic versions of the probe-validation files are provided as Dataset S5. These synthetic datasets replicate the file structure and expected data formats used in the analysis and retain probe-validation status information (e.g., prior synthesis and validation outcomes). Processed summary outputs underlying the main figures and statistical analyses are provided in the Supplementary Datasets.

Code Availability The OTS-Select software developed in this study for mutation selection in tumor-informed ctDNA monitoring is currently available for peer review purpose at the following private link: https://anonymous.4open.science/r/Hiraki_et_al_OTSelect-27FA. The archived public versions correspond exactly to the code used in this study and contain all essential scripts required to fully reproduce the computational steps described herein. The proprietary commercial implementation licensed to Quantdetect, Inc. remains restricted for commercial use.

References

1. Ludwig JA, Weinstein JN. Biomarkers in cancer staging, prognosis and treatment selection. *Nat Rev Cancer*. 2005;5(11):845–56.
2. Nakamura Y, Watanabe J, Akazawa N, Hirata K, Kataoka K, Yokota M, et al. ctDNA-based molecular residual disease and survival in resectable colorectal cancer. *Nat Med*. 2024;30(11):3272–83.
3. Kotani D, Oki E, Nakamura Y, Yukami H, Mishima S, Bando H, et al. Molecular residual disease and efficacy of adjuvant chemotherapy in patients with colorectal cancer. *Nat Med*. 2023;29(1):127–34.
4. Tie J, Wang Y, Lo SN, Lahouel K, Cohen JD, Wong R, et al. Circulating tumor DNA analysis guiding adjuvant therapy in stage II colon cancer: 5-year outcomes of the randomized DYNAMIC trial. *Nat Med*. 2025;31(5):1509–18.
5. Reinert T, Henriksen TV, Christensen E, Sharma S, Salari R, Sethi H, et al. Analysis of Plasma Cell-Free DNA by Ultradeep Sequencing in Patients With Stages I to III Colorectal Cancer. *JAMA Oncol*. 2019;5(8):1124–31.
6. Cleveland MH, He HJ, Milavec M, Bae YK, Vallone PM, Huggett JF. Digital PCR for the characterization of reference materials. *Mol Aspects Med*. 2024;96:101256.

7. Diaz LA Jr., Bardelli A. Liquid biopsies: genotyping circulating tumor DNA. *J Clin Oncol*. 2014;32(6):579–86.
8. Vogelstein B, Kinzler KW, Digital PCR. *Proc Natl Acad Sci USA*. 1999;96:9236–41.
9. Gronlund JK, Veigaard C, Juhl-Christensen C, Skou AS, Melsvik D, Ommen HB. Droplet digital PCR for sensitive relapse detection in acute myeloid leukaemia patients transplanted by reduced intensity conditioning. *Eur J Haematol*. 2024;112(4):601–10.
10. Ogaard N, Jensen SO, Orntoft MW, Demuth C, Rasmussen MH, Henriksen TV, et al. Circulating tumour DNA and risk of recurrence in patients with asymptomatic versus symptomatic colorectal cancer. *Br J Cancer*. 2024;131(10):1707–15.
11. Abe M, Hiraki H, Tsuyukubo T, Ono S, Maekawa S, Tamura D, et al. The Clinical Validity of Urinary Pellet DNA Monitoring for the Diagnosis of Recurrent Bladder Cancer. *J Mol Diagn*. 2024;26(4):278–91.
12. Sato KA, Hachiya T, Iwaya T, Kume K, Matsuo T, Kawasaki K, et al. Individualized Mutation Detection in Circulating Tumor DNA for Monitoring Colorectal Tumor Burden Using a Cancer-Associated Gene Sequencing Panel. *PLoS ONE*. 2016;11(1):e0146275.
13. Nishizuka SS, Sato KA, Hachiya T. A Pipeline for ctDNA Detection Following Primary Tumor Profiling Using a Cancer-Related Gene Sequencing Panel. In: Murray SS, editor. *Tumor Profiling: Methods and Protocols*. New York, NY: Springer New York; 2019. pp. 229–41.
14. Pantel K, Alix-Panabières C. Minimal residual disease as a target for liquid biopsy in patients with solid tumours. *Nat Reviews Clin Oncol*. 2025;22(1):65–77.
15. Iwaya T, Endo F, Takahashi F, Tokino T, Sasaki Y, Nishizuka SS. Frequent Tumor Burden Monitoring of Esophageal Squamous Cell Carcinoma With Circulating Tumor DNA Using Individually Designed Digital Polymerase Chain Reaction. *Gastroenterology*. 2021;160(1):463–e54.
16. Nigro JM, Baker SJ, Preisinger AC, Jessup JM, Hostetter R, Cleary K et al. Mutations in the p53 gene occur in diverse human tumour types. *Nature*. 1989;342(7).
17. Soussi T, Bérout C. Assessing TP53 status in human tumours to evaluate clinical outcome. *Nat Rev Cancer*. 2001;1(3):233–40.
18. Underhill HR, Kitzman JO, Hellwig S, Welker NC, Daza R, Baker DN, et al. Fragment Length of Circulating Tumor DNA. *PLoS Genet*. 2016;12(7):e1006162.
19. Sanchez C, Roch B, Mazard T, Blache P, Dache ZAA, Pastor B et al. Circulating nuclear DNA structural features, origins, and complete size profile revealed by fragmentomics. *JCI Insight*. 2021;6(7).
20. Mouliere F, Robert B, Arnau Peyrotte E, Del Rio M, Ychou M, Molina F, et al. High fragmentation characterizes tumour-derived circulating DNA. *PLoS ONE*. 2011;6(9):e23418.
21. Lebedev L, Akopyants N, Azhikina T, Shevchenko Y, Potapov V, Stecenko D, et al. Oligonucleotides containing 2-aminoadenine and 5-methylcytosine are more effective as primers for PCR amplification than their nonmodified counterparts. *Genet Anal Biomol Eng*. 1996;13:15–21.

