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Liquid Biopsy for *EGFR* Mutation Detection in NSCLC: Evaluation of Plasma ctDNA and Comparison with Plasma exoDNA

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ABSTRACT

Background: Accurate detection of actionable EGFR mutations is essential for guiding targeted therapy in NSCLC. Liquid biopsy approaches using ctDNA and exoDNA offer noninvasive alternatives for molecular profiling. This study evaluated the diagnostic performance of nested PCR combined with sanger sequencing for detecting common EGFR mutations (exon 19 deletions and the L858R point mutation) in plasma samples from Iranian NSCLC patients.

Methods: In this retrospective observational study, blood samples were collected from 30 NSCLC patients with confirmed EGFR mutations. ctDNA was extracted from plasma and analyzed using nested PCR followed by sanger sequencing. Specificity was assessed in 20 EGFR–wild-type NSCLC patients serving as controls. Diagnostic performance was further evaluated in relation to clinicopathological factors.

Results: EGFR mutations were detected in plasma ctDNA in 63.3% of patients. Detection sensitivity was significantly associated with tumor stage but was independent of mutation subtype, age, sex, or smoking status. The assay showed high specificity, with no false-positive results in control samples (95% CI: 83.9–100.0%). Although exoDNA analysis demonstrated a higher sensitivity than ctDNA (76.6% vs. 63.3%), this difference was not statistically significant. Notably, combined analysis of ctDNA and exoDNA increased overall detection sensitivity to 80%.

Conclusions: Nested PCR with sanger sequencing represents a reliable rule-in strategy for EGFR mutation detection in plasma. Integrating ctDNA and exoDNA analyses substantially improves sensitivity and may enhance noninvasive molecular diagnostics in NSCLC. ***DOI: 10.61882/ibj.5018***

Keywords: Exome sequencing, FKBP10 protein, Frameshift mutation, Osteogenesis imperfecta

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List of abbreviations
ctDNA: circulating tumor DNA; **ddPCR:** droplet digital PCR; **EGFR:** epidermal growth factor receptor; **exoDNA:** exosomal DNA; **Nested PCR:** nested polymerase chain reaction; **NGS:** next-generation sequencing; **NSCLC:** non-small cell lung cancer

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INTRODUCTION

Thanks to new advancements in noninvasive molecular techniques, the approach toward cancer management is shifting. ctDNA and exoDNA have emerged as potential noninvasive biomarkers for early cancer detection and guiding targeted therapies[1].

ctDNA is a small fragment of DNA generated by the cells and released into the circulation through necrosis, apoptosis, or passive release from viable cells. In cancer, ctDNA comes from tumors and harbors somatic mutations[2]. This behavior presents ctDNA as an important biomarker for early cancer detection, enhancing patient treatment outcomes and overcoming the limitations of conventional screening approaches, such as low sensitivity and the necessity of invasive confirmatory procedures. Analyzing ctDNA for mutations, copy number variations, and DNA methylation status can improve early-stage cancer identification, as demonstrated in previous research on lung, colorectal, and breast cancers[3]. However, low ctDNA concentrations and methodological challenges prevent its wider application in early cancer diagnosis, though these barriers could be overcome with new advanced technologies[4].

Exosome is an extracellular vesicle that transports biomolecules, including DNA, RNA, and proteins and have recently emerged as stable carriers that may serve as cancer biomarkers[5]. ExoDNA, a source of diagnostic material, harbors tumor-specific genomic alterations and could be useful in cancer diagnosis[6]. Both ctDNA and exoDNA hold significant potential as noninvasive biomarkers for guiding targeted cancer therapies. These noninvasive biomarkers can provide real-time evidence of the tumor's molecular profile and its response to treatment, thereby enabling the optimization and modification of therapeutic strategies to improve clinical outcomes[7].

