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ORIGINAL ARTICLE

Detection of circulating tumor DNA with ultradeep sequencing of plasma cell-free DNA for monitoring minimal residual disease and early detection of recurrence in early-stage lung cancer

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Abstract

Background: In early-stage non–small cell lung cancer (NSCLC), recurrence is frequently observed. Circulating tumor DNA (ctDNA) has emerged as a noninvasive tool to risk stratify patients for recurrence after curative intent therapy. This study aimed to risk stratify patients with early-stage NSCLC via a personalized, tumor-informed multiplex polymerase chain reaction (mPCR) next-generation sequencing assay.

Methods: This retrospective cohort study included patients with stage I–III NSCLC. Recruited patients received standard-of-care management (surgical resection with or without adjuvant chemotherapy, followed by surveillance). Whole-exome sequencing of NSCLC resected tissue and matched germline DNA was used to design patient-specific mPCR assays (Signatera, Natera, Inc) to track up to 16 single-nucleotide variants in plasma samples.

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Results: The overall cohort with analyzed plasma samples consisted of 57 patients. Stage distribution was 68% for stage I and 16% each for stages II and III. Presurgery (i.e., at baseline), ctDNA was detected in 15 of 57 patients (26%). ctDNA detection presurgery was significantly associated with shorter recurrence-free survival (RFS; hazard ratio [HR], 3.54; 95% confidence interval [CI], 1.00–12.62; $p = .009$). In the postsurgery setting, ctDNA was detected in seven patients, of whom 100% experienced radiological recurrence. ctDNA positivity preceded radiological findings by a median lead time of 2.8 months (range, 0–12.9 months). Longitudinally, ctDNA detection at any time point was associated with shorter RFS (HR, 16.1; 95% CI, 1.63–158.9; $p < .0001$).

Conclusions: ctDNA detection before surgical resection was strongly associated with a high risk of relapse in early-stage NSCLC in a large unique Asian cohort. Prospective studies are needed to assess the clinical utility of ctDNA status in this setting.

KEYWORDS
circulating tumor DNA, early-stage lung cancer, liquid biopsy, non–small cell lung cancer

INTRODUCTION

Globally, lung cancer remains the leading cause of cancer-related death. Almost half of patients are diagnosed with early-stage (localized or locally advanced) disease.[1] The proportion of patients diagnosed with early-stage disease is increasing over time,[2] and lung cancer screening will likely result in further stage migration. Consequently, interventions to improve cure rates in this patient population are crucial.

The management of early-stage lung cancer involves multidisciplinary input with definitive local therapy (surgical resection or radiotherapy) with or without adjuvant radiotherapy or systemic therapy.[3] There is also emerging evidence for the use of neoadjuvant systemic therapy.[4] Despite improvements in preoperative staging and surgical and radiotherapy techniques, relapse rates remain high.[5] For several decades, platinum-doublet chemotherapy was the standard-of-care adjuvant therapy for stage IB (tumor size, ≥4 cm) to IIIA resected non–small cell lung cancer (NSCLC).[6] More recently, however, osimertinib in resected stage II–IIIA *EGFR*-mutated NSCLC[7] and pembrolizumab and atezolizumab after adjuvant chemotherapy in resected stage IB–IIIA NSCLC[8,9] have been approved by the US Food and Drug Administration as adjuvant therapies. Improved disease-free survival has also been observed with adjuvant alectinib compared to chemotherapy in resected stage IB–IIIA *ALK*-rearranged NSCLC.[10] With increasing opportunities in drug development to enhance cure rates, there is an urgent need for effective biomarkers to improve risk stratification and monitor treatment efficacy to guide therapeutic decisions.[11]

Circulating tumor DNA (ctDNA) has emerged as a potentially effective noninvasive biomarker to detect or monitor minimal residual disease (MRD) or micrometastases.[12] Postsurgical ctDNA status (positive or negative) may allow for adaptive therapeutic strategies, with the potential escalation or de-escalation of adjuvant systemic therapies, respectively. Moreover, ctDNA detection may precede radiological identification, which emphasizes the potential utility of ctDNA assessment to guide clinical decision-making. However, ctDNA detection in early-stage NSCLC is difficult, with a limited sensitivity of standard assays as well as variable performance across different NSCLC subtypes.[13]

In this study, we performed longitudinal ctDNA assessment via a personalized, tumor-informed multiplex polymerase chain reaction (mPCR) next-generation sequencing (NGS) assay (Signatera, bespoke mPCR NGS assay) to identify presurgical ctDNA status and MRD posttherapy to stratify patients into a high versus low risk of recurrence. We then evaluated the association of ctDNA status with recurrence-free survival (RFS).