22. Prosnyak MI, Veselovskaya SI, Myasnikov VA, Efremova EJ, Potapov VK, Limborska SA, et al. Substitution of 2-Aminoadenine and 5-Methylcytosine for Adenine and Cytosine in Hybridization Probes Increases the Sensitivity of DNA Fingerprinting. *Genomics*. 1994;21:490–4.
23. Lebedev Y, Akopyants N, Azhikina T, Shevchenko Y, Potapov V, Stecenko D, et al. Oligonucleotides containing 2-aminoadenine and 5-methylcytosine are more effective as primers for PCR amplification than their nonmodified counterparts. *Genet Anal Biomol Eng*. 1996;13:15–21.
24. Fujisawa R, Iwaya T, Endo F, Idogawa M, Sasaki N, Hiraki H, et al. Early dynamics of circulating tumor DNA predict chemotherapy responses for patients with esophageal cancer. *Carcinogenesis*. 2021;42(10):1239–49.
25. Sasaki T, Iwaya T, Yaegashi M, Idogawa M, Hiraki H, Abe M, et al. Impact of Sensitive Circulating Tumor DNA Monitoring on CT Scan Intervals During Postoperative Colorectal Cancer Surveillance. *Ann Surg Open*. 2025;6(1):e549.
26. Sasaki N, Iwaya T, Chiba T, Fujita M, Ju Z, Endo F, et al. Analysis of mutational and proteomic heterogeneity of gastric cancer suggests an effective pipeline to monitor post-treatment tumor burden using circulating tumor DNA. *PLoS ONE*. 2020;15(10):e0239966.
27. Tamura D, Abe M, Hiraki H, Sasaki N, Yashima-Abo A, Ikarashi D, et al. Postoperative recurrence detection using individualized circulating tumor DNA in upper tract urothelial carcinoma. *Cancer Sci*. 2024;115(2):529–39.
28. Yaegashi M, Iwaya T, Sasaki N, Fujita M, Ju Z, Siwak D, et al. Frequent post-operative monitoring of colorectal cancer using individualised ctDNA validated by multiregional molecular profiling. *Br J Cancer*. 2021;124(9):1556–65.
29. Butler JM. *Forensic DNA typing: biology, technology, and genetics of STR markers*. Elsevier; 2005.
30. Henriksen TV, Drue SO, Frydendahl A, Demuth C, Rasmussen MH, Reinert T, et al. Error Characterization and Statistical Modeling Improves Circulating Tumor DNA Detection by Droplet Digital PCR. *Clin Chem*. 2022;68(5):657–67.
31. Vessies DCL, Linders TC, Lanfermeijer M, Ramkisoensing KL, van der Noort V, Schouten RD, et al. An Automated Correction Algorithm (ALPACA) for ddPCR Data Using Adaptive Limit of Blank and Correction of False Positive Events Improves Specificity of Mutation Detection. *Clin Chem*. 2021;67(7):959–67.
32. Musso M, Bocciardi R, Parodi S, Ravazzolo R, Ceccherini I. Betaine, dimethyl sulfoxide, and 7-deaza-dGTP, a powerful mixture for amplification of GC-rich DNA sequences. *J Mol Diagn*. 2006;8(5):544–50.
33. Corless BC, Chang GA, Cooper S, Syeda MM, Shao Y, Osman I, et al. Development of Novel Mutation-Specific Droplet Digital PCR Assays Detecting TERT Promoter Mutations in Tumor and Plasma Samples. *J Mol Diagn*. 2019;21(2):274–85.
34. McGrath JP, Capon DJ, Smith DH, Chen EY, Seeburg PH, Goeddel DV, et al. Structure and organization of the human Ki-ras proto-oncogene and a related processed pseudogene. *Nature*. 1983;304(11):501–6.

35. Tsuchida N, Murugan AK, Grieco M. Kirsten Ras* oncogene: Significance of its discovery in human cancer research. *Oncotarget*. 2016;7(29):46717–33.
36. Stetson D, Ahmed A, Xu X, Nuttall BRB, Lubinski TJ, Johnson JH et al. Orthogonal Comparison of Four Plasma NGS Tests With Tumor Suggests Technical Factors are a Major Source of Assay Discordance. *JCO Precision Oncol*. 2019(3):1–9.
37. Bos MK, Nasserinejad K, Jansen M, Angus L, Atmodimedjo PN, de Jonge E, et al. Comparison of variant allele frequency and number of mutant molecules as units of measurement for circulating tumor DNA. *Mol Oncol*. 2021;15(1):57–66.
38. Pereira B, Chen CT, Goyal L, Walmsley C, Pinto CJ, Baiev I, et al. Cell-free DNA captures tumor heterogeneity and driver alterations in rapid autopsies with pre-treated metastatic cancer. *Nat Commun*. 2021;12(1):3199.
39. Hayes DF. Defining Clinical Utility of Tumor Biomarker Tests: A Clinician's Viewpoint. *J Clin Oncol*. 2020;39(3):238–49.
40. Joseph L, Cankovic M, Caugthon S, Chandra P, Emmadi R, Hagenkord J, et al. The Spectrum of Clinical Utilities in Molecular Pathology Testing Procedures for Inherited Conditions and Cancer: A Report of the Association for Molecular Pathology. *J Mol Diagn*. 2016;18(5):605–19.
41. Merker JD, Oxnard GR, Compton C, Diehn M, Hurley P, Lazar AJ, et al. Circulating Tumor DNA Analysis in Patients With Cancer: American Society of Clinical Oncology and College of American Pathologists Joint Review. *J Clin Oncol*. 2018;36(16):1631–41.
42. Sorensen CG, Karlsson WK, Pommergaard HC, Burcharth J, Rosenberg J. The diagnostic accuracy of carcinoembryonic antigen to detect colorectal cancer recurrence - A systematic review. *Int J Surg*. 2016;25:134–44.
43. Baugh EH, Ke H, Levine AJ, Bonneau RA, Chan CS. Why are there hotspot mutations in the TP53 gene in human cancers? *Cell Death Differ*. 2018;25(1):154–60.
44. Morris JPt, Wang SC, Hebrok MKRAS, Hedgehog. Wnt and the twisted developmental biology of pancreatic ductal adenocarcinoma. *Nat Rev Cancer*. 2010;10(10):683–95.
45. Cancer Genome Atlas Research Network. Electronic address aadhe, Cancer Genome Atlas Research N. Integrated Genomic Characterization of Pancreatic Ductal Adenocarcinoma. *Cancer Cell*. 2017;32(2):185–203. e13.
46. Hayashi Y, Fujita K, Nojima S, Tomiyama E, Matsushita M, Koh Y, et al. TERT C228T mutation in non-malignant bladder urothelium is associated with intravesical recurrence for patients with non-muscle invasive bladder cancer. *Mol Oncol*. 2020;14(10):2375–83.
47. Fishbein L, Leshchiner I, Walter V, Danilova L, Robertson AG, Johnson AR, et al. Comprehensive Molecular Characterization of Pheochromocytoma and Paraganglioma. *Cancer Cell*. 2017;31(2):181–93.
48. Radovich M, Pickering CR, Felau I, Ha G, Zhang H, Jo H, et al. The Integrated Genomic Landscape of Thymic Epithelial Tumors. *Cancer Cell*. 2018;33(2):244–58. e10.

