Real-World Utilization and Performance of Circulating Tumor DNA Monitoring to Predict Recurrence in Solid Tumors

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ABSTRACT

PURPOSE Circulating tumor DNA (ctDNA) is a novel biomarker to monitor treatment response and predict cancer recurrence. However, the real-world performance of ctDNA monitoring is not well characterized in underrepresented populations such as the Southeast Asians.

METHODS This retrospective analysis included patients with cancer who had commercial ctDNA tests (K-Track, Gene Solutions, Vietnam) between August 2022 and December 2023. A personalized tumor-informed ctDNA assay was performed for 623 patients and 815 plasma samples to quantify ctDNA before and after treatment. Clinical data of minimum 6 months after the last ctDNA test were available for 263 early-stage patients to analyze the prognostic value of ctDNA.

RESULTS In the early-stage I-III, preoperative ctDNA detection rates were 66.7%, 84.6%, 54.3%, 52.6%, 93.3%, and 75.0% for lung, colorectal, breast, gastric, liver, and ovarian cancers, respectively. After surgery, 84.4% (38/45) of patients with recurrence had ctDNA detected in the plasma, while 96.3% (210/218) of patients with no recurrence had negative results. Postoperative ctDNA positivity significantly increased the risk of recurrence ($P < .001$) in lung (hazard ratio [HR], 71.3 [95% CI, 17.6 to 287.8]), colorectal (HR, 44.3 [95% CI, 11.3 to 173.2]), breast (HR, 37.6 [95% CI, 3.09 to 456.8]), and gastric (HR, >100 [95% CI, 26.9 to >100.0) cancers. In the metastatic stage IV, pretreatment ctDNA detection rates were 80.0%, 87.7%, 73.3%, 70.6%, 91.7%, and 81.8% for lung, colorectal, breast, gastric, liver, and ovarian cancers, respectively. Case studies were presented to demonstrate utilization of ctDNA at all cancer stages.

CONCLUSION ctDNA was a strong prognostic biomarker to monitor patients during cancer management.

ACCOMPANYING CONTENT

Appendix

Data Sharing Statement

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INTRODUCTION

Cancer incidence in Asia accounts for almost 50% of global cases and the burden of cancer continues to rise rapidly, partly because of population aging.[1,2] Cancer mortality showed a decreasing trend in some developed countries in the region, but the mortality rate remains high in Southeast Asia,[1,2] with one of the top reasons being cancer recurrence. Current cancer monitoring during and after treatment primarily relies on imaging methods and blood-based tumor markers such as carcinoembryonic antigen (CEA), which have limited sensitivity and specificity to detect residual cancer.[3] Circulating tumor DNA (ctDNA) is short DNA fragments released from dying cancer cells into the bloodstream. It has emerged as a noninvasive biomarker that allows real-time monitoring for treatment response and detects post-treatment minimal residual disease.[4,5] Despite solid evidence establishing the strong prognostic value of ctDNA in prospective clinical trials, its adoption in routine clinical practice meets with several challenges, such as complex methodologies, insufficient evidence of clinical utility, and financial burden for patients.[6,7] Further analysis of the utilization and performance of ctDNA monitoring in real-world cancer management is imperative to demonstrate the reliability and effectiveness of the test in clinical decision making.

For underrepresented patient populations such as the Southeast Asians, clinical validation data of ctDNA monitoring is even more scarce, despite the high cancer prevalence and mortality rate. This could be attributed to accessibility and affordability issues of current sophisticated ctDNA technologies, particularly for developing countries.

CONTEXT

Key Objective

Is circulating tumor DNA (ctDNA) testing feasible, reliable, and accurate to monitor treatment response and detect recurrence in patients with cancer in Southeast Asia?

Knowledge Generated

In this real-world multicenter study, ctDNA testing showed high pretreatment detection rates, including cases of low-quality pathologic specimens. Postoperative ctDNA could detect recurrence several months before clinical diagnosis with high sensitivity and high specificity in multiple types of solid tumors.

Relevance *(Y. Chavarri-Guerra)*

The relevance of this study lies in its investigation of the efficacy of ctDNA testing as a biomarker among Southeast Asian patients with cancer, a demographic often underrepresented in clinical research. This research showed that ctDNA monitoring not only accurately identifies treatment responses and the risk of recurrence but also performs well in cases with limited pathologic specimen quality; this manuscripts also offers insights that could enhance personalized cancer management in diverse populations.*

Plain Language Summary *(M. Lewis)*

ctDNA can detect the presence of cancer in the bloodstream, and a Southeast Asian study showed that it is potentially useful in testing a variety of cancers both before and after treatment, when it could also signal recurrence. That said, results could vary based on the organ where the cancer started (for instance, it was most sensitive for cancers starting in the liver but less sensitive for cancers arising in the stomach).†

*Relevance section written by *JCO Oncology Advances* Associate Editor Yanin Chavarri-Guerra, MD, MSc, FASCO.
†Plain Language Summary written by *JCO Oncology Advances* Associate Editor Mark Lewis, MD.

Moreover, the implementation of genomic profiling and next-generation sequencing has been challenging in the region, because of health budget constraint, lack of trained personnel and certified laboratories, as well as varying quality of pathologic tissue specimens necessitating protocol optimization.[6,8] Lack of awareness among health care professionals and high rate of loss to follow-up also discourage implementation of clinical trials using ctDNA to monitor patients longitudinally.[8,9] Therefore, the lack of data further delays the adoption of this new innovative tool in local practice.

We previously developed an affordable personalized tumor-informed assay, K-Track, to monitor ctDNA in multiple solid tumors and validated the test in prospective clinical trials to predict cancer recurrence.[10-13] To our knowledge, this retrospective study is the first to characterize the status of real-world utilization and clinical performance of ctDNA testing in Southeast Asia, mainly in Vietnam.

METHODS

Patients and Sample Collection

This retrospective multicenter real-world study included 670 patients who had either lung, colorectal, breast, gastric, liver, or ovarian cancer and had at least one ctDNA test (K-Track, Gene Solutions) between August 2022 and December 2023. After exclusion of 47 patients because of incomplete clinical information, 623 patients were included in the analysis. In the cohort of early-stage cancer, inclusion criteria were patients diagnosed with stage I-III cancer, eligible for curative-intent surgery, and having ctDNA test prescribed at any time point deemed suitable by the treating physicians. For a subset of these patients, the clinical follow-up data of minimum 6 months after the last ctDNA test were provided by the physicians and hence used to analyze the prognostic value of ctDNA to predict recurrence. Clinical recurrence and/or metastasis were verified by radiographic (computed tomography [CT] scan and magnetic resonance imaging imaging) or histopathologic evidence. In the cohort of metastatic cancer, inclusion criteria were patients diagnosed with stage IV cancer and having ctDNA tested before starting any treatment. Selected patients with clinical follow-up data of minimum 6 months after the last post-treatment ctDNA test were analyzed only for case studies. It is important to note that both the ctDNA results and clinical data were preexisting and hence analyzed retrospectively. All patient demographics are provided in Appendix Table A1, and the study design and ctDNA analysis workflow are illustrated in Figure 1 (created with BioRender.com).