MATERIALS AND METHODS

Study population and sample collection

This retrospective cohort study included patients diagnosed with resectable stage I–III NSCLC at the National Cancer Centre Singapore (NCCS) between May 2013 and June 2019. Recruited patients received standard-of-care management according to local guidelines involving baseline staging with positron emission tomography/computed tomography (PET/CT) scans, magnetic resonance brain imaging, and surgical resection with or without adjuvant chemotherapy, followed by surveillance with regular CT scans. Plasma samples were collected at routine follow-up visits pre- and serially postsurgery in all patients (Figure 1). Serial postsurgery plasma samples were collected at routine follow-up visits where patient consent for further plasma sample collection was obtained according to individual clinician-

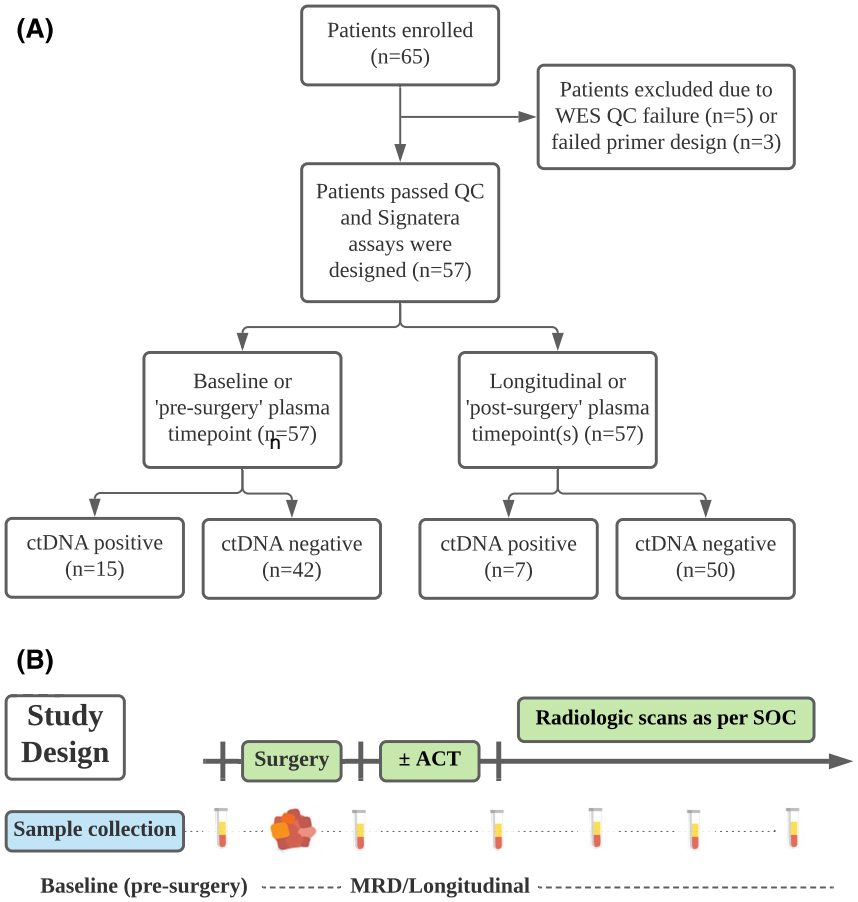

FIGURE 1 Patient enrollment and study design. (A) Consolidated Standards of Reporting Trials diagram showing patient enrollment, assay performance, and ctDNA analysis. (B) Study schema showing sample collection according to SOC management. ACT indicates adjuvant chemotherapy; ctDNA, circulating tumor DNA; MRD, minimal residual disease; QC, quality control; SOC, standard of care; WES, whole-exome sequencing.