Some techniques, including PCR-based techniques, NGS, ddPCR, allele-specific real-time PCR, mass spectrometry, and loop-mediated isothermal amplification, can be employed for mutation detection in ctDNA and exoDNA. All these methods differ in their sensitivities and specificity[8]. Hence, there is a need to develop affordable techniques that are highly sensitive and specific when using noninvasive biomarkers in cancer. While state-of-the-art methods like NGS or ddPCR offer superior sensitivity for ctDNA detection, their widespread adoption in resource-limited settings is severely hindered by high costs, long turnaround times, and complex infrastructure requirements. These constraints often lead to significant diagnostic delays or the complete non-utilization of liquid biopsy. Therefore, developing cost-effective, rapid, and highly accessible diagnostic strategies for initial screening is a critical priority.

The combination of nested PCR and sanger sequencing offers a pragmatic solution that can be swiftly implemented in routine molecular laboratories. Acknowledging its inherent sensitivity limitations, this study was designed to specifically evaluate the clinical utility of nested-PCR and sanger sequencing as affordable, first-line screening tools for affirmative roll-in diagnosis in the NSCLC patient population. In this context, the present study investigated the common driver mutations in the *EGFR* gene in Iranian NSCLC patients using nested PCR, followed by direct sequencing on plasma ctDNA. Additionally, drawing from the results of our previous study on plasma exoDNA[9], we compared the sensitivity of ctDNA and exoDNA for detecting *EGFR* mutations and evaluated the potential advantages of combining these biomarkers for enhanced mutation detection.

MATERIALS AND METHODS

Study design and patients

This research was a retrospective observational study, which was carried out between December 2022 and February 2024 at Masih Daneshvari Hospital in Tehran, Iran. Initially, newly diagnosed NSCLC patients with stage II-IV were screened for common *EGFR* mutations in exons 18-21. Subsequently, 30 patients with *EGFR* mutations confirmed by tissue biopsy were selected for mutation assessment in plasma ctDNA. Additionally, 20 *EGFR*-wild-type NSCLC patients were included as controls to evaluate the specificity of the method.

Sample collection

Patients provided blood samples before receiving any systemic treatment. A 5 mL blood sample was collected from each patient using EDTA-containing tubes. Plasma was separated within 24 hours of collection through centrifugation at 1600 ×g for 10 minutes, followed by a second centrifugation at 2000 ×g for an additional 10 minutes. The obtained plasma was divided into aliquots and preserved at -80 °C until the extraction of ctDNA.

ctDNA extraction

The MagCore® Plasma DNA Extraction Kit (RBC Bioscience, Taiwan) was used to isolate ctDNA from plasma following the manufacturer's instructions. Initially, 20 µL of proteinase K (10 mg/mL) was added to the MagCore® sample tubes and mixed with 1200 µL of plasma. The mixture was then left at room temperature for 10 to 20 minutes and subsequently centrifuged at 20,000 ×g for 10 minutes. Once centrifugation was finished, the purified plasma was

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placed into a new tube for further analysis. The prepared sample tube was placed in the appropriate well of the T-Rack. Additionally, the elution tube and tip plus holder set (HF16, Compact) or pipette tip (Super, Plus) were positioned in their respective wells on the T-Rack. Finally, the Code 105 program was executed on the MagCore®. The extracted ctDNA was stored at -80 °C for further investigation.

ExoDNA extraction and mutation detection

Exosome isolation, characterization, and DNA extraction were conducted as previously established[9]. Briefly, exosomes were isolated from plasma using the miRCURY Exosome Isolation Kit (Qiagen, Germany). The isolated vesicles were characterized to confirm their identity through multiple approaches: morphological analysis by transmission electron microscopy, size distribution profiling by dynamic light scattering, and surface marker expression of tetraspanins (CD9, CD63, and CD81) by flow cytometry. ExoDNA was subsequently extracted from the characterized exosomes using the XCF™ Exosomal DNA Isolation Kit (System Biosciences, USA), and its concentration and purity were assessed using a Thermo Scientific™ NanoDrop™. Finally, mutations in the *EGFR* gene were detected via nested-PCR, followed by sanger sequencing.