49. Robertson AG, Shih J, Yau C, Gibb EA, Oba J, Mungall KL, et al. Integrative Analysis Identifies Four Molecular and Clinical Subsets in Uveal Melanoma. *Cancer Cell*. 2017;32(2):204–20. e15.
50. Horie S, Saito Y, Kogure Y, Mizuno K, Ito Y, Tabata M et al. Pan-cancer comparative and integrative analyses of driver alterations using Japanese and international genomic databases. *Cancer Discov*. 2024.
51. Vyse S, Huang PH. Targeting EGFR exon 20 insertion mutations in non-small cell lung cancer. *Signal Transduct Target Ther*. 2019;4:5.
52. Robichaux JP, Le X, Vijayan RSK, Hicks JK, Heeke S, Elamin YY, et al. Structure-based classification predicts drug response in EGFR-mutant NSCLC. *Nature*. 2021;597(7878):732–7.
53. McGranahan N, Swanton C. Clonal Heterogeneity and Tumor Evolution: Past, Present, and the Future. *Cell*. 2017;168(4):613–28.
54. Abbosh C, Frankell AM, Harrison T, Kisistok J, Garnett A, Johnson L, et al. Tracking early lung cancer metastatic dissemination in TRACERx using ctDNA. *Nature*. 2023;616(7957):553–62.
55. Murtaza M, Dawson SJ, Tsui DW, Gale D, Forshew T, Piskorz AM, et al. Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. *Nature*. 2013;497(7447):108–12.
56. Newman AM, Bratman SV, To J, Wynne JF, Eclov NC, Modlin LA, et al. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. *Nat Med*. 2014;20(5):548–54.
57. Kinde I, Wu J, Papadopoulos N, Kinzler KW, Vogelstein B. Detection and quantification of rare mutations with massively parallel sequencing. *Proc Natl Acad Sci U S A*. 2011;108(23):9530–5.
58. Cohen JD, Douville C, Dudley JC, Mog BJ, Popoli M, Ptak J, et al. Detection of low-frequency DNA variants by targeted sequencing of the Watson and Crick strands. *Nat Biotechnol*. 2021;39(10):1220–7.
59. Garcia-Murillas I, Abbott CW, Cutts RJ, Boyle SM, Pugh J, Keough KC, et al. Whole genome sequencing-powered ctDNA sequencing for breast cancer detection. *Ann Oncol*. 2025;36(6):673–81.