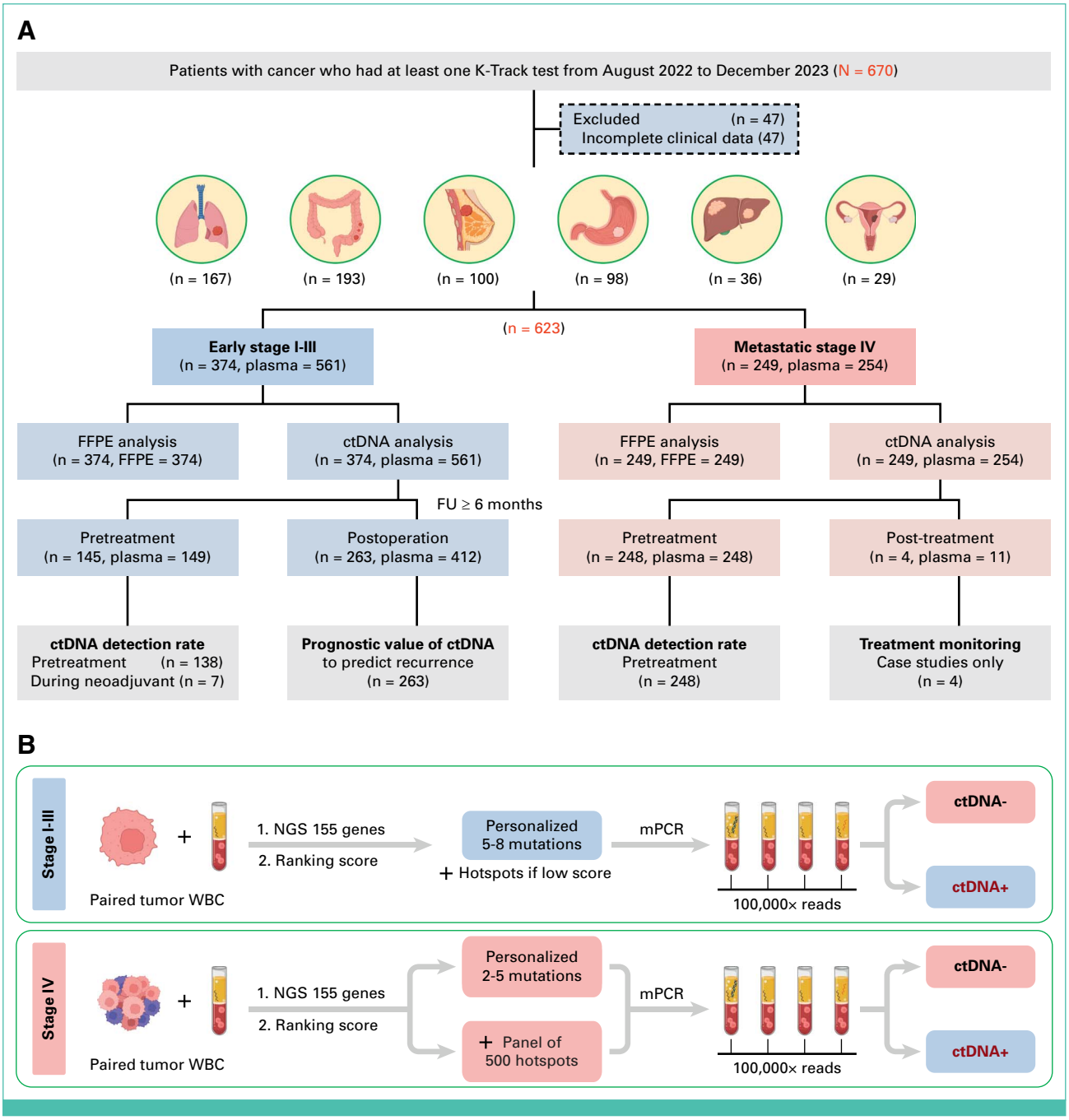

FIG 1. Study design and ctDNA analysis workflow. (A) The number of patients with cancer, their tissue, and serial plasma samples that were included in the study. The patients had at least one commercial ctDNA test from August 2022 to December 2023 and for the analysis of prognostic value of ctDNA, selected patients were followed up for at least 6 months from the last ctDNA test. (B) For patients at early stage I-III, paired tumor FFPE and WBC DNA samples were sequenced to identify tumor-specific mutations in 155 cancer-associated genes. Top personalized mutations with the highest ranking scores were used to detect ctDNA in plasma samples by mPCR and ultradeep sequencing. For patients at metastatic stage IV, besides personalized mutations, an additional panel of approximately 500 hotspot mutations specific to each cancer type was added to the analysis. ctDNA, circulating tumor DNA; FFPE, formalin-fixed paraffin-embedded; mPCR, multiplex PCR; PCR, polymerase chain reaction.

The study was approved by the institutional ethics review board of the Medical Genetics Institute for the analysis of deidentified genomic and clinical data (approval No.: 03/2023/CT-VDTYH; 03/2024/CT-VDTYH). All patients provided written informed consent. The ctDNA test provider remained blinded to the clinical outcomes until final data compilation.

Tumor Variant and Plasma ctDNA Analysis

Formalin-fixed paraffin-embedded (FFPE) and matching WBCs samples were processed following the established procedure of the K-Track assay.[10,11,13] Tumor-specific variant ranking, plasma ctDNA, and limit of detection analysis were described previously[10,11,13] and in Appendix 1 Methods.

Statistical Analysis

For comparisons of categorical variables including ctDNA and imaging test frequency and interval among different groups, Fisher's exact test was used. Disease-free survival (DFS) was measured from the time of curative-intent treatment to clinically diagnosed recurrence or cancer-related death. Cox proportional hazard regression was used to calculate the hazard ratio (HR) of postoperative ctDNA status. Survival curves were estimated by the Kaplan–Meier method and log-rank test. All statistical analyses were conducted using GraphPad Prism, and significance was determined at $P < .05$.

RESULTS

Study Design and Cohorts

This retrospective study included 623 patients with lung (n = 167), colorectal (n = 193), breast (n = 100), gastric (n = 98), liver (n = 36), and ovarian (n = 29) cancers (Fig 1A). In the cohort of early-stage cancer, there were 374 patients, with median age 60 years and TNM classification of mainly stage III (23.6%) and stage II (16.8%) followed by stage I (12.8%; Appendix Table A1). A total of 374 FFPE and 561 plasma samples were analyzed, in which 149 plasma samples were collected before any treatment to evaluate ctDNA detection rate; 412 samples from 263 patients were tested after surgery and had clinical data for at least 6 months after the last ctDNA test, and therefore were analyzed for the prognosis value of ctDNA to predict recurrence (Fig 1A). Among these 263 patients, 45 of them were diagnosed with local recurrence (23/45) and distant metastasis (22/45; Appendix Table A1). In a separate cohort of metastatic cancer, there were 249 patients, with median age 60 years and all provided plasma samples before the start of any treatment to evaluate ctDNA detection rate. Post-treatment ctDNA results and clinical information were analyzed as case studies for four patients only.

All FFPE and plasma samples were subjected to the commercial K-Track workflow to identify all tumor-specific variants (Fig 1B). Our scoring algorithm was used to select top mutations for each patient, which were then used to detect ctDNA in the plasma using bespoke multiplex polymerase chain reaction and ultradeep sequencing (Fig 1B). For some early-stage patients without highly scored mutations and for all metastatic patients, we added a panel of approximately 500 hotspot mutations to detect plasma mutations absent from the corresponding FFPE. For analytical validation of ctDNA detection, Seraseq ctDNA reference materials titrated at VAFs of 0.5%, 0.25%, 0.1%, 0.05%, 0.025%, and 0.00% were used. The observed VAFs were comparable with the expected VAFs, with high repeatability and reproducibility (Appendix Fig A1A). Variants could be detected at variant allele frequency (VAF) levels down to 0.025%, and the specificity using the reference with 0.00% VAF was 99.1% (Appendix Fig A1B).

Real-World Utilization of ctDNA Monitoring

We first sought to understand the clinical context that ctDNA tests were being prescribed in real-world practice. ctDNA was prescribed at all phases of cancer management (Fig 2A). Particularly after curative-intent surgery, majority of ctDNA testing was performed at 2-4 weeks after surgery (90.0%) compared with postadjuvant therapy (10.0%). During follow-up, routine surveillance accounted for 82.9% of the tests, besides suspected cases (17.1%; Fig 2A). The frequency and interval of ctDNA testing were next analyzed by cancer types and patient risk factors. In colorectal, breast, gastric, liver, and ovarian cancers, most physicians ordered ctDNA test for only one time (69.7%, 75.0%, 68.5%, and 88.9%, respectively; Fig 2B). For lung and liver cancers, more than 50% of patients had ctDNA tested for at least 2 times. The testing frequency did not seem to be affected by disease stage and previous ctDNA results (Fig 2B). For patients having at least two ctDNA tests, majority repeated ctDNA test every 3-6 months (Fig 2C). The interval between consecutive tests was not affected by disease stage, but probably by the initial ctDNA results. Although the majority of patients (76.1%) with negative ctDNA results waited for 3-6 months or longer to test again, 52.6% of those with positive ctDNA results repeated the test in <3 months (Fig 2C).

We then compared the frequency and interval of imaging that patients received after knowing their ctDNA results. The proportion of ctDNA-positive patients (79.6%) having their next imaging performed in <3 months after the ctDNA test was significantly higher than that of ctDNA-negative patients (11.2%; Fig 2D). This trend applied to all cancer stages and cancer types. Majority of those with ctDNA negativity seemed to follow standard of care to have imaging performed at 3-6 months (28.5%) or more than 6 months (60.3%) after ctDNA testing (Fig 2D).

Performance of ctDNA Monitoring in Early-Stage Cancer

In pretreatment plasma samples, the ctDNA detection rates were 66.7%, 84.6%, 54.3%, 52.6%, 93.3%, and 75.0% for lung, colorectal, breast, gastric, liver, and ovarian cancers, respectively (Fig 3A), with VAF levels mostly below 1% (Fig 3B). There were 4.0% (15/374) tumor samples with no highly scored mutations, and the addition of hotspot panels helped detect ctDNA in 46.7% (7/15) of these samples, hence reducing the false-negative rate in pretreatment samples (Fig 3A). There was no correlation between VAF of a mutation

