# Potential utility of cell-free deoxyribonucleic acid for detection of early-stage resectable non-small cell lung cancer

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## ABSTRACT

**Introduction: Cell-free DNA (cfDNA) has emerged as a promising non-invasive biomarker for cancer detection, including non-small cell lung cancer (NSCLC). While these approaches have demonstrated high diagnostic accuracy in controlled settings, their performance in real-world clinical populations remains uncertain. This study aimed to evaluate the diagnostic performance of a plasma cfDNA assay for early-stage NSCLC detection and to explore its association with clinicopathological parameters.**

**Materials and Methods: This was a single-center, cross-sectional study involving 61 patients with radiologically suspected lung lesions, of whom 52 were confirmed NSCLC. Plasma samples were analyzed using a shallow genome-wide sequencing-based cfDNA assay incorporating fragmentomic and copy number features. cfDNA results were compared against histopathological diagnosis to determine sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). Associations between cfDNA detection and clinical variables were evaluated using appropriate statistical tests.**

**Results: The cfDNA assay demonstrated a sensitivity of 75% and a PPV of 84.8% with a specificity of 22.2% and NPV of 13.3%. cfDNA detection rates increased with advancing disease stage, although a relatively high sensitivity was observed even in stage I disease (79.4%). No statistically significant associations were found between cfDNA positivity and conventional markers of tumour burden, including tumour size, serum carcinoembryonic antigen, and SUVmax.**

**Conclusion: The cfDNA assay evaluated in this study demonstrated limited clinical utility as a standalone screening tool due to its low specificity and NPV in a real-world early-stage NSCLC population. Future efforts should focus on improving assay specificity through the incorporation of multi-omic tumour-specific biomarkers and validation that includes early-stage lung cancer cases and benign pulmonary conditions.**

**KEYWORDS:**
*cfDNA, early diagnosis, screening, non-small-cell lung cancer (NSCLC), lung cancer detection*

## INTRODUCTION

Lung cancer remains a leading cause of cancer-related mortality worldwide due to a preponderance of late-stage diagnosis in a majority of cases. Early diagnosis is critical, as curative-intent therapy and prognosis are largely stage-dependent. Low-dose computed tomography (LDCT) screening creates an impactful stage-shift with a reduction in lung cancer-specific mortality but is hampered by cost, false positives and accessibility issues. Presently, tissue biopsy is the gold standard for diagnosis however procedures are invasive and subjective due to spatial and temporal tumour heterogeneity. Yield may also be insufficient for molecular profiling resulting in concurrent or repeated sampling with additional risk and cost to the patient.

Liquid biopsy approaches enable minimally invasive detection of tumour-derived genetic and epigenetic alterations on cell-free DNA (cfDNA) and have potential applications in early diagnosis, disease monitoring and treatment stratification.[1] Multi-cancer early detection (MCED) and single-cancer early detection (SCED) strategies are emerging approaches aimed at improving cancer diagnosis at earlier, more treatable stages through more minimally invasive analysis of cfDNA. MCED assays use cfDNA-based fragmentation patterns, methylation signatures, or mutation profiles to detect signals from multiple tumour types in a single blood test.[2-4] In contrast, SCED approaches focus on detecting a single cancer type with higher sensitivity for a specific disease. In lung cancer, cfDNA-based SCED strategies include targeted mutation panels and methylation or fragmentomic-based classifiers designed specifically for early-stage lung cancer detection. These lung-specific cfDNA assays may complement LDCT, helping to reduce false-positive rates and improve diagnostic confidence in screening settings.

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To date, there is a paucity of data regarding the role of cfDNA for primary diagnosis or risk stratification of indeterminate pulmonary nodules (IPN), a small focal lung lesion identified on imaging that may represent a precursor of lung cancer but cannot be definitively classified as benign or malignant at initial detection, which requires further evaluation. Much of the current literature focuses on postoperative monitoring for molecular residual disease (MRD), response to therapy including surgery, and relapse prediction.[5-7] It is believed that the low tumour burden and reduced DNA shedding in early-stage disease limits its sensitivity for detection. However, efforts incorporating ultra-deep sequencing methodology or a multi-modal approach coupled with machine learning classification models may have the potential to enhance the detection of early lung cancers.