determined follow-up schedules. Plasma samples were collected as whole blood in EDTA tubes and processed by centrifugation to obtain plasma and subsequently stored at −80°C. Tissue samples were obtained from formalin-fixed, paraffin-embedded (FFPE) resection specimens. DNA from paired peripheral blood mononuclear cells of the same patient collected at the time of surgery was obtained for sequencing as a germline control. All patients provided written informed consent under the National Lung Cancer Research Study (SingHealth Centralised Institutional Review Board; reference number 2018/2963). Patient demographics, detailed treatment histories, and clinical outcomes were collected from the medical record (with a data cutoff of March 2021).

Personalized mPCR-based NGS assay for ctDNA detection

Whole-exome sequencing (WES) of resected tissue and matched germline DNA from each patient was used to design patient-specific mPCR-based NGS assays (Signatera) to track up to 16 single-nucleotide variants (SNVs) in plasma samples. As previously published,[14] a median cell-free DNA concentration of 6.46 ng/mL was extracted from a median of 3.5 mL (range, 1.4–4.6 mL) of plasma, and libraries were prepared via end repair, A tailing, and ligation with custom adapters. Libraries were then amplified by mPCR, barcoded, pooled, and sequenced on an NGS platform (Illumina HiSeq 2500) to achieve the deduplicated on-target average coverage of >180× for tumor tissue and 50× for the associated matched normal sample. Plasma samples that contained at least two of 16 variants with a confidence score above a predefined algorithm threshold were denoted as ctDNA positive. Plasma samples deemed ctDNA positive were quantified, and ctDNA concentration was reported as mean tumor molecules per milliliter of plasma. The relationship between ctDNA status and clinical recurrence as detected radiologically was evaluated.

Statistical analysis

Patient demographics were summarized via descriptive statistics. RFS was determined via Kaplan–Meier survival analysis with a Cox

proportional hazards model. Hazard ratios (HRs) and 95% confidence intervals (CIs) are displayed with an associated log-rank *p* value. A value of $p < .05$ was considered statistically significant. Odds ratios for ctDNA positivity were analyzed via logistic regression (STATA 16.1).

RESULTS

Patient and sample characteristics

There were 65 patients identified for this study, with eight patients (12%) excluded because of WES quality control (QC) failure or failed primer design (Figure 1). Consequently, the overall cohort with analyzed plasma samples consisted of 57 patients with a median age of 60 years (range, 43–83 years), of which 60% were male, 54% were never smokers, and 84% had adenocarcinoma (Table 1; Table S1). There were two patients (4%) with *ALK*-rearranged and 27 patients (47%) with *EGFR*-mutated NSCLC (*EGFR* exon 19 deletion, $n = 10$; *EGFR* exon 20 insertion, $n = 2$; *EGFR* L858R, $n = 12$; other *EGFR* mutations, $n = 3$; Table S2). Stage distribution was 68% for stage I and 16% each for stages II and III. There were 15 patients (26%) who received adjuvant platinum-doublet chemotherapy for pathologic stage II or III disease. There were five patients (9%) who received adjuvant radiotherapy, all of whom were pathologic stage III and had also received adjuvant chemotherapy either sequentially ($n = 4$) or in combination ($n = 1$). A total of $n = 165$ plasma samples were collected, with all patients having a presurgery (i.e., baseline) plasma sample and a median of two (range, one to four) plasma samples longitudinally. After a median follow-up of 40.8 months (range, 4.9–82.3 months), 15 patients (26%) had relapsed and nine patients (16%) had died. Of the 15 patients who relapsed, eight patients (53%) had biopsies to histologically confirm recurrence, whereas the remaining patients were diagnosed with recurrence on the basis of imaging findings alone. Patterns and sites of recurrence are shown in Table S3.

TABLE 1 Baseline patient and disease characteristics.