***EGFR* mutation detection in plasma ctDNA**

Four exons (18-21) were evaluated using primers designed in our previous study[9]. *EGFR* mutation detection in ctDNA was conducted through nested PCR, followed by sanger sequencing. The external and internal nested PCR and the sanger sequencing were performed according to the protocol outlined in our earlier research[9].

Statistical analysis

All statistical analyses were carried out using SPSS version 26.0 (IBM Corp., Armonk, NY, USA). To examine the association between clinical factors and the detection of *EGFR* mutations in plasma ctDNA, we employed binary logistic regression analysis. Results were expressed as OR and their respective 95% CIs. The sensitivity of plasma ctDNA was assessed and expressed in terms of the proportion of patients with detectable *EGFR* mutations in plasma in comparison to tissue biopsy results. Specificity was calculated as the proportion of controls without detectable *EGFR* mutations in plasma ctDNA. Sensitivity and specificity were reported, along with their 95% CI calculated using the Clopper-Pearson method. To formally compare the diagnostic sensitivities of the ctDNA and exoDNA methods, the Chi-square test was employed. Combined sensitivity for ctDNA and exoDNA was also calculated to assess their complementary diagnostic performance. A p value < 0.05 was considered statistically significant.

RESULTS

Characteristics of participants

Thirty patients with stage II-IV NSCLC and confirmed *EGFR* mutation were enrolled in this study. The mean age of participants was 56.5 ± 11.3 years (range: 27-75), and 63.4% were female. Adenocarcinoma was the predominant histological subtype (90%), while the remaining 10% had squamous cell carcinoma. Most patients (70%) presented with advanced-stage disease (stage III-IV), 70% had exon 19 deletion, and 73.3% were non-smokers. Table 1 provides an overview of the demographic and clinical features of the participants.

***EGFR* mutation detection in plasma ctDNA**

Of the 30 patients with *EGFR* mutations confirmed by tissue biopsies, 19 (63.3%) also had detectable mutations in their plasma ctDNA. The detection sensitivity of ctDNA was 63.3% (95% CI: 43.9%-80.2%). Among the 20 *EGFR*-wild-type NSCLC control patients, no false-positive results were observed, yielding high specificity (95% CI: 83.9%-100.0%).

Table 1. The characteristics of participants

Characteristics	No. of participants (%)
Gender	
Female	19 (63.4)
Male	11 (36.6)
Age	
Median	56.5
Range	27-75
Smoking status	
Smoker	8 (26.6)
Non-smoker	22 (73.3)
Tumor histology	
Adenocarcinoma	27 (90)
Squamous cell carcinoma	3 (10)
Mutation type	
Exon 19 deletion	21 (70)
L858R mutation	9 (30)
Tumor Stage	
stage II	9 (30)
stage III-IV	21 (70)

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Binary logistic regression analysis

We performed binary logistic regression analysis to examine the association between various clinical factors and the detection of *EGFR* mutations in plasma ctDNA. The results are summarized in Table 2.

Age and gender

Mutations were detected in 70% of patients aged <50 compared to 54% of those aged 0 years (Fig. 1). Increasing age was not significantly associated with the likelihood of detecting *EGFR* mutations in ctDNA (OR = 0.85; 95% CI: 0.28-2.55; p = 0.76). Similarly, no statistically significant association was observed between gender and detection rates, which were 63.5% in men and 63.0% in women (OR = 1.09; 95% CI: 0.36-3.33; p = 0.88).

Tumor stage

Mutations were detected in 76.2% of patients with stage III-IV disease compared to 33.3% of those with stage II (Fig. 1). Advanced tumor stage (III-IV) was significantly associated with an increased likelihood of detecting *EGFR* mutations in ctDNA (OR = 3.91; 95% CI: 1.17-9.77; p = 0.04). This finding indicates that patients with advanced-stage disease have a higher likelihood of detecting *EGFR* mutations in their ctDNA compared to those with stage II disease.