We conducted a prospective, observational, cross-sectional, real-world evidence study to evaluate the diagnostic performance of a commercially available cfDNA assay for the screening and diagnosis of early-stage non-small cell lung cancer (NSCLC) in predominantly asymptomatic individuals who presented with suspicious lung pathology on imaging, suggestive of potentially resectable disease.

## MATERIALS AND METHODS

### Patient recruitment and clinical procedures

From January 2025 to February 2026, we enrolled 61 patients from Sunway Medical Centre referred for surgical evaluation and further management of suspicious lung nodules on PET-CT imaging suggestive of possible early-stage lung cancer. Some patients had a pre-operative tissue biopsy confirming NSCLC or benign pathology. Serum carcinoembryonic antigen (CEA), a common lung cancer biomarker, was measured for most patients pre-operatively. Patients underwent PET-CT imaging to determine the clinical stage, tumour size as well as F-18 fluorodeoxyglucose (FDG)-based metabolic activity of the tumour using maximum standardized uptake value (SUVmax) measurement. All eligible patients underwent surgical resection after appropriate staging, with baseline cfDNA testing performed pre-surgically. Histopathological examination of the resected specimen was performed in all cases.

### Blood collection, cfDNA extraction and next-generation sequencing

A 10ml peripheral blood draw was obtained from each patient on the evening before the operation, using a commercially available SCED assay named SPOT-MAS Lung (Gene Solutions).[2] Plasma was separated through a two-step centrifugation process, followed by cfDNA extraction. The extracted and purified cfDNA was then subjected to next-generation sequencing (NGS) using the DNBSEQ-G400 system (MGI) with shallow sequencing coverage (0.5x), integrating fragmentomic, nucleosome footprinting, copy number alterations (CNAs), and 4-mer end motif analyses, according to the manufacturer's protocol.[8]

### Statistical analyses

Diagnostic performance of the cfDNA assay was evaluated using histopathological findings as the reference standard. Statistical analyses were performed using 2 × 2 contingency tables comparing cfDNA results with corresponding histopathology outcomes. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and overall diagnostic accuracy were calculated to determine the performance of the cfDNA assay in detecting cancer. Sensitivity was defined as the proportion of histopathologically confirmed positive cases correctly identified by cfDNA analysis, whereas specificity represented the proportion of histopathologically negative cases correctly classified as negative by cfDNA testing. PPV and NPV were calculated to assess the probability that positive and negative cfDNA results reflected the true disease status, respectively. Diagnostic accuracy was defined as the proportion of correctly classified cases among all evaluated samples. Sensitivity was also calculated according to the clinical stage of the cancers.

Continuous variables, including CEA level, tumour size, and SUVmax were summarized using the median and interquartile range (IQR). The associations between cfDNA status and these clinicopathological parameters were evaluated using the Mann-Whitney U test for continuous variables and Fisher's exact test for categorical variables, as appropriate. For subgroup analyses, predefined cut-off values were applied to dichotomize variables: CEA level (≥5 ng/mL vs. <5 ng/mL), tumour size (≥3 cm vs. <3 cm), and SUVmax (≥5 vs. <5). The association between categorized variables and cfDNA positivity was further assessed using Fisher's exact test. A two-tailed p-value of $<0.05$ was considered statistically significant. Statistical analyses were performed using GraphPad PRISM version 10.6 (GraphPad Software, San Diego, CA, USA).

### Ethics approval

This study was approved by the Sunway Medical Centre Independent Research Ethics Committee (SREC) with the approval number 044/2025/IND/ER.

## RESULTS

### Patient cohort and diagnostic classification

61 patients were enrolled in this study. Mean patient age was 60 years with a majority being female (63.9%), of Chinese ethnicity (68.9%) and never smokers (70.5%). 52 patients had pathological confirmation of a NSCLC, and 9 patients had non-NSCLC conditions such as granulomatous inflammation, tuberculosis, carcinoid tumours, focal pneumonia, and lymphocytic infiltrations.