Characteristic ($N = 57$)	
Age, median (range), years	60 (43–83)
Gender, No. (%)	
Female	23 (40)
Male	34 (60)
Ethnicity, No. (%)	
Chinese	47 (82)
Indian	3 (5)
Malay	5 (9)
Other ethnicity[a]	2 (4)
Histologic diagnosis, No. (%)	
Adenocarcinoma	48 (84)
Squamous cell carcinoma	4 (7)
Other histology[b]	5 (9)
Staging (AJCC, 7th ed.), No. (%)	
IA	31 (54)
IB	8 (14)
IIA	5 (9)
IIB	4 (7)
IIIA	8 (14)
IIIB	1 (2)
Smoking history, No. (%)	
Never	31 (54)
Ex-smoker	15 (26)
Current smoker	11 (19)
EGFR mutation status, No. (%)	
EGFR mutated	27 (47)
EGFR wild type	22 (39)
Unknown or not tested	8 (14)
ALK rearrangement status, No. (%)	
ALK rearranged	2 (4)
ALK negative	47 (82)
Unknown or not tested	8 (14)
Adjuvant chemotherapy, No. (%)	
No	42 (74)
Yes	15 (26)
Adjuvant radiotherapy, No. (%)	
No	52 (91)
Yes	5 (9)
Follow-up, median (range), months	40.8 (4.9–82.3)

Abbreviation: AJCC, American Joint Committee on Cancer.

[a]Other ethnicity includes White ($n = 1$) and United Arab Emirates ($n = 1$).

[b]Other histology includes ciliated muconodular papillary tumor of the lung ($n = 1$), lymphoepithelial-like carcinoma ($n = 2$), large cell neuroendocrine carcinoma ($n = 1$), and sarcomatoid carcinoma ($n = 1$).

Baseline or presurgery ctDNA detection is associated with shorter RFS

Baseline or presurgery plasma samples ($n = 57$) were collected on the day of surgery in 40 of 57 (70%) patients (median, day of surgery; mean, 0.5 months before surgery; range, 0–3.4 months before surgery). Presurgical ctDNA positivity (at least two of 16 SNVs detected)

was associated with shorter RFS (HR, 3.54; 95% CI, 1.00–12.62; $p = .009$; Figures 2A and 3), with a median of seven (range 2–16) variants detected per patient. Of 27 patients with *EGFR*-mutated NSCLC, 14 patients (52%) had *EGFR* mutations as part of the bespoke assay because selection was restricted to SNVs. In the overall cohort, *ERBB2* and *KRAS* mutations were included as part of the bespoke assay in one patient (2%) each. ctDNA detection at baseline was associated with smoking history, squamous histology, and higher stage (Figure S1).

Longitudinal postsurgery ctDNA detection precedes radiological recurrence

In the postsurgical setting, plasma samples ($n = 108$) were collected serially. All patients had at least one plasma time point collected after surgery, with the first time point tested at a median of 9.9 months (range, 2.7–56.3 months; interquartile range [IQR], 4.0–19.8 months) postsurgery. In patients who received adjuvant therapies, the first plasma time point was collected after adjuvant therapy. The second surveillance time point was collected in 34 patients (median, 17.2 months; range, 6.6–58.9 months; IQR, 10.0–29.7 months), third time point in 13 patients (median, 25.5 months; range, 11.6–55.7 months; IQR, 16.5–32.6 months), and fourth time point in four patients (median, 26.2 months; range, 19.1–37.0 months; IQR, 22.1–31.3 months). ctDNA was detected in seven patients (Table S1), of whom 100% (positive predictive value, 100%) experienced radiological recurrence at a median of 15.5 months (range, 4.9–43.0 months). ctDNA positivity preceded radiological findings by a median lead time of 2.8 months (range, 0–12.9 months; Figure 3). Longitudinally, ctDNA detection at any time point postsurgery was also associated with shorter RFS (HR, 16.10; 95% CI, 1.63–158.9; $p < .0001$; Figure 2B). The absence of ctDNA detection longitudinally was associated with favorable outcomes, with a negative predictive value of 84% (42 of 50 patients).