Figures

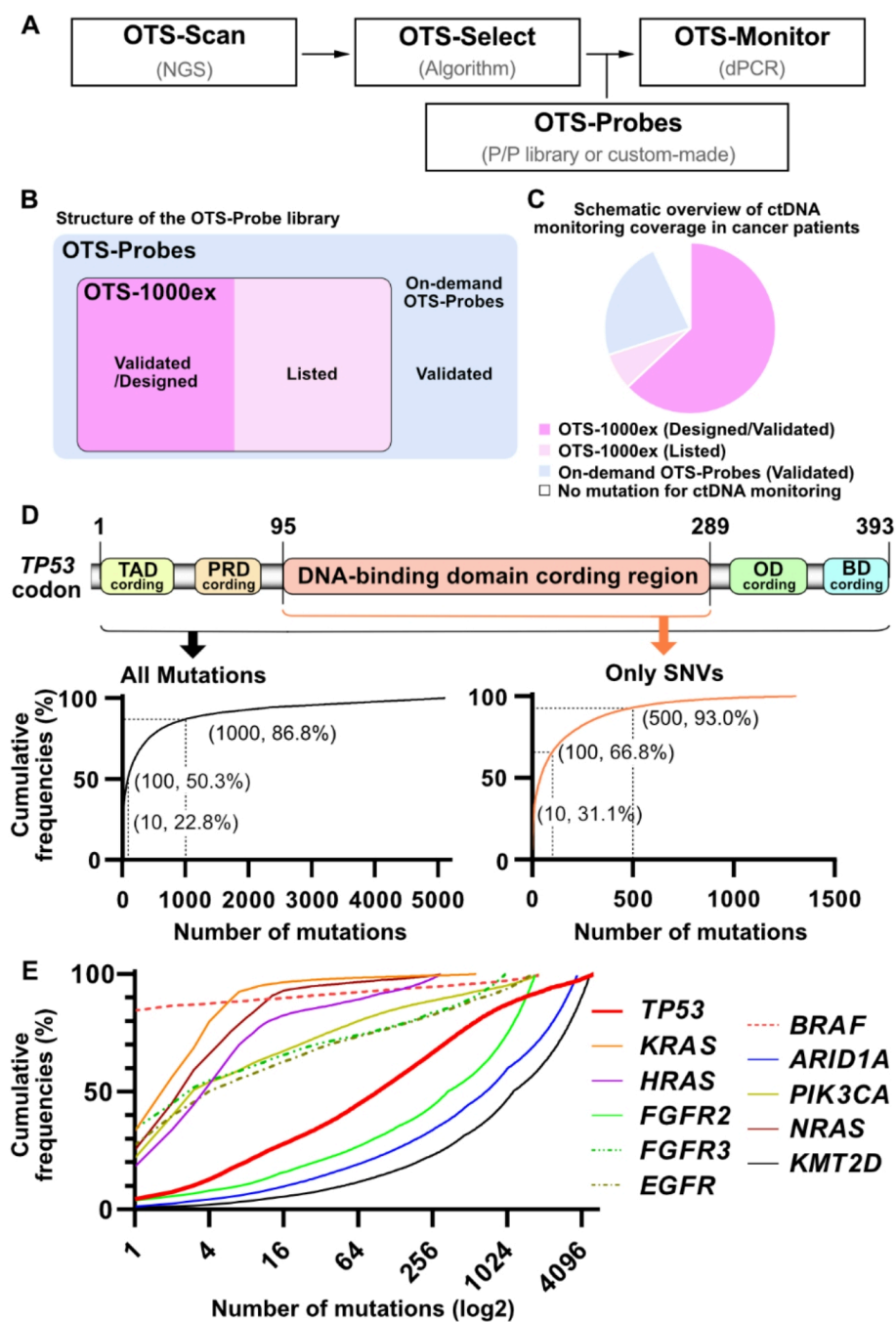


Figure 1

Overview of the OTS-Assay workflow and design concept of OTS-Probes. (A) The workflow of the OTS-Assay. (B) The structure of the OTS-Probe library. (C) The schematic overview of ctDNA monitoring coverage in cancer patients by OTS-Probes. (D) Gene structure of *TP53*. TAD: Trans-activation domain, PRD: Proline-rich domain, OD: Oligomerization domain, BD: Basic domain. Cumulative frequency of *TP53* all mutations in the COSMIC database (indicated by the black arrow) and that of *TP53* SNV of the DNA-

binding domain in the COSMIC database (indicated by the orange arrow). (E) Cumulative frequency of *TP53*, *KRAS*, *HRAS*, *FGFR2*, *FGFR3*, *EGFR*, *BRAF*, *ARID1A*, *PIK3CA*, *NRAS*, and *KMT2D* in the COSMIC database.