### Overall Diagnostic Performance of cfDNA

The cfDNA results were evaluated against histopathological examination as the reference standard using a disease confusion matrix to assess diagnostic performance (Table I). The comparison quantified concordance between cfDNA-based classification and tissue histopathology, enabling calculation of sensitivity, specificity, and overall diagnostic accuracy.

Based on the confusion matrix, the analysis revealed that the tested assay demonstrated a sensitivity of 75%, specificity of 22.2%, positive predictive value (PPV) of 84.8%, and negative predictive value (NPV) of 13.3%, with an overall accuracy of 67.2% (Table II).

**Table I: Disease Confusion Matrix**

| | NSCLC (HPE +) | Non-NSCLC (HPE -) | Total |
|---|---|---|---|
| cfDNA positive | 39 (True Positive) | 7 (False Positive) | 46 |
| cfDNA negative | 13 (False Negative) | 2 (True Negative) | 15 |
| Total | 52 | 9 | 61 |

NSCLC= Non-small cell lung cancer, HPE= histopathology examination

**Table II: Overall diagnostic performance of cfDNA**

| Metric | Value (%) |
|---|---|
| Sensitivity | 75 |
| Specificity | 22.2 |
| Positive Predictive Value (PPV) | 84.8 |
| Negative Predictive Value (NPV) | 13.3 |
| Accuracy | 67.2 |

**Table III: cfDNA Sensitivity by Clinical Stage**

| Clinical Stage | True Positive (TP) | False Negative (FN) | Sensitivity (%) |
|---|---|---|---|
| Stage I | 27 | 7 | 79.4 |
| IA | 22 | 4 | 84.6 |
| IB | 5 | 3 | 62.5 |
| Stage II | 5 | 5 | 50 |
| IIA | 0 | 3 | 0 |
| IIB | 5 | 2 | 71.4 |
| Stage III | 7 | 1 | 87.5 |
| IIIA | 5 | 1 | 83.3 |
| IIIB | 2 | 0 | 100 |

**Table IV: Association of cfDNA Results with Clinical and Imaging Parameters**

| Variable | Overall Median (IQR) | cfDNA+ (n=40) Median (IQR) | cfDNA- (n=13) Median (IQR) | p-value (Mann-Whitney) | Cut-off value | cfDNA+ n (%) | cfDNA- n (%) | p-value (Fisher) |
|---|---|---|---|---|---|---|---|---|
| CEA (ng/mL) (n= 49) | 2.6 (1.8-8.2) | 2.7 (1.75- 8.35) | 2.3 (1.9- 5.6) | 0.9421 | ≥5ng/mL | 36 (73.5) | 13 (26.5) | > 0.999 |
| Tumor size(cm) (n= 52) | 2.7 (2.2- 3.9) | 2.7 (2.2- 3.9) | 2.7 (2.1- 4.2) | 0.8984 | ≥3cm | 39 (75) | 13 (25) | > 0.999 |
| SUVmax (n= 49) | 6.6 (2.85- 9.99) | 7.06 (2.85- 10.14) | 6.58 (4.29- 8.94) | 0.7437 | ≥5 | 37 (75.5) | 12 (24.5) | 0.7434 |

IQR= Interquartile range

**Table V: cfDNA Results in Non-NSCLC Patients**

| Diagnosis | Number | cfDNA Positive | cfDNA Negative |
|---|---|---|---|
| Granulomatous inflammation/ tuberculosis (TB) | 3 | 3 | 0 |
| Carcinoid tumour | 2 | 1 | 1 |
| No granuloma, atypical cells or malignancy | 2 | 1 | 1 |
| Focal pneumonia | 1 | 1 | 0 |
| Lymphocytic infiltration | 1 | 1 | 0 |
| Total | 9 | 7 | 2 |

### cfDNA sensitivity by clinical stage

To further stratify the cfDNA detection results, we analyzed the sensitivity by clinical stage as determined pre-operatively using imaging (Table III). The results demonstrated that the sensitivity for stage I disease remains relatively high, given the high representation of these cases.