Smoking status

Mutations were detected in the ctDNA of 87% of smokers compared to 54.5% of non-smokers (Fig. 1). While not reaching statistical significance, smoking status showed a trend toward increased likelihood of mutation detection (OR = 2.06; 95% CI: 0.91-11.41; p = 0.06). However, given the extremely small sample size in the smoker subgroup, this finding has a very wide CI and must be treated purely as an exploratory observation that requires confirmation in more robust studies.

Mutation type and tumor histology

Mutations were detectable in the ctDNA of 61.9% of patients with exon 19 deletions and 75% of those with the L858R mutation. In addition, 63% of patients with squamous cell carcinoma and 66.7% of those with adenocarcinoma had detectable mutations (Fig. 1). Neither mutation type (OR = 1.85; 95% CI: 0.51-8.01; p = 0.50) nor tumor histology (OR = 0.92; 95% CI: 0.21-3.99; p = 0.91) showed a significant association with the likelihood of detecting *EGFR* mutations in ctDNA.

ctDNA sensitivity vs. ExoDNA sensitivity

Statistical analysis showed a consistent, though not statistically significant, trend of higher sensitivity for plasma exoDNA compared to ctDNA. This pattern suggests a possible biological advantage, likely due to the protective environment of exosomes (Fig. 2).

Combined sensitivity of ctDNA and exoDNA

As depicted in Figure 2, ctDNA and exoDNA analysis together provided an overall sensitivity of 80.0% (24/30 patients) for *EGFR* mutations, which was significantly greater than that achieved by either ctDNA (63.3%) or exoDNA (76.6%) alone. These findings indicate the complementary function of these biomarkers in increasing diagnostic sensitivity for actionable mutations in NSCLC.

DISCUSSION

Liquid biopsy has emerged as a revolutionary technique in oncology, enabling patients to undergo diagnostic tests without the constraints of tissue samples. It facilitates the identification of tumor-specific characteristics, thereby aiding in targeted treatment and monitoring the disease[10]. Furthermore, real-time monitoring of tumor dynamics through liquid biopsy can detect resistance mutations and variations in tumor burden. This advantage allows for timely adjustments to treatment methods, which is not feasible with invasive tissue biopsies due to their invasive nature and the necessity for repetition[11].

Previous studies have demonstrated that liquid biopsies exhibit a high level of concordance with tissue biopsies in identifying targetable mutations in NSCLC. For example, Raez et al. have reported that liquid biopsy

Table 2. Binary logistic regression analysis of factors associated with *EGFR* mutation detection in plasma ctDNA of NSCLC patients

Factor	*p* value	OR	95% CI
Age	0.76	0.85	0.28-2.55
Gender	0.88	1.09	0.36-3.33
Tumor stage	0.04	3.91	1.17-9.77
Smoking status	0.06	2.06	0.91-11.41
Mutation type	0.50	1.85	0.51-8.01
Tumor histology	0.91	0.92	0.21-3.99