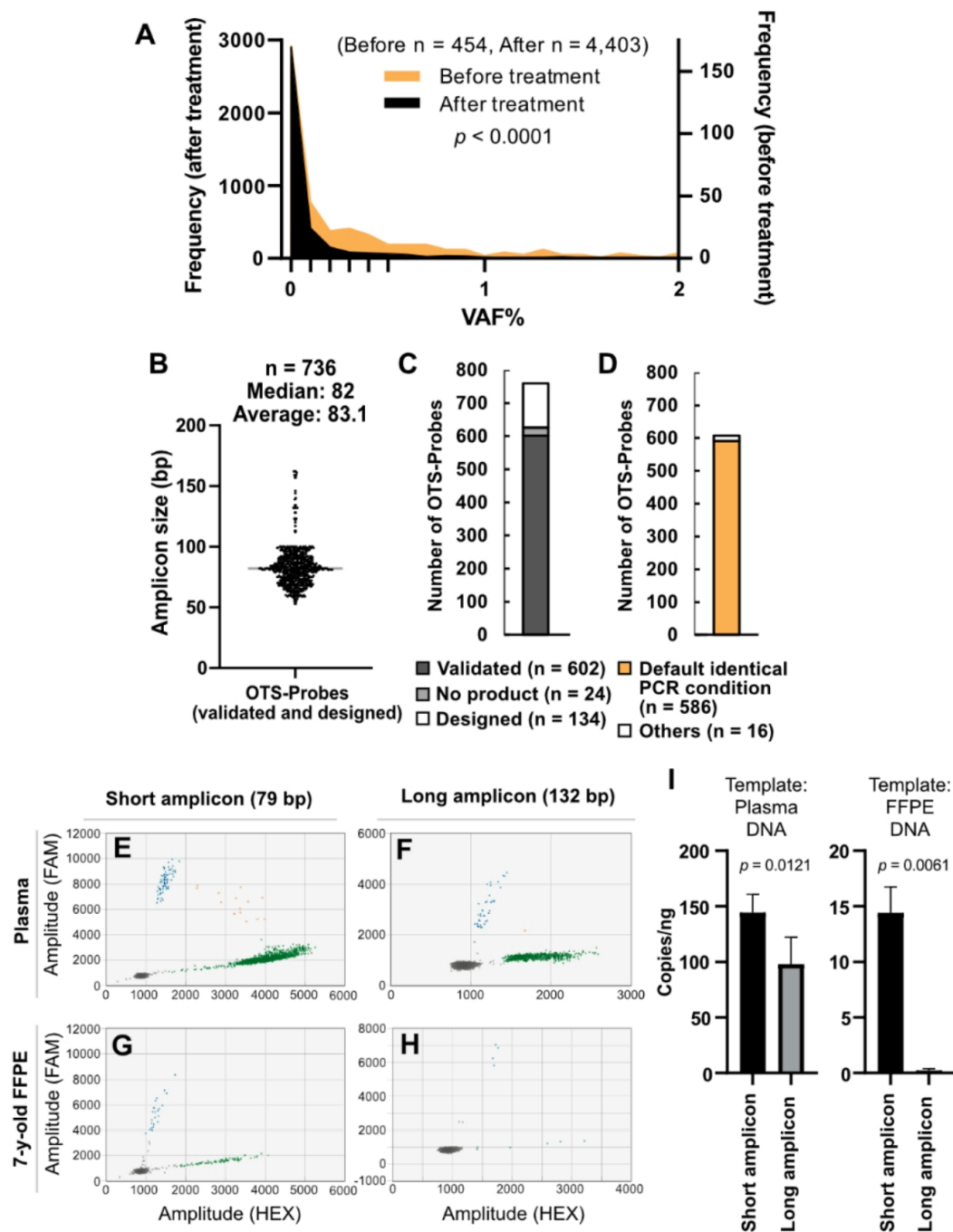


Figure 2

Analytical characteristics of OTS-Probes. (A) Histogram of VAF before and after treatment. Data from the MORIOKA study, OTS-AO study, and other previous studies were integrated(11, 15, 26, 28). Statistically significant differences were assessed by the Mann-Whitney test ($p < 0.0001$). (B) Amplicon size distribution of OTS-Probes. The sequences of OTS-Probes were designed using the Hypercool primer & probe technologyTM (Nihon Gene Research Laboratories Inc., Sendai, Japan) (n = 728). (C) Status of OTS-Probes. Number of OTS-Probes of successfully validated but failed at the first attempt and designed (i.e., not yet PCR tested). (D) Number of OTS-Probes that work default in identical PCR conditions. (E-H) Amplicon size of OTS-Probes and resulting scattergram with different template types. (E) A 2D scatter plot with short amplicon (79 bp) using plasma DNA 9.6 ng. (F) A 2D scatter plot with long amplicon (132 bp) using plasma DNA 9.6 ng. (G) A 2D scatter plot with short amplicon DNA extracted from FFPE stored for 7 years. DNA input was 11 ng. (H) A 2D scatter plot with long amplicon (132 bp) using DNA extracted from FFPE stored for 7 years. DNA input was 11 ng. (I) Averaged dot number per DNA input in two kinds of OTS-Probes with DNA extracted from plasma (left panel) and FFPE (right panel) (n = 4 or 7). Error bars indicate standard deviation. Significant differences between datasets were assessed using the Mann-Whitney test.

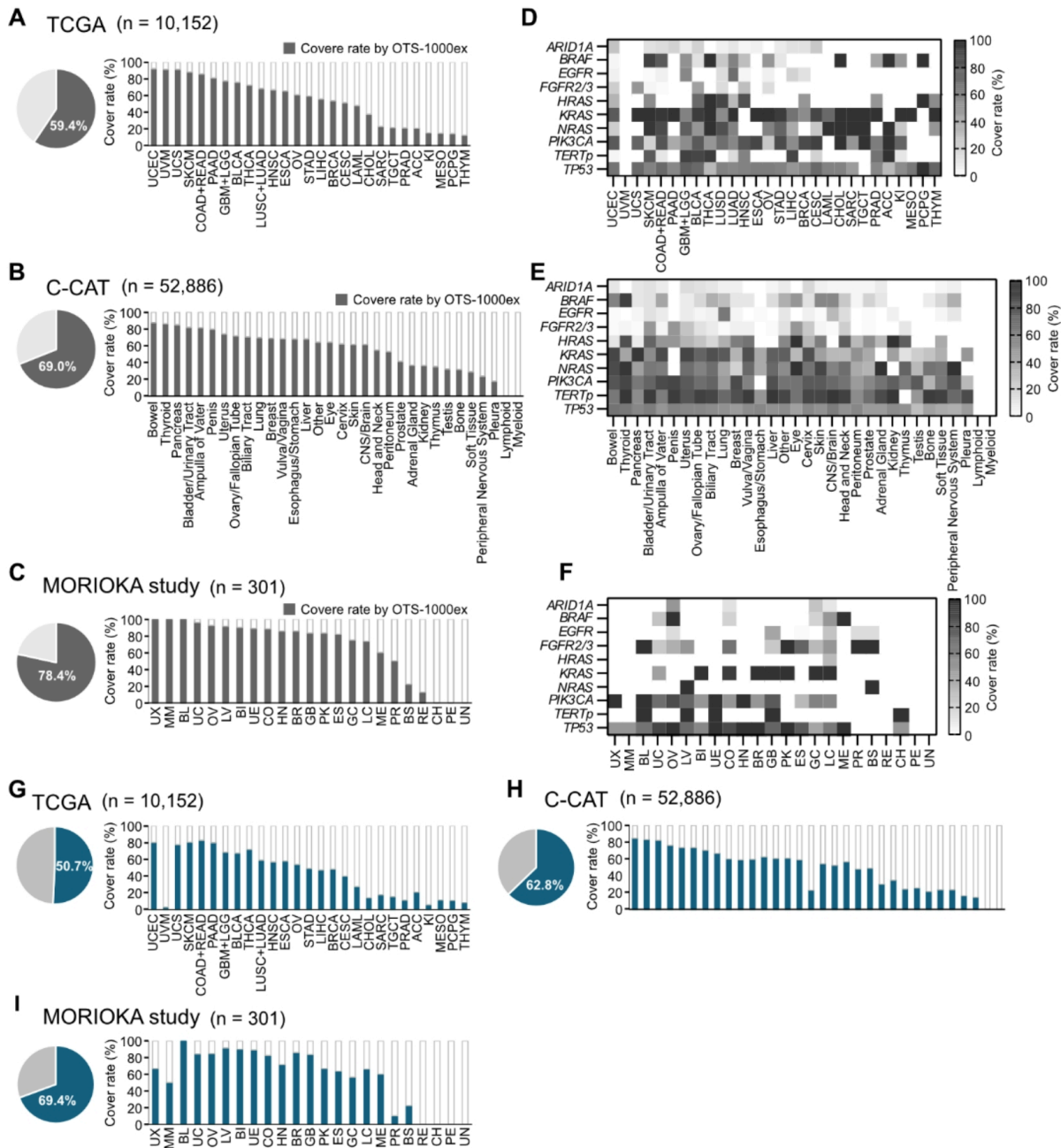


Figure 3

The cover rate by OTS-Probes in three datasets. (A-C) The cover rate by OTS-Probes in terms of organs of origin in TCGA (A) C-CAT (B), and the MORIOKA study (C) sequence datasets. UCEC, Uterine Corpus Endometrioid Carcinoma; UVM, Uveal Melanoma; UCS, Uterine Carcinosarcoma; SKCM, Skin Cutaneous Melanoma; COAD-READ, Colorectal Adenocarcinoma; PAAD, Pancreatic Ductal Adenocarcinoma; GBM, Glioblastoma Multiforme; LGG, Lower Grade Glioma; BLCA, Bladder Urothelial Carcinoma; THCA, Thyroid Papillary Carcinoma; LUSC, Lung Squamous Cell Carcinoma; LUAD, Lung Adenocarcinoma; HNSC, Head and Neck Squamous Cell Carcinoma; ESCA, Esophageal Carcinoma; OV, Ovarian Serous