### Association of cfDNA Results with Selected Clinical Parameters in NSCLC Patients

Tumour size was recorded in all 52 NSCLC patients while baseline serum CEA levels and FDG-PET SUVmax uptake values were available in only 49 patients. Median CEA levels did not differ significantly between groups (2.7 vs 2.3 ng/mL, $p = 0.942$), and the proportion of patients with elevated CEA (≥5 ng/mL) was similar ($p > 0.999$). Likewise, tumour size was comparable (median 2.7 vs 2.7 cm, $p = 0.898$), with no

difference in the proportion of tumours ≥3 cm (p>0.999). SUVmax values showed no significant variation between cfDNA-positive and cfDNA-negative groups (median 7.06 vs 6.58, p=0.744), and categorization by SUVmax ≥5 was not associated with cfDNA status (p=0.743). Overall, these findings indicate that cfDNA positivity was not associated with the tested clinical parameters in this cohort (Table IV).

**cfDNA results in non-NSCLC patients**

cfDNA results for the nine non-NSCLC patients are summarized in Table V. A high proportion of non-NSCLC cases tested cfDNA positive, including all three cases of pulmonary tuberculosis, contributing to the low overall specificity of the assay. False positive findings were observed across various benign conditions.

**Association of cfDNA findings with genomic mutations detected in NSCLC patients**

Forty-eight of the 52 NSCLC patients had genomic tumor mutational sequencing analysis using a targeted panel, of whom 36 were cfDNA positive and 12 were cfDNA negative. Among patients who underwent NGS testing, the most frequently identified mutations were EGFR (n=27), TP53 (n=15), KRAS (n=6), and ALK (n=3), with several other mutations detected at low frequency (results not shown). A subset of the patients (n=6) had no detectable mutations. Categorical analyses performed using predefined thresholds demonstrated no significant association between cfDNA positivity and the presence of an oncogenic mutation (Fisher's exact test, p=0.563).

## DISCUSSION

The diagnostic usage of cfDNA is relatively new and its use in the detection of early-stage NSCLC is still limited. cfDNA appears to be effective for predicting relapse risk and pre-operative positivity is associated with a reduced recurrence-free survival. The presence of postoperative cfDNA, reflecting MRD, is strongly associated with subsequent disease recurrence.[9-11] The performance of cfDNA in early lung cancer detection is influenced by the methods used for its analysis. Several approaches can improve detection sensitivity. Mutation-based detection remains the most conventional method and involves identifying tumour-specific genetic alterations in circulating DNA. However, it requires adequate cfDNA and is therefore limited in early-stage disease due to reduced tumour burden and less DNA shedding. Methylation-based approaches offer a promising alternative that focuses on epigenetic modifications of DNA rather than changes in the DNA sequence. For example, a plasma DNA methylation–based ctDNA assay developed by Liang et al distinguished malignant from benign pulmonary nodules with 79.5% sensitivity and 85.2% specificity in plasma (93.2% specificity in healthy controls), while demonstrating strong performance for early-stage disease (75.0% in stage IA and 85.7% in stage IB), supported by tissue-level accuracy of 92.7% sensitivity and 92.8% specificity.[12] Similarly, Zhao et al developed a multiplex digital methylation-specific PCR platform that improved the detection of low cfDNA levels and had superior performance compared to standard assays.[13] These findings suggest methylation-based approaches may help overcome the limitations of mutation-based methods, particularly in early-stage disease where detectable cfDNA levels are low.

In addition to methylation-based approaches, alternative methods such as fragmentomics can improve cfDNA detection. Fragmentomics analyse patterns in cfDNA fragmentation rather than specific DNA changes. Mathios et al combined fragmentation analysis with clinical risk factors, CEA levels and CT imaging to accurately detect 94% of patients with cancer across various stages and subtypes, including 91% of stage I or II disease.[14] Using whole-genome sequencing to profile cfDNA fragmentomics, the machine learning model developed by another research group achieved exceptionally high accuracy for early lung cancer detection across validation cohorts, while maintaining strong performance at low sequencing depth and demonstrating 83.2% sensitivity for stage I disease and 85.0% for tumors <1 cm.[15]