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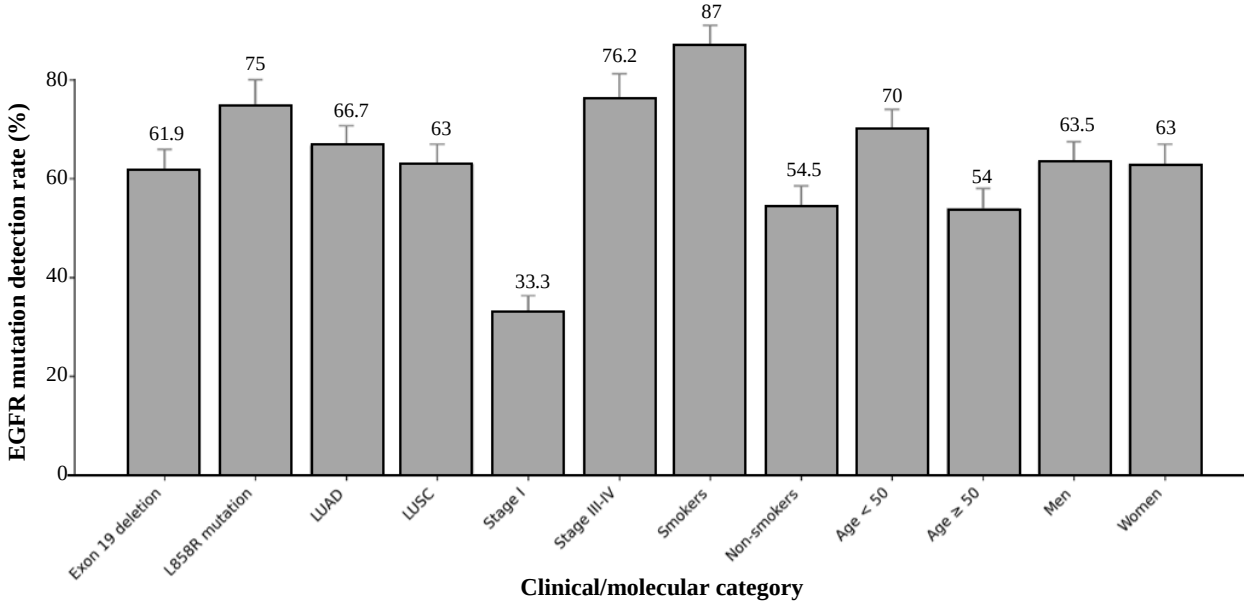

Fig 1. *EGFR* mutation detection rate in the plasma ctDNA of 30 patients with NSCLC: lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC).

results are approximately 97% concordant with tissue biopsy findings in detecting guideline-recommended biomarkers[12]. However, the sensitivity of liquid biopsies can vary based on the factors such as cancer type, tumor stage, and the method employed for mutation detection[13]. Consequently, we investigated the sensitivity of sanger sequencing following nested PCR on plasma ctDNA for detecting *EGFR* actionable mutations in Iranian NSCLC patients. Additionally, we performed a direct comparison of ctDNA and exoDNA by analyzing both biomarkers from the same patient cohort. We also calculated the combined sensitivity of both methods in detecting *EGFR* mutations. While the sensitivity of our method for *EGFR* mutation detection in ctDNA was 63.3%, its major strength resided in high specificity, with no false-positive detections identified. While we acknowledge that this sensitivity is lower than that of advanced technologies like NGS or ddPCR, our nested PCR and sanger sequencing workflow is justified by the critical balance between cost and sensitivity, particularly in resource-limited settings. This strategy makes our method a reliable "Rule-In" tool for highly specific and actionable diagnoses, ensuring patients are not subjected to unnecessary targeted therapies. We explicitly acknowledge that the 63.3% sensitivity prohibits its use as a "Rule-Out" test. Accordingly, our proposed diagnostic method mandates that negative results are confirmed by a more sensitive method to avoid withholding effective therapy. This pragmatic approach prioritizes accessibility and rapid turnaround, thereby facilitating timely molecular diagnosis for two-thirds of patients who would otherwise remain untested; however, negative results still necessitate confirmatory testing to ensure that eligible candidates for effective therapies are not inadvertently excluded. Zhu et al. reported a sensitivity of 82% for detecting *EGFR* mutations in plasma ctDNA from patients with advanced NSCLC using digital droplet PCR[14]. The observed difference in sensitivity (63.3% in our study) can be attributed to varying methodologies employed. Additionally, Yao et al. investigated *EGFR*, *KRAS*, and *PIK3CA* mutations, as well as gene rearrangements, through targeted DNA sequencing of ctDNA in advanced NSCLC, reporting a sensitivity of 70%[15].