Adenocarcinoma; STAD, Gastric Adenocarcinoma; LIHC, Hepatocellular Carcinoma; BRCA, Breast Ductal Carcinoma and Breast Lobular Carcinoma; CESC, Cervical Carcinoma; LAML, Acute Myeloid Leukemia; CHOL, Cholangiocarcinoma; SARC, Sarcoma; TGCT, Testicular Germ Cell Cancer; PRAD, Prostate Adenocarcinoma; ACC, Adrenocortical Carcinoma; KI including Kidney Chromophobe Carcinoma, KICH; Kidney Clear Cell Carcinoma, KIRC; and Kidney Papillary Cell Carcinoma, KIRP; MESO, Mesothelioma; PCPG, Paraganglioma & Pheochromocytoma; THYM, Thymoma. UX, Cervical cancer; MM, Multiple Myeloma; BL, Bladder cancer; UC, Uterine cancer; OV, Ovarian cancer; LV, Liver cancer; BI, Biliary tract cancer; UE, Urothelial carcinoma; CO, Colorectal cancer; HN, Head and neck cancer; BR, Breast cancer; GB, Glioblastoma; PK, Pancreatic cancer; ES, Esophageal cancer; GC, Gastric cancer, LC, Lung cancer; ME, Melanoma; PR, Prostate cancer; BS, Bone and soft tissue tumor; RE, Renal cancer; CH, Cholangiocarcinoma; PE, Pediatric cancer; and UN, Unknown primary cancer. (D-F) The cover rate of gene mutations in terms of organs of origin in TCGA (D), C-CAT (E) and the MORIOKA study (F). (G-I) The cover rate from the available 366 sets of OTS-Probes in terms of organs of origin in TCGA (G), C-CAT (H), and the MORIOKA study (I) sequence datasets.