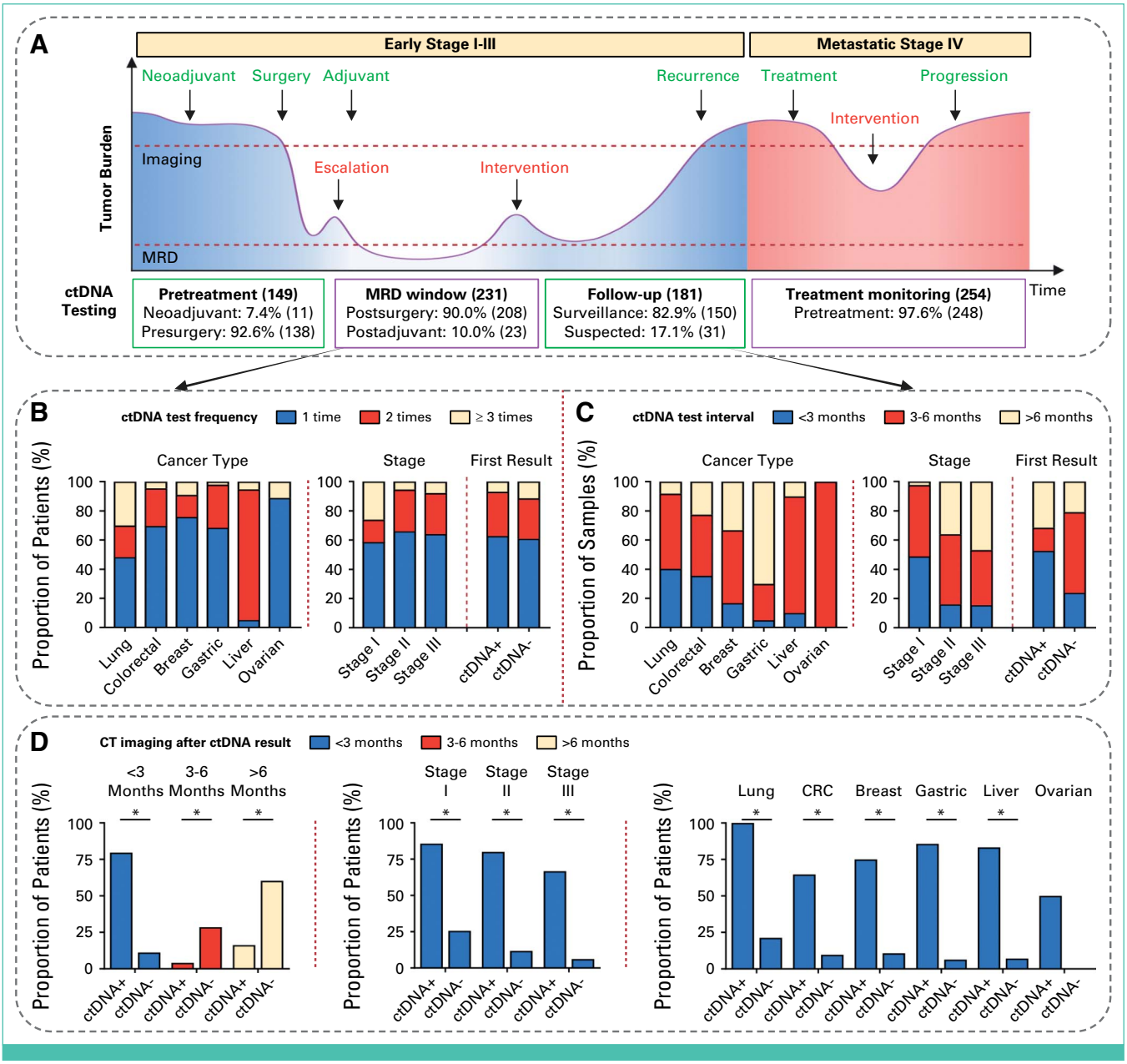

FIG 2. Real-world utilization of ctDNA monitoring. (A) A timeline of ctDNA testing through different phases of cancer management and its potential applications to guide therapy escalation or intervention. In real-world practice, the number of ctDNA tests prescribed by physicians was indicated at each time window. (B) The frequency and (C) the interval of ctDNA testing in early-stage patients after surgery were analyzed by cancer type and presence of risk factors including TNM stage and first ctDNA result. (D) The frequency and interval of imaging that patients received after knowing their ctDNA results. *P < .05, Fisher's exact test. CRC, colorectal cancer; CT, computed tomography; ctDNA, circulating tumor DNA; MRD, minimal residual disease.

in the plasma and its VAF in the matching FFPE (Appendix Fig A2A). Several factors could affect ctDNA detection rate (Appendix Tables A2-A4), including biologic factors such as the low ctDNA-shedding nature and location of the tumors (Appendix Fig A2B), and technical factors such as sample quality and error in polymerase chain reaction and sequencing. In our study, 16.1% of FFPE samples had low DNA quality and 4.2% had low tumor fraction (Appendix Fig A2C). We also frequently observed hemolysis and WBC lysis in plasma samples that were stored and transported for more than 3 days. As WBC lysis leads to gDNA contamination, the sensitivity to detect mutations with VAF <1% declined rapidly as the fraction of cell-free DNA in plasma decreased below 50% (Appendix Fig A2D). For mutations with VAF above 1%, the absolute VAF quantification was reduced as ctDNA was diluted in the presence of gDNA contamination (Appendix Fig A2D).

For postoperative ctDNA monitoring, 84.4% (38/45) of patients with recurrence had ctDNA detected in the plasma,

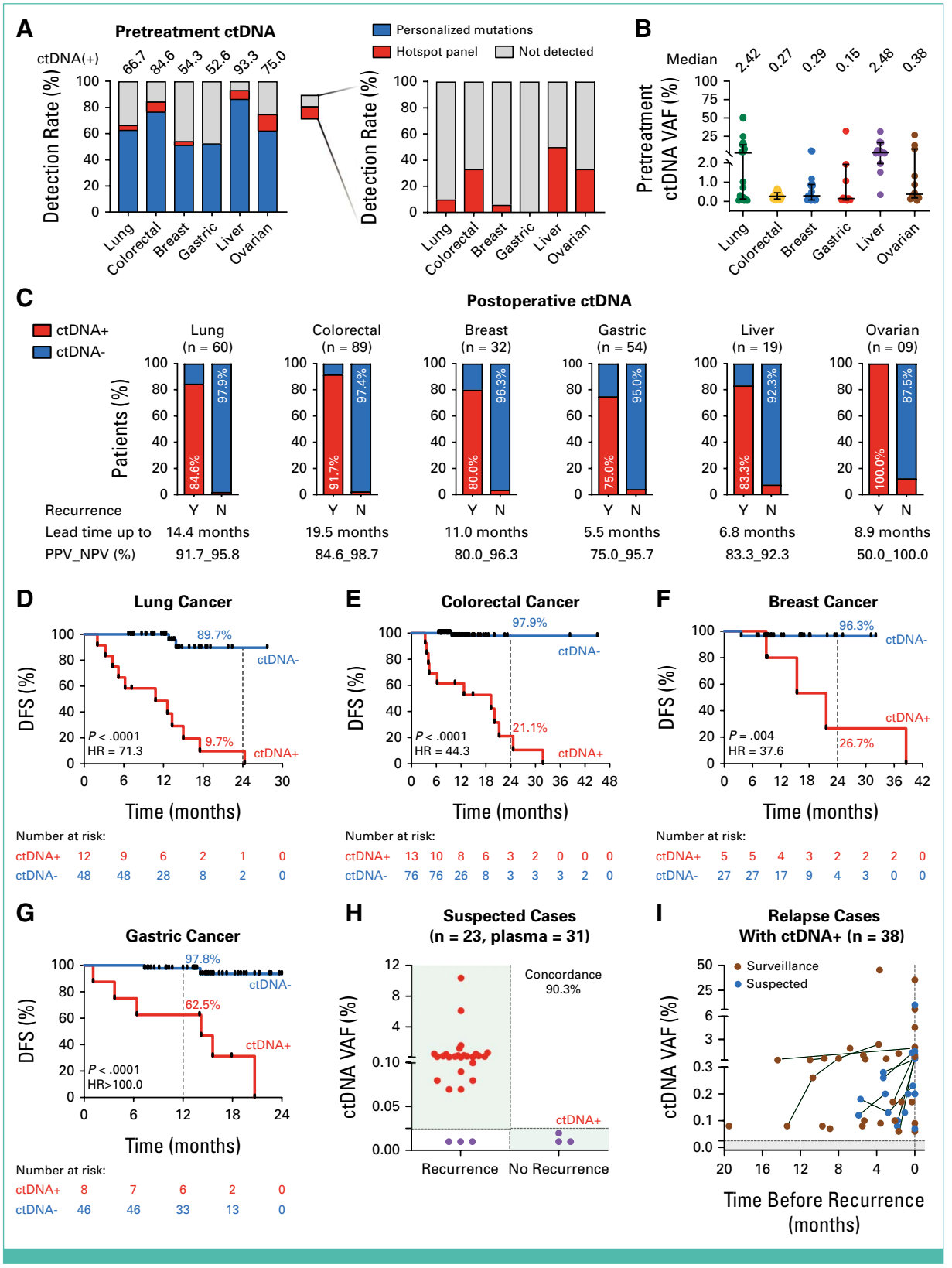

FIG 3. Detection rate and prognostic value of ctDNA in early-stage cancer. (A) ctDNA detection rate in pretreatment plasma among cancer types. The addition of the hotspot panel improved the detection rate for patients with no highly scored mutations. (B) VAF of ctDNA in pretreatment plasma. (C) Sensitivity, specificity, PPV, and NPV of postoperative ctDNA to predict recurrence in different types of cancer and the maximal lead time before clinical diagnosis. (D-G) Kaplan-Meier analysis of DFS for patients stratified by postoperative ctDNA status. (H) For patients suspected of recurrence, ctDNA (continued on following page)