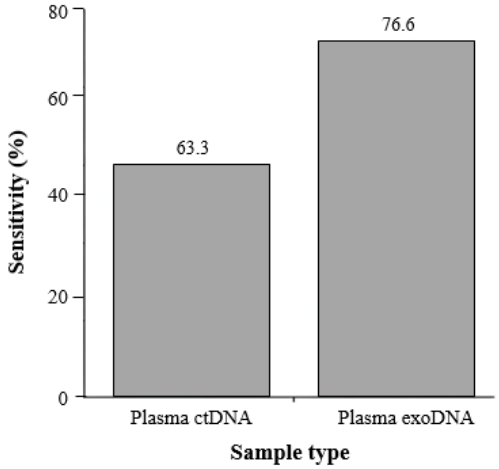

Fig. 2. The sensitivity of ctDNA vs. exoDNA in detecting *EGFR* common mutations.

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These findings indicate that the techniques utilized for mutation detection can significantly influence the sensitivity of ctDNA analysis. However, while digital PCR and NGS have demonstrated higher sensitivities[14,15], these technologies are often complex, costly, and may not be accessible in all clinical or research settings. Our robust, cost-effective nested PCR and sanger sequencing workflow offers a vital alternative for guiding treatment in resource-limited settings. This approach provides a fundamental diagnostic capability that can be used to identify a considerable proportion of patients harboring *EGFR* mutations, thereby supporting more individualized clinical management. Our results further revealed that the sensitivity of mutation detection was significantly associated with tumor stage, but not with tumor histology, mutation type, smoking status, or patient gender and age. Our results are consistent with the findings of a study indicating that tumor stage can influence the sensitivity of ctDNA in detecting *EGFR* mutations in NSCLC[16]. Crucially, this variability in detection is compounded by fundamental biological limitations of ctDNA, including variable tumor shedding rates (especially in early-stage disease) and ctDNA fragmentation patterns. In general, tumor mass tends to increase as NSCLC progresses to advanced stages. This behavior suggests that patients with a higher tumor burden have elevated levels of ctDNA in the bloodstream, thereby increasing the likelihood of identifying the mutation. For instance, a research has demonstrated that patients with stage III and IV NSCLC exhibit higher levels of plasma ctDNA[16], which complicates the effective detection of *EGFR* mutations in these patients compared to those with earlier stages of the disease[17,18]. Regarding smoking status, prior research has yielded inconsistent findings; for example, Tran et al. have found that the sensitivity of plasma ctDNA in detecting *EGFR* mutations was lower in smokers compared to non-smokers[19]. Conversely, Guo et al. have reported no significant association between ctDNA concentration and age, gender, or smoking habits[20]. Although our findings indicated no association between patient age and gender, previous studies have demonstrated that these demographic factors influence the sensitivity of ctDNA for detecting *EGFR* mutations in NSCLC. Specifically, a higher prevalence of mutations and elevated ctDNA levels in younger and female patients may enhance detection sensitivity[21-23]. Acknowledging these factors is crucial for optimizing the outcomes of ctDNA assays in clinical practice and providing targeted therapies that benefit patients.

Our findings indicated a sensitivity of 63.3% for detecting *EGFR* mutations using ctDNA. Notably, our previous study on the same cohort demonstrated a higher sensitivity (76.6%) with plasma exoDNA[9], (Fig. 2) . While this difference did not reach statistical significance, the trend aligns with findings from prior studies. For instance, Kim et al. have found that exoDNA is a more sensitive biomarker than ctDNA and may be useful in detecting *EGFR* mutations in NSCLC patients who exhibit low copy number mutations[24]. Furthermore, Wan et al. have revealed that nanoscale extracellular vesicle-derived DNA is more sensitive than ctDNA for detecting *EGFR* mutations in early-stage NSCLC[25]. Additionally, our analysis revealed that the combined sensitivity of exoDNA and ctDNA, as assessed by nested PCR and direct sequencing, was 80%, surpassing the sensitivity achieved by either biomarker alone. This result aligns with earlier research findings, suggesting a promising path for future research. For example, Castellanos-Rizaldos et al. reported that co-analyzing exosomal RNA/DNA and ctDNA yields significantly higher sensitivity for detecting the *EGFR* T790M mutation compared to analyzing ctDNA in isolation.[26]. Furthermore, Krug et al. have found that the combination of exosomal RNA/DNA and ctDNA is more sensitive than either ctDNA or exosomal RNA/DNA alone in identifying *EGFR* mutations in the plasma of patients with NSCLC[27].