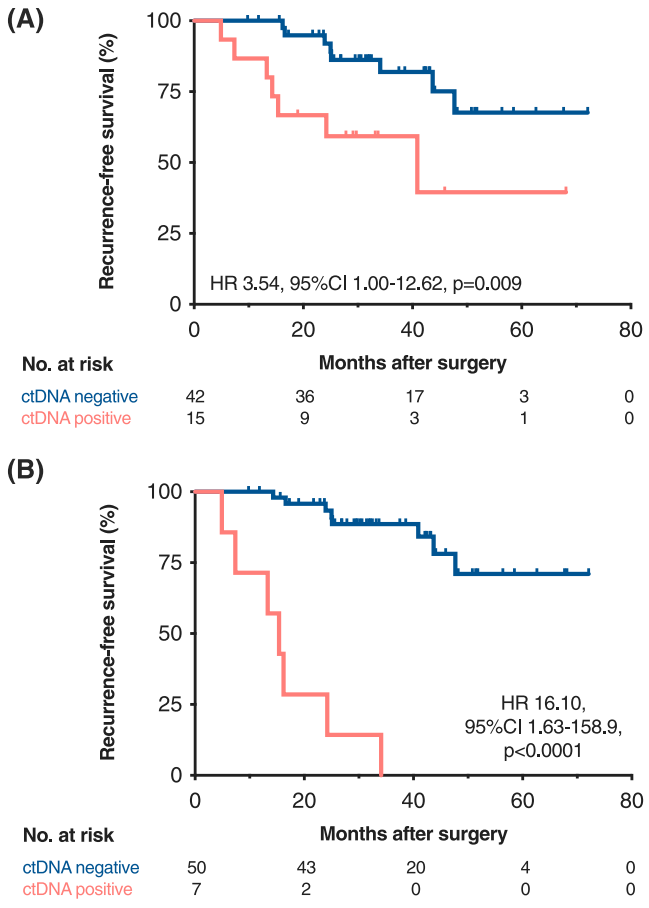

FIGURE 2 Association of ctDNA status with disease recurrence and survival outcomes. (A) ctDNA status at baseline. (B) ctDNA status longitudinally. CI indicates confidence interval; ctDNA, circulating tumor DNA; HR, hazard ratio.

DISCUSSION

Our findings demonstrate that ctDNA detection before resection was strongly associated with a high risk of relapse in early-stage NSCLC. In addition, ctDNA detection during follow-up after surgery identified patients who recurred, with ctDNA positivity preceding radiological findings. This illustrates the potential utility of ctDNA as a noninvasive tool in both pre- and postsurgery plasma samples to risk stratify patients and guide disease surveillance and adjuvant therapy decision-making.

There have been extremely limited studies evaluating ctDNA detection in early-stage lung cancer, and our cohort represents one of the largest cohorts to date and a unique Asian cohort. Consistent with our findings, previous studies have demonstrated that ctDNA detection, either pre- or postsurgery, can be associated with survival outcomes.[14–20] Although these studies had similar sized or smaller cohorts, differences in patient demographics, curative therapies, and inconsistencies with plasma time point collection make cross-study comparisons difficult. Notably, the use of personalized assays, with tumor-specific variants, has overcome the limited sensitivity of standard assays in early-stage lung cancer.[13] Nevertheless, our findings continue to underscore the difficulties of sensitive ctDNA detection in early-stage lung cancer. In contrast to previous studies, our study consisted of a large proportion of never smokers (54%) and patients with known *EGFR* mutations (47%). Further investigation is necessary to understand how these factors could affect the rate of ctDNA detection; it is possible that smoking habits along with ethnic genomic differences could indicate a unique tumor biology characterized by lower ctDNA shed rates. For example, in our study, there was an opposite correlation with ctDNA detection and *EGFR* mutation status compared with the IMpower010 cohort,[18] which also used the Signatera assay. This potentially implicates ethnic genomic differences.[21] In addition, our findings, which indicated that ctDNA detection before surgery was strongly associated with relapse, differ from a similar sized cohort that used the RaDaR assay, which found that ctDNA detection within 1–3 days after surgery was not associated with disease recurrence.[16] Key demographic differences, such