FIG 3. (Continued). results showed concordance of 87.0% with later diagnosis. (I) No correlation between VAF of ctDNA and time to recurrence was observed in both suspected and routine surveillance cases. ctDNA, circulating tumor DNA; DFS, disease-free survival; N, no; NPV, negative predictive value; PPV, positive predictive value; VAF, variant allele frequency; Y, yes.

while 96.3% (210/218) of patients with no recurrence had negative ctDNA results. In lung, colorectal, breast, gastric, liver, and ovarian cancers, ctDNA detection showed high sensitivity to predict recurrence at 84.6%, 91.7%, 80.0%, 75.0%, 83.3%, and 100.0% respectively; and the specificity was 97.9%, 97.4%, 96.3%, 95.0%, 92.3%, and 87.5% respectively (Fig 3C). The lead time of ctDNA detection before clinical diagnosis was up to 14.4, 19.5, 11.0, 5.5, 6.8, and 8.9 months for lung, colorectal, breast, gastric, liver, and ovarian cancers, respectively (Fig 3C). Postoperative ctDNA status was found to be a significant prognostic factor for DFS as ctDNA positivity increased the risk of recurrence by 71.3 times in lung cancer (HR, 71.3 [95% CI, 17.6 to 287.8; Fig 3D), by 44.3 times in colorectal cancer (HR, 44.3 [95% CI, 11.3 to 173.2]; Fig 3E), by 37.6 times in breast cancer (HR, 37.6 [95% CI, 3.09 to 456.8]; Fig 3F), and by >100 times in gastric cancer (HR, >100 [95% CI, 26.9 to >100.0]; Fig 3G). Specifically for 23 patients with suspected but unconfirmed metastasis at the time of ctDNA testing, 31 blood samples were analyzed and the concordance of ctDNA results with later diagnosis was 90.3% (28/31; Fig 3H). Among all relapse patients with ctDNA-positive, there was no relationship between the VAF dynamics of ctDNA and the lead time (Fig 3I).

We next presented case studies to illustrate clinical utilization of ctDNA testing for early-stage cancer (Fig 4). The first patient ZMB501 was a 35-year-old woman diagnosed

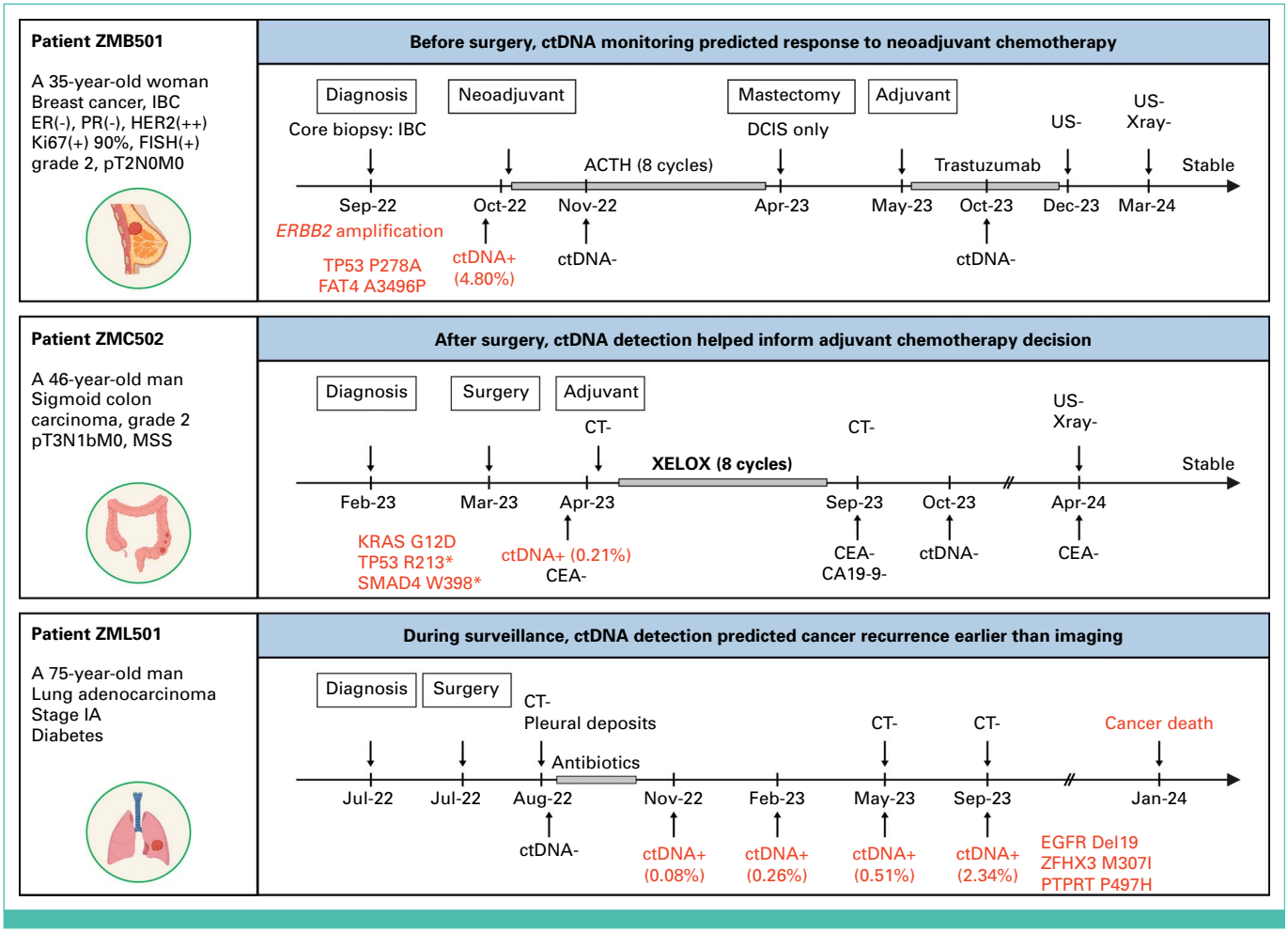

FIG. 4. Case studies showing clinical utilization of ctDNA in early-stage cancer. Case studies demonstrating the use of ctDNA to monitor response to neoadjuvant chemotherapy (ZMB501), to detect postoperative MRD to inform adjuvant chemotherapy decision (ZMC502), and to predict recurrence early during post-treatment surveillance (ZML501). CEA, carcinoembryonic antigen; CT, computed tomography; ctDNA, circulating tumor DNA; DCIS, ductal carcinoma in situ; ER, estrogen receptor; FISH, fluorescent in situ hybridization; HER2, human epidermal growth factor receptor 2; IBC, invasive breast carcinoma; MRD, minimal residual disease; MSS, microsatellite stable; PR, progesterone receptor; US, ultrasound; XELOX, capecitabine plus oxaliplatin.