The current findings should be interpreted within the specific context of our study objective to establish a pragmatic, cost-effective, and highly specific "Rule-In" tool for settings with limited resources, rather than to replace gold-standard quantitative technologies. This strategic focus directly informs the analytical validation priorities. While comprehensive analytical metrics such as the precise limit of detection, precision, and inter-assay variability were not the primary endpoint of this clinical feasibility study, the performance of our chosen method is well-understood. The nested PCR/sanger sequencing method is a widely recognized qualitative method with an established and inherently higher limit of detection (typically in the range of 5-10% variant allele frequency) compared to ultra-sensitive techniques like ddPCR. This characteristic is not a limitation of execution but a defining feature of the method, and it directly and expectedly underpins our observed clinical sensitivity of 63.3%. Furthermore, it is important to contextualize this methodological choice within the practical realities of the healthcare environment. In many resource-limited settings, including Iran, access to advanced genomic platforms like NGS or ddPCR is severely restricted due to financial constraints, international sanctions limiting equipment acquisition and reagent availability, and insufficient technical infrastructure. Therefore, the development and

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validation of accessible, affordable, and reliable alternatives is not merely an academic exercise but a critical necessity to bridge the diagnostic gap and ensure equitable cancer care. Crucially, for its intended role as a 'Rule-In' test, the most critical analytical performance metric is specificity. Our method achieved high specificity (100%, 95% CI: 83.9%–100.0%), which serves as a robust and direct measure of its analytical validity for ensuring reliable positive calls. Accordingly, any negative result obtained from this assay should be corroborated using a more sensitive analytical method. Furthermore, the single-center, retrospective design and the limited cohort size mean that the promising exploratory trends, such as those related to smoking status, require validation in larger, multi-center prospective studies to confirm their generalizability.

CONCLUSION

This study serves as a proof-of-concept, validating the use of nested PCR coupled with sanger sequencing as a highly specific and affordable liquid biopsy methodology for the detection of actionable *EGFR* mutations in the specific context of resource-limited settings such as Iran. The findings demonstrate that this approach provides a critical diagnostic capability where advanced genomic technologies are not routinely accessible. While the demonstrated sensitivity of 63.3% is inherently constrained by the analytical limits of the platform, the assayexceptional specificity establishes it as a reliable "Rule-In" tool for this setting, ensuring that positive results are unequivocally actionable. The consistently observed trend of superior sensitivity with exoDNA, coupled with the significant enhancement in detection sensitivity (80%) achieved through combined ctDNA/exoDNA analysis, further supports the potential of this multi-analyte approach and lays the groundwork for future optimization. Future efforts are warranted to build upon this proof-of-concept; these attempts should focus on technical refinement, validating the integrated approach in larger, multi-center prospective cohorts within similar resource-constrained environments, and formally assessing its impact on clinical pathways. This foundational work represents a necessary step toward achieving more equitable and personalized management of NSCLC patients in diverse healthcare settings.

DECLARATIONS

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Ethical approval

The study was approved by the Ethics Committee of the Pasteur Institute of Ira, Tehran, Iran (ethical code: IR.PII.REC.1401.038).

Consent to participate

All participants filled out the written informed consent forms.

Consent for publication

All authors reviewed the results and approved the final version of the manuscript.

Authors' contributions

PM, MDO, AD, AK, MMJ, NK contributed to designing the research, interpreting the data, and preparing the manuscript. All authors revised the manuscript.

Data availability

All data produced or examined in the course of this study are contained within this published article.

Competing interests

The authors declare that they have no competing interests.

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Supplementary information

The online version does not contain supplementary material.

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