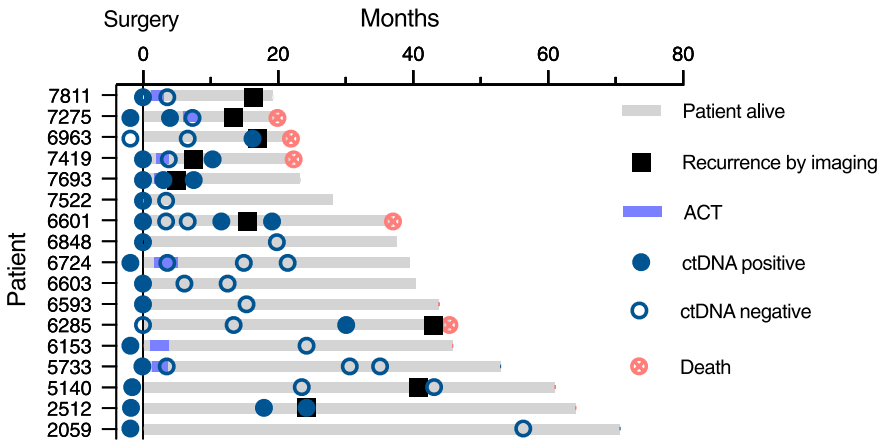

FIGURE 3 Swimmer's plot of ctDNA assay results and clinical outcomes in patients with positive baseline or longitudinal ctDNA. ACT indicates adjuvant chemotherapy; ctDNA, circulating tumor DNA.

as the proportion of never smokers (54% vs. 9%), adenocarcinoma histology (84% vs. 63%), and *EGFR* mutation status (47% vs. not reported), may potentially account for these findings. This further emphasizes the importance of understanding the clinical and genomic heterogeneity of lung cancer and the need to judiciously apply assays with different methodologies. In particular, clinical stratification based on the presence of oncogenic driver alterations may be crucial. For example, the absence of ctDNA detection postsurgery at an MRD time point in resected stage IB–IIIA *EGFR*-mutated or *ALK*-rearranged NSCLC could suggest a potential role in deescalating adjuvant systemic therapies with chemotherapy and/or targeted therapy (with osimertinib or alectinib, respectively). Overall, the aforementioned clinical parameters are needed for optimal clinical decision-making and prognostication, and the presence of ctDNA informs the necessity of further treatment. However, prospective studies are still needed to fully evaluate the clinical utility of ctDNA detection at the MRD time point.

Limitations of our study include a relatively small cohort and sample size, long recruitment time period, and lack of standardized time points for plasma collection across patients, in particular the first time point postsurgery. In patients who received adjuvant therapy, the first plasma time point postsurgery was after adjuvant therapy, which limited the potential utility and interpretation of the result to aid in clinical decision-making for the use of adjuvant therapy itself. Nevertheless, given the scarcity of published evidence evaluating ctDNA assays in early-stage lung cancer, our study provides important data that may guide future prospective studies and clinical trials. Last, although our study used a tumor-informed personalized assay, there were eight patients (12%) who were excluded because of tissue WES QC failure or failed primer design. Given the retrospective nature of the study, it is important to mention that it is not uncommon to observe compromised tissue quality in archival FFPE specimens. Although the WES QC failure rate observed in this study is similar to that reported previously by Razavi et al.,[22] this presents a significant challenge, especially for tumors with lower cellularity. We expect that failure to sequence tumor tissue will lessen as pathological macro- and microdissection techniques improve over time. In addition, although tumor-naive assays have also been described,[23,24] tumor-informed approaches provide potentially enhanced specificity and sensitivity as well as ease of performing serial testing.[25] Longitudinal ctDNA monitoring has been demonstrated to increase the sensitivity of detecting recurrence in several tumor types, including lung cancer.[14,15,25,26]

In conclusion, with increasing evidence for adjuvant therapies in early-stage NSCLC, ctDNA detection may represent an important biomarker to risk stratify patients and improve survival outcomes.[27] Ultimately, however, prospective studies are needed to fully evaluate the clinical utility of ctDNA status to guide disease surveillance and management in patients with lung cancer.