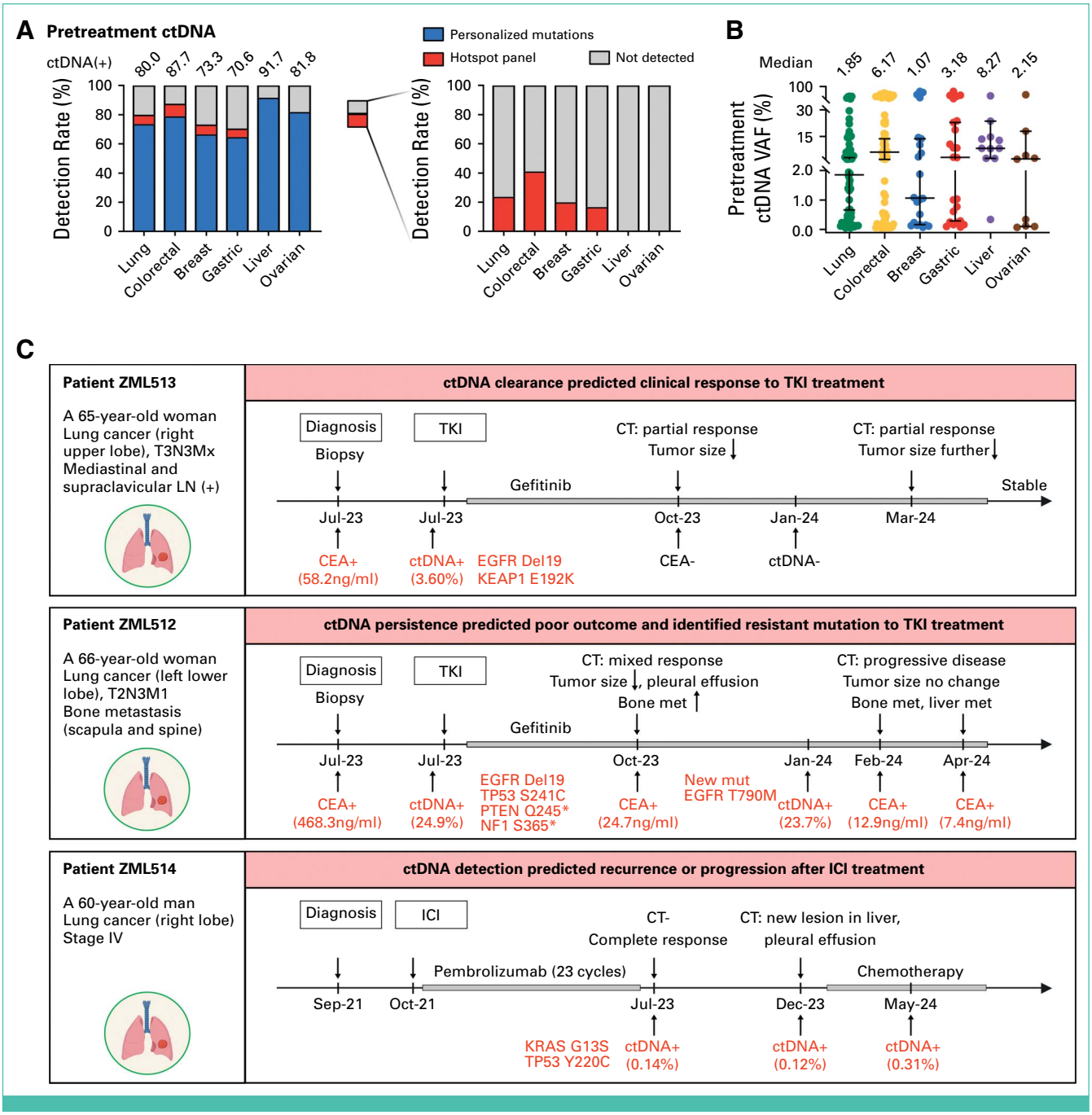

FIG 5. Detection rate and clinical utilization of ctDNA in metastatic cancer. (A) ctDNA detection rate in pretreatment plasma among cancer types. The addition of the hotspot panel improved the detection rate. (B) VAF of ctDNA in pretreatment plasma. (C) Case studies demonstrating the use of ctDNA to monitor response to targeted TKIs (ZML513 and ZML512), while also identifying resistant mutation(s) to treatment (ZML512), and to predict recurrence or progression after treatment with ICI. CT, computed tomography; ctDNA, circulating tumor DNA; ICI, immune checkpoint inhibitor; TKI, tyrosine kinase inhibitor; VAF, variant allele frequency.

with invasive breast carcinoma, estrogen receptor–negative, progesterone receptor–negative, human epidermal growth factor receptor 2–positive (+) by immunohistochemistry (IHC), fluorescent in situ hybridization–positive (FISH+), pT2N0M0. Genomic profiling of the core biopsy identified *ERBB2* amplification, supporting the IHC and FISH results. Pretreatment ctDNA was detected at VAF of 4.80%, with two tumor-derived mutations TP53 P278A and FAT4 A3496P. During neoadjuvant chemotherapy, ctDNA was cleared. After mastectomy, pathologic evaluation of surgical specimens found disappearance of invasive disease and only residual ductal carcinoma in situ, suggesting pathologic complete response. At the 12-month visit, her ctDNA was negative, and the patient remained stable at the 21-month visit. The use of ctDNA to predict neoadjuvant response was further demonstrated in patient ZMC501 who had rectal cancer

pT4bN2M0; his postneoadjuvant persistence of ctDNA correlated with the clinical evaluation of pathologic partial response (Appendix Fig A3A). The second patient ZMC502 was a 46-year-old man, diagnosed with sigmoid colon carcinoma, pT3N1bM0—low-risk stage III. At 4 weeks after surgery, his CEA was negative but ctDNA was positive at 0.21%. This risk factor was taken into consideration for adjuvant therapy decision, and he then received eight cycles of capecitabine plus oxaliplatin, which cleared ctDNA and the patient remained stable (Fig 4). Finally, ZML501 was a 75-year-old man diagnosed with stage IA lung adenocarcinoma. At 4 months after surgery, ctDNA was positive at 0.08% and the VAF gradually increased for 1 year despite no clinical symptoms and all normal CT scans. He later died of cancer metastasis, 14 months after his first positive ctDNA result (Fig 4). This case demonstrated the utility of ctDNA testing to detect recurrence early during surveillance.

Performance of ctDNA Monitoring in Metastatic Cancer

In patients with metastatic cancer, pretreatment ctDNA detection rates were 80.0%, 87.7%, 73.3%, 70.6%, 91.7%, and 81.8% for lung, colorectal, breast, gastric, liver, and ovarian cancers, respectively (Fig 5A). Similar to the early stage, the addition of hotspot panel reduced false negativity in ctDNA detection rate. The median VAFs of ctDNA in the metastatic stage were higher compared with those in the early stage for all cancer types except lung cancer (Fig 5B).

We next presented case studies showing the clinical utilization of ctDNA testing to detect actionable/resistant mutations and monitor treatment response (Fig 5C). The first patient ZML513 was a 65-year-old woman, diagnosed with lung cancer T3N3Mx and prescribed with gefitinib as EGFR Del19 was detected in both biopsy FFPE and plasma samples. ctDNA was cleared after 6 months of treatment, corresponding with the partial response evaluated by RECIST 1.1 criteria at the 9-month visit (Fig 5C). By contrast, the second patient ZML512 was a 66-year-old woman, diagnosed with lung cancer T2N3M1 and bone metastasis. She was also treated with gefitinib after EGFR Del19 was found in both FFPE and plasma samples. During treatment, CEA level kept decreasing, but ctDNA was persistent and positive for a new resistant mutation EGFR T790M. CT scan concluded progressive disease as the bone and liver metastases progressed (Fig 5C). Unlike the above two patients, in patient ZML511, a 71-year-old man with stage IV lung cancer, there was a discrepancy between the mutations detected in FFPE (KRAS G12V, 21%; and EGFR L858R, 4%) and plasma (only KRAS G12V, 0.11%). The patient was prescribed osimertinib, but after 4 months, ctDNA remained positive with KRAS G12V and a new TP53 mutation; CT scan confirmed progressive disease (Appendix Fig A3B). Finally, a patient ZML514 was a 60-year-old man, diagnosed with lung cancer stage IV (Fig 5C). He had completed 23 cycles of pembrolizumab before stopping the treatment because of financial reasons. At that point, his CT scans were all negative, suggesting a complete response; ctDNA result was, however, positive at 0.14%. Six months later, the patient presented with pleural effusion and a new lesion in liver, and was diagnosed with recurrence.

DISCUSSION

In this retrospective real-world study, we first evaluated when and how often ctDNA testing was being used in clinical practice. Although the samples included in the study provided only a snapshot of all commercial samples, the analysis indicated that ctDNA was indeed being used throughout all phases of cancer management. However, majority of the patients had ctDNA tested for only one time, much less frequently compared with other blood biomarkers such as CEA. This was likely because of the high cost of the assay not yet covered by insurance. For patients repeating the ctDNA test, it was mostly done every 3-6 months, aligning with the recommended follow-up schedule. It has been consistently demonstrated that longitudinal monitoring of ctDNA with appropriate frequency helped avoid false-negative or false-positive results.[3,14,15]

In the cohort of early-stage cancer, our results showed that ctDNA was a strong prognostic biomarker with high sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) to predict recurrence ahead of clinical diagnosis by imaging or pathology. Despite our fairly streamlined and cost-effective design compared with multiple platforms using whole-exome sequencing and more mutations to track,[15-17] our ctDNA detection rate and performance to detect recurrence in all cancer types were noninferior to previous clinical trials.[16-25] In comparison with the limited reports of real-word ctDNA monitoring, our assay performance was also comparable. Specifically, Martin et al[26] reported sensitivity, specificity, PPV, and NPV of ctDNA to predict recurrence in early-stage lung cancer at 80.0%, 95.8%, 88.9%, and 92.0%, respectively, while ours were 84.6%, 97.9%, 91.7%, and 95.8%, respectively. Similarly, Maddalena et al[27] examined the real-world utilization of ctDNA in a large cohort of patients with colorectal cancer, and showed the sensitivity and specificity to detect molecular recurrence at 92.5% and 83.6%, respectively, while our data, albeit in a much smaller cohort, were 91.7% and 97.4%, respectively. Furthermore, we observed that patients with ctDNA-positive results had subsequent imaging performed earlier than those with negative results. Although this potentially helped with earlier recurrence detection for timely intervention, it might also result in unnecessary imaging and confusion when the lead time between molecular and clinical recurrence was long. Therefore, future practice guidelines of how to manage patients on the basis of ctDNA test results is critical to balance the risks and benefits.

We then compared the real-world performance with our own data in previous prospective clinical trials and found that the detection rates of ctDNA in both pretreatment and relapse

samples were slightly lower in the real world compared with the clinical trial setting.[10-12] This was expected because of much less frequent blood sampling as well as varying quality of FFPE and blood samples after storage and transportation. To overcome the challenges, we built a hotspot mutation panel for each type of cancer and found that it substantially improved the ctDNA detection rate. For metastatic cancer, this panel was also used to screen for resistant mutations arising after a period of treatment. Interestingly, we observed the use of ctDNA in many patients suspected of recurrence because of equivocal imaging result, which was an unique scenario not usually investigated in prospective clinical trials. ctDNA result showed a high concordance rate of 90.3% with subsequent diagnosis of these patients, supporting the use of ctDNA to assist future clinical decision making.