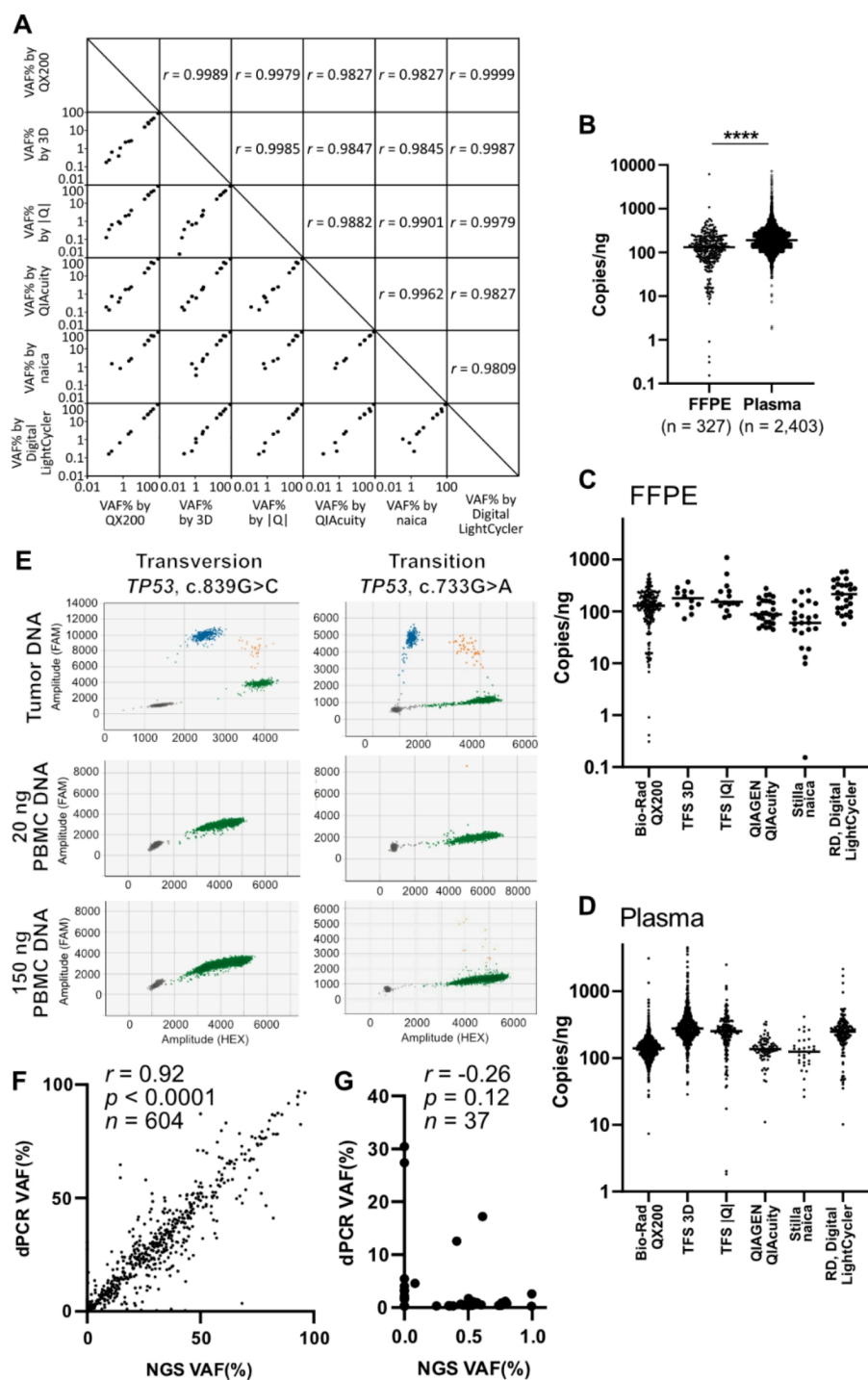


Figure 4

Analytical evaluation of OTS-Probes. (A) Concordance of VAF in six digital PCR (dPCR) systems. The dPCR systems, QX200 Droplet Digital PCR System (Bio-Rad Laboratories), QuantStudio 3D Digital PCR (Thermo Fisher Scientific, Waltham, MA, USA), QuantStudio Absolute Q (Thermo Fisher Scientific), QIAcuity Digital PCR System (QIAGEN, Hilden, Germany), Crystal Digital PCR Naica system (Stilla Technologies), and Digital LightCycler System (Roche Diagnostics, Rotkreuz, Switzerland), were

evaluated by using the same sample and primers/probes. The DNA samples were extracted from human tumors or plasma specimens (n = 13-18). The Spearman correlation test was used to analyze data. (B) Comparison of copy number per DNA input between FFPE and plasma. Data were obtained from six types of dPCR systems. Differences between FFPE (n = 273) and plasma (n = 2,131) were analyzed using the t-test (****p < 0.0001). (C) Comparison of copy number per FFPE DNA input among six dPCR systems. (D) Comparison of copy number per plasma DNA input among six dPCR systems. (E) DNA input and false-positive PCR products. The scatter plots with tumor DNA, 20 ng of peripheral blood mononuclear cells (PBMC) DNA, and 150 ng PBMC DNA, and probes targeting the transition mutation *TP53* c.733G>A and transversion mutation *TP53* c.839G>C. (F) Concordance of variant allele frequency between NGS and dPCR. VAF% of all mutations were obtained from both NGS and dPCR (n = 604). (G) A scatter plot of VAF% under 1% in NGS (n = 37). The Spearman correlation test was used to analyze data.

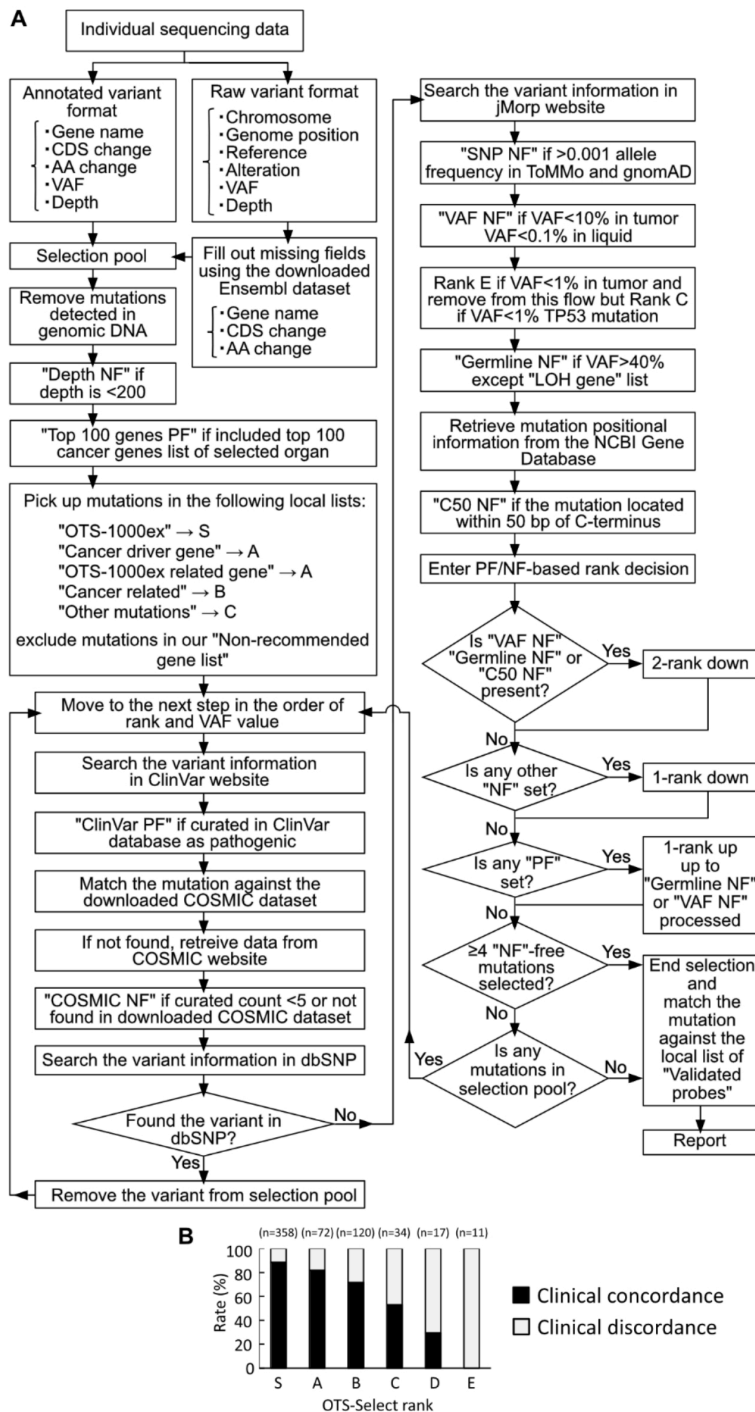


Figure 5

The algorithm “OTS-Select” selects the target mutation for ctDNA monitoring. (A) The input data of sequence must have a mutated gene name, coding DNA sequence (CDS) change, amino acid change, and VAF. This algorithm selects and ranks the mutation from S to E (i.e., S, A, B, C, D, and E). NF, negative flag; PF, positive flag; SNP, single nucleotide polymorphism; LOH, loss of heterozygosity; OTS-1000ex, off-the-shelf probe library including more than 1000 mutations; ClinVar, Clinical variant interpretation

database by NCBI (<https://www.ncbi.nlm.nih.gov/clinvar/>); COSMIC, the Catalogue Of Somatic Mutations In Cancer (<https://cancer.sanger.ac.uk/cosmic>); NCBI, National Center for Biotechnology Information; C50 NF, negative flag within 50 bp counts from the last nucleotide of the c-terminus; jMorp; Japanese Multi Omics Reference Panel; Genome Aggregation Database (gnomAD). (B) Clinical concordance by OTS-Select ranking. Clinical concordant was defined as either detection of ctDNA at least once during the observation period, or consistently negative ctDNA results with no tumor detected by CT scan.