Our real-world evidence study is the first in Malaysia to evaluate the diagnostic performance of a commercially available plasma cfDNA assay for the detection of early-stage NSCLC in a cohort of mostly asymptomatic individuals who presented with an incidental or screening-detected lung nodule suggestive of a possible underlying early-stage resectable NSCLC. Some patients had histological confirmation of lung cancer pre-surgery, from a CT-guided percutaneous biopsy. We explored the association of baseline cfDNA positivity with clinicopathological parameters and tumour genomic profiles. Our findings demonstrated that the assay had reasonable sensitivity (75%) and a relatively high positive predictive value (PPV) (84.8%) for moderate detection of NSCLC in largely asymptomatic individuals with indeterminate visible pathology on a PET-CT imaging. Hence the assay may help personalize surveillance scan intervals, lower the threshold for confirmatory tissue biopsy or proceed with upfront surgical resection after appropriate staging. However, the low specificity (22.2%) and negative predictive value (NPV) (13.3%) mean a negative cfDNA result could not reliably exclude an underlying lung cancer and thus is presently not suitable as a standalone screening modality or a substitute for physician acumen based on clinical risk profiling and morphology of the lung nodule. The low specificity we observed may be attributable to the detection of non-tumour-derived cfDNA, particularly in patients with inflammatory or infectious pulmonary conditions which can contribute to background genomic noise and false-positive signals. Interestingly, there was no significant association of cfDNA results with clinical parameters such as baseline CEA, tumour size, metabolic SUVmax uptake on FDG-PET imaging, and tumour mutation signatures.

The detection of cfDNA in our study utilized a multimodal cfDNA assay with shallow sequencing coverage which combines fragmentomic, nucleosome, end-motif, and copy number alteration analyses.[8] This assay has a previously reported sensitivity of 94% and 90% in the discovery and validation cohorts respectively, with a specificity of 92% for both. These reported values differ significantly from the results of our real-world patient cohort, and this discrepancy may be due to the varied characteristics of the lung cancer patients in their study compared to ours. In terms of disease

stage, only 6% and 6.8% of their discovery and validation cohorts respectively comprised early-stage (stage I, II) patients.[8] In contrast, our study involved patients with potentially resectable NSCLC, with a majority (83%) having early-stage disease. Furthermore, no staging information was available for 58.6% and 46.6% of lung cancer patients in their discovery and validation cohorts respectively. This lack of accurate staging data and their low proportion of early-stage NSCLC patients in both their training and validation cohorts, may have hindered the accuracy of the advanced machine learning models used in this assay to detect early-stage NSCLC. Additionally, the non-lung cancer cases used as a control in the study by Nguyen et al were all healthy individuals, which differs largely from our patient cohort who had visible lung pathology, including benign lung conditions.[8] The assay does not detect NSCLC or lung cancer-specific markers solely, but rather cfDNA fragmentation patterns, copy number alterations, nucleosome positioning, and genome-wide cfDNA signatures, all surrogates or indirect signals of abnormal cellular processes, and not tumour-specific signals or mutations. Besides malignant tumours, cfDNA may be released in benign conditions through various cellular mechanisms including apoptosis, necrosis, autophagy and phagocytosis[16] and therefore may be detected with granulomatous inflammation,[17-18] tuberculosis,[19-20] focal pneumonia,[21-22] and carcinoid tumours.[23] Hence, the markedly low specificity we observed is likely attributable to the inclusion of patients with non-malignant but biologically active pulmonary conditions. Unlike healthy controls, these conditions are characterized by increased cellular turnover, inflammation, and tissue injury, all of which can lead to the release of cell-free DNA with altered fragmentation patterns and genomic features that may overlap with those observed in malignancy. Consequently, the assay may erroneously misclassify such cases, contributing to the high false-positive rate and low specificity we observed.

Our study highlights a key limitation of cfDNA-based assays when applied in clinically heterogeneous populations, where distinguishing malignancy from inflammatory or benign conditions is inherently more challenging than in case-control settings involving healthy individuals. Our study population reflects a more heterogeneous, real-world cohort of patients who often present with an IPN, including many with benign pulmonary diseases that can confound cfDNA detection. In countries like Malaysia, with a high prevalence of inflammatory or infectious lung diseases, existing cfDNA-based assays may lack critical specificity and require further refinement with more diverse training and validation datasets to generate more accurate and discriminatory ensemble machine learning models. Our findings reiterate the importance of external validation in diverse patient populations prior to clinical implementation of any assay for population-based screening and detection of early-stage NSCLC.