In the cohort of metastatic cancer, the detection rates and the VAF levels of ctDNA were generally higher than the early stage for all examined types of cancer. This was likely attributed to the high tumor burden and the presence of cancer cells in circulation and distant organs at the advanced stage.[28] Nonetheless, it should be noted that a significant proportion of patients with metastatic cancer still had ctDNA undetected in the plasma; or if detected, the VAF level of ctDNA could be relatively small. The major biological reasons were the low ctDNA-shedding nature of tumors such as adenocarcinoma of the lung,[29] or location of metastatic lesions such as the peritoneum, bone, and brain.[28,30] Technical problems often involved biased sampling and tumor heterogeneity, particularly with small biopsy samples. Therefore, the sensitivity of a ctDNA assay could be improved by the addition of hotspot mutation panels like in our study, or by combination with other nonmutation features of ctDNA such as methylation and fragment length.[15,31] Recently, Gouda et al[32] proposed LB-RECIST, or liquid biopsy-RECIST, that integrated the dynamic changes in ctDNA with current RECIST criteria to predict tumor response. The potential advantages of incorporating ctDNA, as also demonstrated in our case studies, are to predict therapeutic response earlier than imaging,[33,34] to recognize pseudoprogression in immune checkpoint inhibitor treatment,[35,36] and in the case of tyrosine kinase inhibitor therapies, to identify resistant mutations to inform therapeutic intervention.[37]

The major limitations of our study were a relatively small number of patients and blood samples collected at different time points, as well as short follow-up time. This could lead to overestimation of our test performance and the hazard ratios for ctDNA-positive patients. Moreover, the retrospective design with limited number of patients per cancer type led to missing clinical information and possibly sampling bias; hence, the ctDNA performance might not be generalizable, especially across different cancer subtypes. Nevertheless, we demonstrated robust ctDNA performance and provided compelling evidence for its clinical utilization throughout different stages of cancer management. As the first study, to our knowledge, of real-world utilization of ctDNA in Southeast Asia, this result hopefully contributes to the mounting evidence of ctDNA monitoring globally and helps raise awareness of ctDNA as well as precision medicine in developing countries.

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DISCLAIMER

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DATA SHARING STATEMENT

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AUTHOR CONTRIBUTIONS

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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APPENDIX 1. METHODS

Tumor Variant Calling and Ranking

Genomic DNA from formalin-fixed paraffin-embedded (FFPE) and matching WBC samples were processed following the established procedure of the K-Track assay.[10,11] Briefly, DNA libraries were hybridized with a custom capture probe set targeting 155 cancer-associated genes (Integrated DNA Technologies, Singapore; Appendix Table A2) that were the most frequently mutated in the top 10 common solid tumor types. For somatic variant calling, sequencing data were analyzed using the Genome Analysis Tool Kit in tumor-normal mode, combined with the Genome Aggregation Database and an in-house panel of 800 normal samples to filter sequencing artifacts, germline variants, and clonal hematopoiesis of intermediate potential variants.[10,11,13] All nonsynonymous mutations were then ranked by a proprietary scoring algorithm[10,11,13] to identify the top driver and clonal mutations to be used for tracking circulating tumor DNA (ctDNA) in the plasma. The maximum ranking score for a mutation was set at 25, and a score <5 was considered low confidence.

Plasma ctDNA Analysis

Cell-free DNA (cfDNA) was isolated from plasma samples using the MagMAX Cell-Free DNA Isolation Kit (Thermo Fisher, Waltham, MA) as previously described.[10,11] cfDNA fragments were amplified using the KAPA HiFi DNA Polymerase (Roche, Basel, Switzerland) and targeted primer pairs (PhuSa Biochem, Vietnam). Besides top-ranked personalized mutations above, a panel of hotspot mutations specific to the cancer type was used in a separate multiplex polymerase chain reaction (PCR) reaction, amplifying approximately 500 hotspot mutations in 37 cancer-associated genes (Appendix Table A2). The panel was added for early-stage cases if no mutation in the tumor achieved a ranking score above 5, and for all metastatic cases. Amplified cfDNA fragments were sequenced on the NextSeq 2000 system (Illumina) and those with < 100,00× coverage were considered unsuccessful and rerun to achieve the minimum read depth. A plasma sample that had at least one mutation with variant allele frequency (VAF) above limit of detection (LOD) was defined as ctDNA-positive. Mean VAF of a sample was calculated as mean of all positive mutations if present. If no mutations were detected, mean VAF was the means of all tracked mutations.

For analytical validation, the LOD was determined using the Seraseq ctDNA Mutation Mix v2 (SeraCare, Milford, MA), which was titrated at 0.5%, 0.25%, 0.1%, 0.05%, 0.025%, and 0.00% VAFs. DNA input of 3-10 ng at each VAF level was used in 7-plex PCR reactions (Appendix Figure A1). Each reaction was run as either duplicates or triplicates on the same day and repeated for three different days. The observed VAF values were compared with the expected VAF values for each mutation to evaluate the analytical performance of the assay, including LOD and interassay and intra-assay coefficients of variation.

TABLE A1. Patient Demographics in the Study

Early Stage I-III	N = 374
Age at diagnosis, years (range)	60 (21-95)
<60, No. (%)	193 (51.6)
≥60, No. (%)	180 (48.1)
Sex, No. (%)	
Female	205 (54.8)
Male	169 (45.2)
Cancer type, No. (%)	
Lung cancer	86 (23.0)
Adenocarcinoma	14
Unknown	72
Colorectal cancer	112 (30.0)
Colon	46
Rectum	7
Unknown	59
Breast cancer	70 (18.7)
Hormone receptor–positive HER2–	10
Hormone receptor–positive HER2+	8
Hormone receptor–negative HER2+	5
Hormone receptor–negative HER2–	3
Unknown	44
Gastric cancer	64 (17.1)
Liver cancer	24 (6.4)
Left lobe	6
Right lobe	11
Unknown	7
Ovarian cancer	18 (4.8)
TNM stage, No. (%)	
I	48 (12.8)
II	63 (16.8)
III	89 (23.8)
Recurrence rate, No. (%)	
Lung cancer	13/60 (21.7)
Colorectal cancer	12/89 (13.5)
Breast cancer	5/32 (15.6)
Gastric cancer	8/54 (14.8)
Liver cancer	6/19 (31.6)
Ovarian cancer	1/9 (11.1)
Recurrence site, No. (%)	
Local	23 (51.1)
Distant	22 (48.9)
Metastatic stage IV	N = 249
Age at diagnosis, years (range)	60 (31-97)
<60, No. (%)	105 (42.2)
≥60, No. (%)	144 (57.8)
Sex, No. (%)	
Female	114 (45.8)

(continued on following page)

TABLE A1. Patient Demographics in the Study (continued)

Early Stage I-III	N = 374
Male	135 (54.2)
Cancer type, No. (%)	
Lung cancer	81 (32.5)
mEGFR	54 (66.7)
mKRAS	15 (18.5)
Colorectal cancer	81 (32.5)
mKRAS	40 (49.4)
Breast cancer	30 (12.1)
mPIK3CA	14 (46.7)
Gastric cancer	34 (13.7)
Liver cancer	12 (4.8)
Ovarian cancer	11 (4.4)

Abbreviations: HER2, human epidermal growth factor receptor 2; *m*, mutated.

TABLE A2. List of Cancer-Associated Genes

150 genes: full coding regions							
ABL1	*BRAF*	*DNMT3A*	*FOXA1*	*KMT2D*	*NOTCH2*	*RBM10*	*TP53*
ACVR2A	*BRCA1*	*DROSHA*	*FOXP1*	*KRAS*	*NRAS*	*RET*	*TRRAP*
ADGRV1	*BRCA2*	*EBF1*	*GATA3*	*LPP*	*NSD1*	*RNF213*	*TSC1*
AFF3	*CACNA1E*	*EGFR*	*GNAS*	*LRP1B*	*NSD2*	*RNF43*	*TSC2*
AKT1	*CAMTA1*	*EP300*	*GPHN*	*MAP2K4*	*NTRK3*	*ROBO2*	*TSHR*
ALB	*CASP8*	*EPHA3*	*GRIN2A*	*MAP3K1*	*PBRM1*	*ROS1*	*TSPOAP1*
ALK	*CDH1*	*EPHA5*	*HLA-A*	*MDM2*	*PDE4DIP*	*RSPO2*	*VHL*
AMER1	*CDK4*	*EPHB1*	*HNF1A*	*MDM4*	*PDGFRA*	*SETD2*	*ZFHX3*
ANK2	*CDKN1A*	*ERBB2*	*HRAS*	*MED12*	*PIK3CA*	*SF3B1*	*ZNF521*
APC	*CDKN2A*	*ERBB3*	*IDH1*	*MET*	*PIK3R1*	*SMAD2*	*ZNF536*
AR	*CHD4*	*ERBB4*	*IDH2*	*MGA*	*PREX2*	*SMAD4*	
ARID1A	*CHEK2*	*ERCC2*	*IL6ST*	*MLH1*	*PTCH1*	*SMARCA4*	
ARID1B	*COL11A1*	*ESR1*	*JAK1*	*MSH6*	*PTEN*	*SOX9*	
ARID2	*CREBBP*	*FAT1*	*JAK2*	*mTOR*	*PTPN13*	*SPOP*	
ATM	*CTNNA2*	*FAT4*	*KDM6A*	*MYCN*	*PTPRB*	*SPTA1*	
ATR	*CTNNB1*	*FBN2*	*KDR*	*NCOR1*	*PTPRD*	*STAG2*	
ATRX	*DDR2*	*FBXW7*	*KEAP1*	*NCOR2*	*PTPRT*	*STK11*	
AXIN1	*DGCR8*	*FGFR2*	*KIT*	*NF1*	*RAD51B*	*TBX3*	
BAP1	*DICER1*	*FGFR3*	*KMT2A*	*NFE2L2*	*RAF1*	*TCF7L2*	
BCOR	*DMD*	*FHIT*	*KMT2C*	*NOTCH1*	*RB1*	*TERT*	