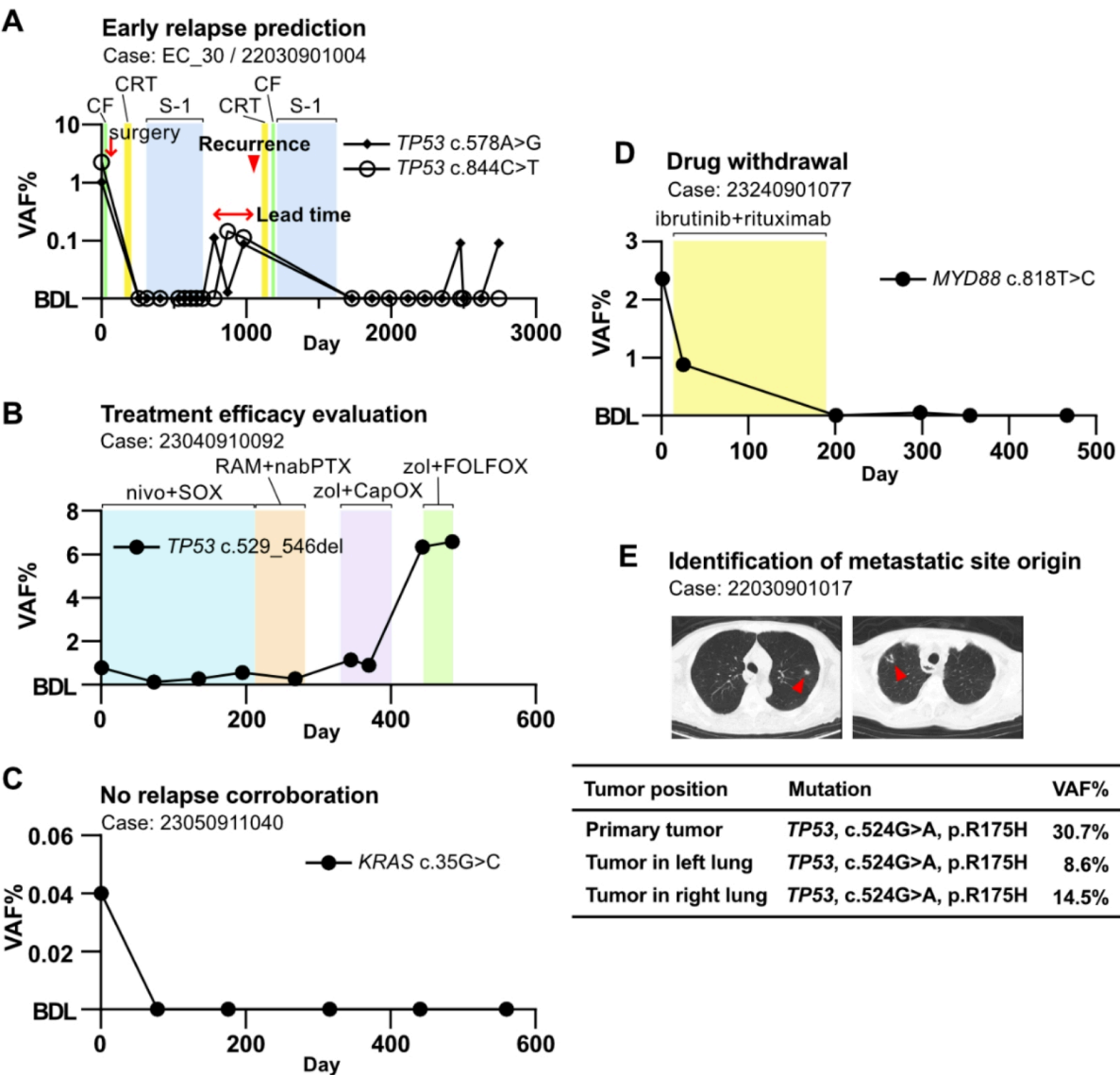


Figure 6

The validity and potential application of ctDNA monitoring. (A) VAF dynamics of an esophagus cancer case with recurrence representing early relapse prediction. (B) VAF dynamics of a gastric cancer case

with multiple chemotherapy representing treatment efficacy evaluation. (C) Post-treatment (i.e., surgery and chemotherapy) VAF dynamics in a colon cancer case representing no relapse corroboration. Although no treatment was administered during the observation period, the ctDNA detected at the initial time point spontaneously declined to undetectable levels thereafter. (D) VAF dynamics in a Waldenström macroglobulinemia patient who discontinued treatment due to adverse events, with continued follow-up to monitor for potential re-elevation of ctDNA level. (E) An esophageal cancer case with two tumors in the left and right lungs. CRT, chemoradiotherapy; CF, cisplatin + 5-fluorouracil; nivo+SOX, nivolumab + S-1 + oxaliplatin; RAM+nabPTX, ramucirumab + nab-paclitaxel; zol+CapOX, zolbetuximab + capecitabine + oxaliplatin; zol+FOLFOX, zolbetuximab + leucovorin + 5-fluorouracil + oxaliplatin.

Supplementary Files

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