When stratified by clinical stage, we observed cfDNA detection rates generally increased with advancing disease stage, consistent with published data suggesting that tumour burden influences cfDNA shedding, either through apoptosis, necrosis, or active secretion. Notably, the sensitivity observed in stage I disease (79.4%) remained relatively high, encouragingly indicating that cfDNA may still be detectable in a subset of early-stage patients despite lower tumour burden. However, the variability in detection rates, particularly in stage II disease, is likely due to our small sample size, and the results should be interpreted with caution. Interestingly, we found no significant association between cfDNA positivity and conventional markers of tumour burden, such as serum CEA levels, tumour size, and FDG-PET SUVmax values. These findings contrast with the expectation that larger or more metabolically active tumours would release greater quantities of cfDNA. A previous study demonstrated a significant association between cfDNA shedding and tumour size and volume in surgically resected oncogenic-driven NSCLC.[24] Our results suggests that cfDNA release is not solely dependent on tumour burden but rather a complex, multifactorial process influenced by tumour biology, including cellular turnover, necrosis, apoptosis, and vascular invasion. Tumours can exhibit biological heterogeneity; wherein certain tumours exhibit low cfDNA shedding despite substantial size or metabolic activity.[25] Tumours with high proliferative rates or increased apoptotic and necrotic activity may release greater amounts of DNA into the circulation, independent of size. Conversely, small tumours with aggressive biological behavior may shed detectable ctDNA, whilst others are non-shedders.[26] Tumour location, vascular access, and host-related factors such as cfDNA clearance mechanisms may further influence circulating ctDNA levels.[27] Taken together, these findings highlight that ctDNA is not simply a surrogate of tumour burden but reflects the tumour biology and microenvironment.

With respect to genomic profiling, tumour sequencing revealed a spectrum of mutations, most commonly involving EGFR, TP53, and KRAS. However, overall mutation burden was not significantly associated with cfDNA detection. This lack of association may be explained by discordance between tumour tissue and plasma cfDNA, as not all tumour-derived mutations are shed into the bloodstream at detectable levels. Additionally, technical limitations, including assay sensitivity and detection thresholds, may contribute to this discrepancy. Given the complexity of cfDNA biology, tissue-based genomic alterations may not be easily extrapolated to plasma-based detection.

## LIMITATIONS

The strength of our study is the heterogeneous clinical cohort, which included patients with both NSCLC and various benign pulmonary conditions, reflecting real-world settings. Furthermore, we correlated the diagnostic performance of the cfDNA assay with critical clinical, molecular, and imaging features, and importantly, using the gold-standard histopathological tissue confirmation as a reference standard, in all cases. Study limitations include the relatively small sample size and single-center study design.

## CONCLUSION

cfDNA offers a promising non-invasive approach for early cancer detection, however our findings suggest limited utility of this particular cfDNA assay presently, as a screening or

diagnostic tool for detection of early-stage NSCLC as the low specificity and NPV, means a negative result cannot reliably exclude an underlying malignancy. Given the relatively high PPV, a positive result may guide decision making with regards to thresholds for a continued 'wait and watch' approach, confirmatory tissue biopsy or upfront surgical resection after appropriate staging. The high false positive rate however limits its standalone diagnostic applicability. The low specificity and NPV suggest that the assay is currently not suitable as a standalone screening modality and should not replace clinical judgement. The false negatives observed in 13 NSCLC patients were a particular concern as clinician reliance on the biomarker result might have prompted a conservative approach with possible interim disease progression.

cfDNA detection appears to capture a complex and distinct aspect of tumour biology, and its clinical utility will depend on continued advancements in assay design and a deeper understanding of its underlying biological determinants. Future efforts should focus on improving assay specificity, potentially through the incorporation of more tumour-specific biomarkers, refinement of bioinformatic filtering strategies, and integration with other diagnostic modalities such as imaging or protein biomarkers. Diverse training and validation patient cohorts incorporating both more early-stage lung cancer cases as well as benign pulmonary conditions are likely to enhance the diagnostic performance of future iterations of the assay to facilitate impactful real-world use.

## CONFLICT OF THE INTERESTS

The authors have no conflicts of interest to disclose.

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