37 genes: approximately 500 hotspot mutations							
ACVR2A	*ARID1A*	*CDH1*	*FBXW7*	*IDH2*	*NRAS*	*RB1*	*TP53*
AKT1	*AXIN1*	*CTNNB1*	*GATA3*	*KEAP1*	*PIK3CA*	*SMAD4*	*ZFHX3*
ALK	*BRAF*	*EGFR*	*GNAS*	*KRAS*	*POLE*	*STK11*	
AMER1	*BRCA1*	*ERBB2*	*HRAS*	*LRP1B*	*PPP2R1A*	*TCF7L2*	
APC	*BRCA2*	*ESR1*	*IDH1*	*MET*	*PTEN*	*TERT*	

TABLE A3. Potential Causes of Inaccurate ctDNA Results

Potential Causes	Proposed Solutions
False positive (ctDNA-positive but no recurrence)	
Technical issue	
Mutation is from CHIP	WBC sequencing and advanced bioinformatics
Mutation is benign or not cancer-associated	Check VAF of ctDNA after 3-6 months
Error from PCR and noise from sequencing (occur at low VAF)	Check VAF of ctDNA after 3-6 months
Biologic issue	
Few residual cancer cells do not progress to recurrence	Check VAF of ctDNA after 3-6 months
Cancer cells are dormant and/or follow-up time is too short	Check VAF of ctDNA after 3-6 months
Test frequency	
ctDNA test before or during treatment	Repeat the test after treatment completion
False negative (ctDNA-negative but recurrence)	
Technical issue	
ctDNA signal is below LOD	Technical limitation of the assay design
Mutation is not covered by the gene panel	Add hotspot panel to increase coverage
Tumor sampling is not representative (eg, Biopsy)	Add hotspot panel to increase coverage
Poor FFPE sample quality: fail to identify mutations	Add hotspot panel to increase coverage
Poor blood sample quality: low ctDNA fraction because of WBC lysis, or low cfDNA input	Collect blood sample again
Biologic issue	
Low ctDNA-shedding tumors (cancer type, location, dormancy)	Clinical limitation of ctDNA-based method
Test frequency	
Previous ctDNA test is more than 6 months before	Repeat the test every 3-6 months

Abbreviations: CHIP, clonal hematopoiesis; cfDNA, cell-free DNA; ctDNA, circulating tumor DNA; FFPE, formalin-fixed paraffin-embedded; LOD, limit of detection; PCR, polymerase chain reaction; VAF, variant allele frequency.

TABLE A4. Comparison of Pretreatment ctDNA Detection Rate in Multiple Studies

Cancer	Study	Design	Cohort, No.	Method	ctDNA Detection
Lung	This study	Real-world	I-III (n = 86) IV (n = 81)	Tumor: 155-gene cfDNA: 5-plex	I-III: 66.7% IV: 80.0%
	Abbosh et al,[30] 2023 (Natera)	Clinical trial	I-III (n = 197)	Tumor: WES cfDNA: 200-plex	I-III: 65.7%
	Chen et al,[38] 2024	Clinical trial	I-III (n = 181)	Tumor: WES cfDNA: panel	I-III: 41.7%
CRC	This study	Real-world	I-III (n = 112) IV (n = 81)	Tumor: 155-gene cfDNA: 5-plex	I-III: 84.6% IV: 87.7%
	Kotani et al,[39] 2023 (Natera)	Clinical trial	II – IV (n = 1,039)	Tumor: WES cfDNA: 16-plex	II-IV: 91.3%
	Reinert et al,[40] 2019	Clinical trial	I-III (n = 125)	Tumor: WES cfDNA: 16-plex	I-III: 88.5%
Breast	This study	Real-world	I-III (n = 70) IV (n = 30)	Tumor: 155-gene cfDNA: 5-plex	I-III: 54.3% IV: 73.3%
	Cutts et al,[41] 2024 (Natera)	Clinical trial	Hormone receptor–positive, HER2+, TNBC (n = 48)	Tumor: WES cfDNA: 16-plex	64.5%
	Garcia-Murillas et al,[42] 2023 (Invitae)	Clinical trial	Hormone receptor–positive, HER2+, TNBC (n = 61)	Tumor: WES cfDNA: 48-plex	67.8%
Liver	This study	Real-world	I-III (n = 24) IV (n = 12)	Tumor: 155-gene cfDNA: 5-plex	I-III: 93.3% IV: 91.7%
	Zhu et al,[20] 2022	Clinical trial	BCLC 0/A (n = 31) BCLC B (n = 10)	Tumor: WES cfDNA: 197	BCLC0-B: 63.4%
	Cai et al,[18] 2019	Clinical trial	Unknown (n = 34)	Tumor: WES cfDNA: panel	100.0%
Gastric	This study	Real-world	I-III (n = 64) IV (n = 34)	Tumor: 155-gene cfDNA: 5-plex	I-III: 52.6% IV: 70.6%
	Yuan et al,[43] 2023	Clinical trial	II-III (n = 100)	Tumor + cfDNA: 425 genes	II-III: 38.0%
	Jang et al,[16] 2020	Clinical trial	I–III (n = 46)	Tumor + cfDNA: 1,021 genes	I-III: 46.3%
Ovarian	This study	Real-world	I-III (n = 18) IV (n = 11)	Tumor: 155-gene cfDNA: 5-plex	I-III: 75.0% IV: 81.8%
	Wang et al,[44] 2024	Clinical trial	I-IV (n = 34)	Tumor: WES cfDNA: 40-plex	I-IV: 88.2%
	Hou et al,[25] 2022	Clinical trial	I-IV (n = 69)	Tumor: WES cfDNA: 16-plex	I-II: 60.0% III-IV: 72.0%

Abbreviations: BCLC, the Barcelona Clinic Liver Cancer staging; cfDNA, cell-free DNA; CRC, colorectal cancer; ctDNA, circulating tumor DNA; HER2, human epidermal growth factor receptor 2; TNBC, triple negative breast cancer; WES, whole exome sequencing.

A Seraseq Reference

Expected VAF (%)	Observed VAF (%)	Repeatability		Reproducibility		Total	
		SD	CV (%)	SD	CV (%)	SD	CV (%)
0.50	0.42	0.11	24.05	0.07	13.99	0.09	19.02
0.25	0.22	0.07	34.68	0.04	18.83	0.06	26.76
0.10	0.08	0.04	52.60	0.02	30.56	0.03	41.58
0.05	0.04	0.04	83.20	0.02	49.23	0.03	66.21
0.025	0.024	0.02	84.04	0.01	46.54	0.01	65.29

B Seraseq Reference

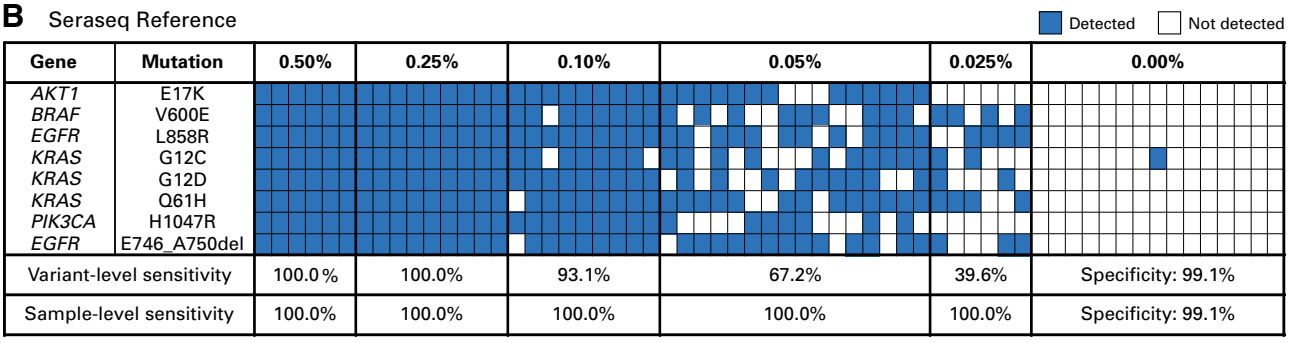

FIG A1. Analytical validation of the K-Track assay. (A) Analysis of VAF, repeatability, and reproducibility of the K-Track assay using Seraseq reference materials. (B) Titration series for Seraseq reference materials to determine the LOD at 0.025%. If one variant had VAF ≥0.025%, the sample was called positive. CV, coefficient of variation; LOD, limit of detection; SD, standard deviation; VAF, variant allele frequency.