AUTHOR CONTRIBUTIONS

Aaron C. Tan: Conceptualization, investigation, writing–original draft, writing–review and editing, data curation, formal analysis, and resources. **Gillianne G. Y. Lai:** Resources, data curation, formal analysis, writing–review and editing, and investigation. **Stephanie P. L. Saw:** Investigation, writing–review and editing, formal analysis, data curation, and resources. **Kevin L. M. Chua:** Resources, data curation, formal analysis, writing–review and editing, and investigation. **Angela Takano:** Investigation, writing–review and editing, formal analysis, data curation, and resources. **Boon-Hean Ong:** Resources, data curation, formal analysis, writing–review and editing, and investigation. **Tina P. T. Koh:** Investigation, writing–review and editing, formal analysis, data curation, and resources. **Amit Jain:** Resources, data curation, formal analysis, writing–review and editing, and investigation. **Wan Ling Tan:** Investigation, writing–review and editing, formal analysis, data curation, and resources. **Quan Sing Ng:** Investigation, writing–review and editing, formal analysis, data curation, and resources. **Ravindran Kanesvaran:** Investigation, writing–review and editing, formal analysis, data curation, and resources. **Tanujaa Rajasekaran:** Resources, data curation, formal

analysis, writing–review and editing, and investigation. **Ekaterina Kalashnikova:** Resources, data curation, formal analysis, writing–review and editing, and investigation. **Derrick Renner:** Resources, data curation, formal analysis, writing–review and editing, and investigation. **Sumedha Sudhaman:** Investigation, writing–review and editing, formal analysis, data curation, and resources. **Meenakshi Malhotra:** Writing–review and editing, investigation, formal analysis, data curation, and resources. **Himanshu Sethi:** Investigation, writing–review and editing, formal analysis, data curation, and resources. **Minetta C. Liu:** Investigation, writing–review and editing, formal analysis, data curation, and resources. **Alexey Aleshin:** Conceptualization, investigation, writing–review and editing, formal analysis, data curation, and resources. **Wan-Teck Lim:** Investigation, formal analysis, data curation, resources, and writing–review and editing. **Eng-Huat Tan:** Investigation, formal analysis, data curation, resources, and writing–review and editing. **Anders J. Skanderup:** Investigation, data curation, resources, formal analysis, and writing–review and editing. **Mei-Kim Ang:** Investigation, formal analysis, data curation, resources, and writing–review and editing. **Daniel S. W. Tan:** Conceptualization, supervision, writing–review and editing, resources, data curation, formal analysis, and investigation.

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CONFLICT OF INTEREST STATEMENT

Aaron C. Tan reports personal fees from Amgen, Bayer, and Pfizer outside the submitted work. Gillianne G. Y. Lai reports personal fees from Amgen and grants from Merck, AstraZeneca, Pfizer, Bristol-Myers Squibb, and Roche outside the submitted work. Stephanie P. L. Saw reports personal fees from Pfizer, AstraZeneca, MSD, and Bayer and nonfinancial support from MSD outside the submitted work. Kevin L. M. Chua reports personal fees from Roche, AstraZeneca, PeerVoice, Takeda Pharmaceuticals, Regeneron Pharmaceuticals, Seagen, MSD, and Varian outside the submitted work. Boon-Hean Ong reports personal fees from AstraZeneca, Medtronic, Roche Health Solutions, and Bristol-Myers Squibb; nonfinancial support from Johnson & Johnson; personal fees and nonfinancial support from Stryker; and personal fees from MSD outside the submitted work. Quan Sing Ng reports serving on advisory boards for Boehringer Ingelheim and Merck. Ravindran Kanesvaran reports personal fees from MSD, Bristol-Myers Squibb, Eisai, Bayer, Ipsen, Astellas Pharma, and Novartis outside the submitted work. Wan-Teck Lim reports grants from Bristol-Myers Squibb and Boehringer Ingelheim and personal fees from Merck, Roche, Pfizer, Taiho, and AstraZeneca outside the submitted work. Daniel S. W. Tan reports grants from AstraZeneca, ACM Biolabs, Bayer, Pfizer, and Amgen and personal fees from Novartis, Boehringer Ingelheim, Bayer, GlaxoSmithKline, Janssen, Amgen, MSD, Pfizer, Roche, AstraZeneca, Takeda Pharmaceuticals, DKSH, and C4 Therapeutics during the conduct of the study. The other authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data generated in this study are not publicly available because of patient privacy but are available on reasonable request from the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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