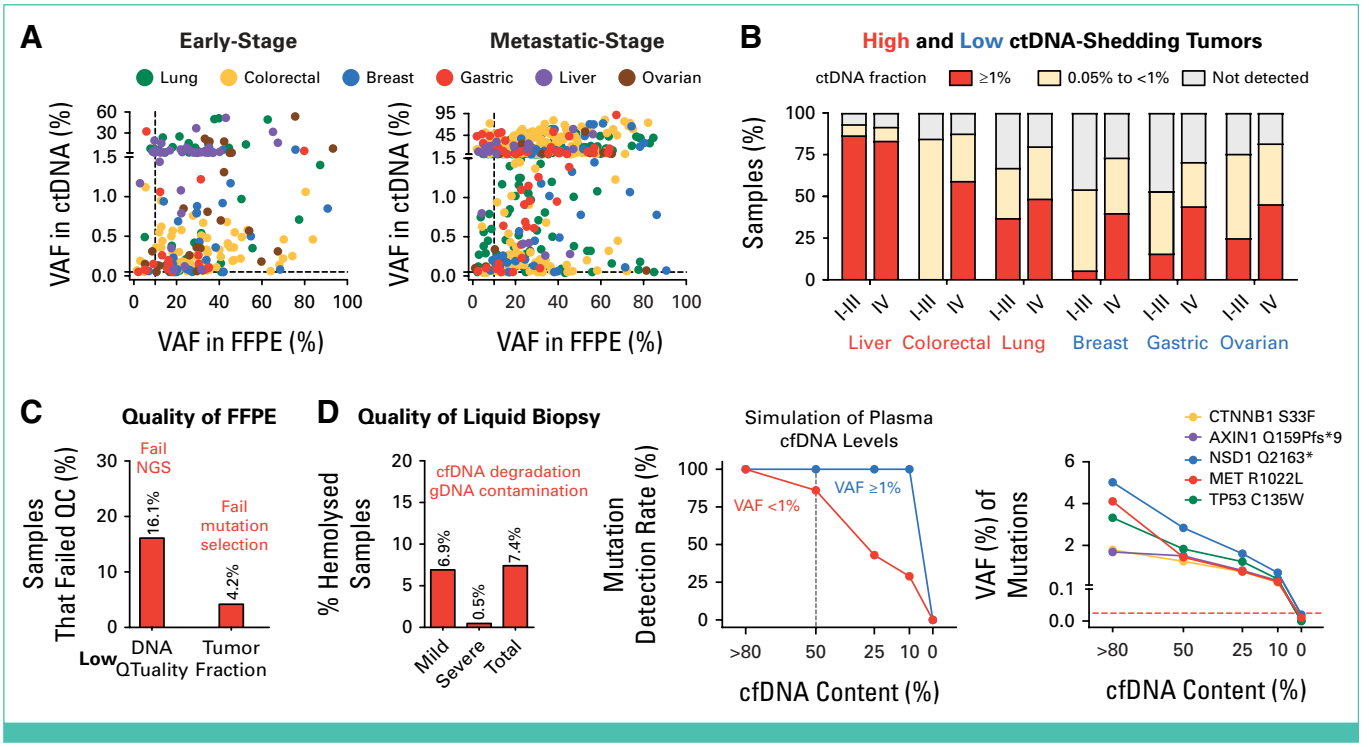

FIG A2. Factors affecting sensitivity to detect ctDNA. (A) There was no correlation between VAF of a mutation in the FFPE and its VAF in the plasma. (B) ctDNA fractions found in plasma samples of the high- and low-ctDNA-shedding tumor types. (C) Quality of FFPE in this real-world cohort. Low DNA quality caused samples to fail depth of NGS, while low tumor fraction (<20%) or biased sampling could result in failure to identify cancer-specific mutations. (D) Quality of liquid biopsy in this real-world cohort. A proportion of samples with hemolysis had gDNA contamination because of WBC lysis and cfDNA degradation. A simulation test showing that reduced cfDNA content because of gDNA contamination lowered the sensitivity to detect variants at VAF below 1%. The absolute quantification of VAF for detected variants was reduced as the cfDNA fraction reduced. cfDNA, cell-free DNA; ctDNA, circulating tumor DNA; FFPE, formalin-fixed paraffin-embedded; NGS, next-generation sequencing; VAF, variant allele frequency.

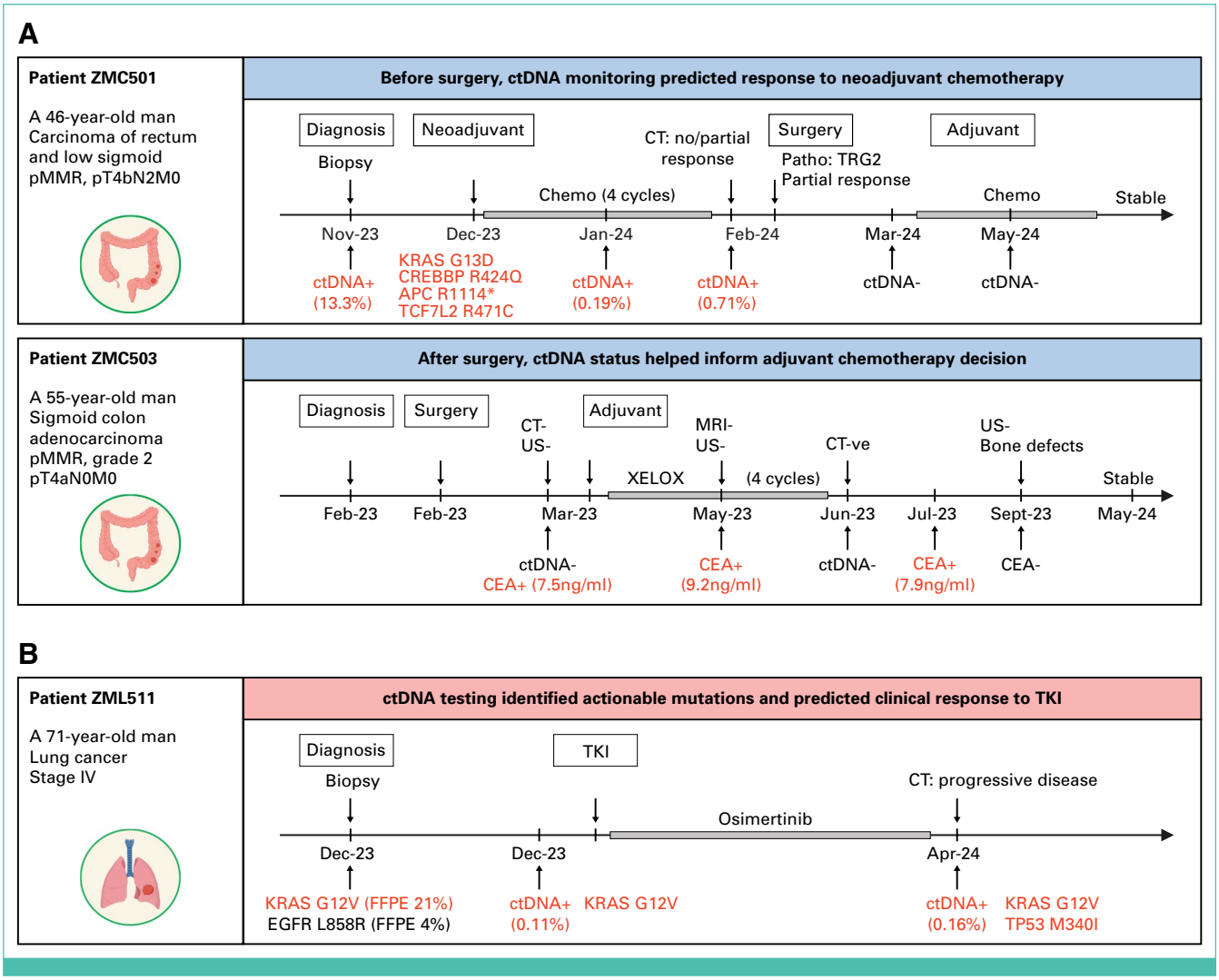

FIG A3. Case studies illustrating clinical utilization of ctDNA. (A) Case studies demonstrating the use of ctDNA to monitor response to neoadjuvant chemotherapy (ZMC501) and to detect postoperative minimal residual disease to inform adjuvant chemotherapy decision (ZMC503) in early-stage cancer. (B) A case study demonstrating the use of ctDNA to detect actionable mutations and monitor response to targeted TKI (ZML511) in metastatic cancer. CEA, carcinoembryonic antigen; CT, computed tomography; ctDNA, circulating tumor DNA; FFPE, formalin-fixed paraffin-embedded; MRI, magnetic resonance imaging; TKI, tyrosine kinase inhibitor; US, ultrasound; XELOX, capecitabine plus oxaliplatin.