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Assessing the Sensitivity of Nested PCR Followed by Direct Sequencing on Exosomal DNA for EGFR Mutation Detection in NSCLC

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ABSTRACT

Background: Early and minimally invasive detection of EGFR mutations in NSCLC patients is a promising tool to select patients for targeted therapy in order to improve their prognosis. This study aimed to identify a sensitive, cost-effective, and easily accessible noninvasive method for detecting the EGFR-targetable mutations in the plasma exoDNA of patients with NSCLC.

Methods: This retrospective observational study was conducted over 10 months, from December 2022 to October 2023, at Masih Daneshvari Hospital in Tehran, Iran. A total of 30 patients with stage II-IV NSCLC and targetable mutation in the EGFR gene were included in the study. Nested PCR and Sanger sequencing were used to evaluate EGFR mutations in the DNA extracted from circulating exosomes.

Results: The study found a sensitivity of 76.6% for EGFR mutation detection on exoDNA compared to tissue results. No significant impact was observed based on tumor staging, histology, mutation type, smoking status, gender, or age.

Conclusion: Therapeutically targetable driver mutations in the EGFR gene can be accurately detected using nested PCR followed by direct sequencing of plasma exoDNA from patients with NSCLC. This approach facilitates timely and more personalized treatment for NSCLC patients, ultimately improving patient prognosis. Additionally, this method reduces the reliance on invasive tissue biopsies and their associated complications.
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List of Abbreviations:
ctDNA: circulating tumor DNA; **DLS:** dynamic Light Scattering; **EGFR:** epidermal growth factor receptor; **exoDNA:** exosomal DNA; **NGS:** next-generation sequencing; **NSCLC:** non-small cell lung cancer; **PCR:** polymerase chain reaction; **TEM:** transmission Electron Microscopy; **TKI:** tyrosine kinase inhibitor

INTRODUCTION

According to the World Health Organization (2021), lung cancer is a leading cause of cancer-related deaths, with approximately 12 million incidences of new cases and 1.8 million deaths each year. Among these cases, NSCLC is prevalent in about 85% of individuals. NSCLC is often diagnosed at a later stage, leading to increased therapeutic complexities and lower survival probabilities[1]. Recent advancements in understanding the molecular intricacies of various cancers and the impact of oncogenic driver mutations have paved the way for targeted therapies[2].

EGFR gene mutations are considered targetable drivers in NSCLC, and identifying these mutations has completely altered the approach toward NSCLC treatment[3]. Mutations like exon 19 deletion and also L858R point mutation in exon 21 make patients sensitive to TKIs and improve their prognosis[4]. EGFR genetic testing typically involves tissue biopsies, which are accurate but risky due to their invasive nature. Some complications of tissue biopsy include bleeding, infections, and patient distress, especially in advanced stage of disease. Additionally, challenges such as limited tissue availability and the potential for false-negative outcomes due to tumor heterogeneity or low tumor cellularity can further complicate the procedure[5]. This challenge highlights the importance of more specialized and less invasive diagnostic methods to detect targetable mutations in NSCLC. Liquid biopsy, as a noninvasive method, facilitates the early detection of cancer through genetic testing. This technique mainly utilizes ctDNA or exoDNA from a blood sample, eliminating the need for tissue biopsies[6]. Necrotic or apoptotic dead cancer cells release their ctDNA into the bloodstream, while ExoDNA originates from living tumor cells that secrete small extracellular vesicles called exosomes. Such exosomes contain molecular material that reflects the genomic profile of the tumor[7].

ctDNA is among the commonly used techniques for detecting EGFR mutations in NSCLC; thus, more sensitive technical structures taking cognizance of ctDNA have already been integrated into clinical practice[8]. Compared to ctDNA, exoDNA is a more effective biomarker for cancer detection because of its unique genetic indicators and gene expression profile[9]. It detects mutated tumor DNA in the blood more accurately than ctDNA, especially in early-stage patients, thereby providing a comprehensive reflection of tumor heterogeneity. Lipid bilayer membrane of exosomes protects the encapsulated DNA, allowing for storage at 4 °C for 48-96 hours or at -70 °C for extended periods while retaining organic integrity[10]. Considering the potential of exoDNA as a reliable source for identifying the molecular characteristics of tumors, this study was designed to investigate the sensitivity of nested PCR followed by direct sequencing in identifying EGFR mutations on plasma exoDNA in patients with NSCLC. The aim was to identify a sensitive, cost-effective, and easily accessible noninvasive method for detecting targetable EGFR gene mutations in NSCLC patients. Furthermore, the study analyzed detection sensitivity concerning age, male-to-female ratio, smoking history, and tumor histology, both generally and by stage. The present research seeks to address the existing gap in the literature regarding liquid biopsy strategies and their potential clinical applicability in the future. Such integration could have significant implications for the prognosis and management of NSCLC patients by enhancing the accessibility, timeliness, and minimally invasive nature of genetic testing.

MATERIALS AND METHODS

Study design and sample size

The present retrospective and observational study was conducted over a period of 10 months, from December 2022 to October 2023, at Masih Daneshvari Hospital in Tehran, Iran. A total of 110 patients diagnosed with NSCLC (stages II-IV) and aged ≥18 years were included in the study and evaluated for EGFR gene mutations. Diagnostic tissue samples were obtained through gross-needle biopsy or surgery. Among the 110 patients, 30 were positive for EGFR-targetable mutations, which is the focus of this study. Blood samples (approximately 5 mL in EDTA tubes) were collected at the time of diagnosis or before the initiation of therapy. The samples were then centrifuged for 15 minutes at 3000 ×g and a temperature of 4 °C. The resulting supernatant was transferred to a new tube and stored at -80 °C until further analysis.

Exosome isolation

The miRCURY Exosome Isolation Kit for serum and plasma (Qiagen, Germany) was used to isolate exosomes following the manufacturer's protocol. Initially, 1700 µL of plasma was centrifuged at 10,000 ×g for 5 minutes, and then the pellet was removed. Afterwards, 17 µL of thrombin was added to the supernatants, gently vortexed, and incubated at 15-25 °C for 5 minutes. The samples were centrifuged again under the same conditions, and 1400 µL of the supernatant was collected for exosome purification. Subsequently, 560 µL of precipitation buffer was added to the sample, vortexed, and left at 4 °C overnight. Samples were then centrifuged twice at 500 ×g for 5 minutes to ensure the complete removal of the

supernatant. Finally, the pellets were resuspended in 240 µL of resuspension buffer and mixed by vortexing.

Exosome characterization

Transmission electron microscopy

A negative stained sample was prepared using a 400 mesh formvar-coated copper grid and 2% phosphotungestic acid. A drop of the sample was placed on the formvar-coated grid. After 10 minutes, excess liquid was blotted with filter paper, and then distilled water was added and blotted again. Finally, a drop of 2% phosphotungstic acid solution was added. After 2 minutes, the excess liquid was blotted, and the sample examined using an 80 kilovolt Transmission Electron Microscope (Zeiss EM 900, Germany).

Dynamic light scattering

The size distribution of nanoparticles was evaluated using the DLS technique. Briefly, 25 µL of samples were mixed with phosphate buffered saline to reach a total volume of 500 µL. The mixture was then measured using a SZ-100 Nanoparticle Size Analyzer (Horiba, Japan) with a dynamic range of 0.3 nm -8 µm.

Flow cytometry

The presence of tetraspanins CD9, CD63, and CD81 on the surface of exosomes was analyzed using flow cytometry. A 100 µL of sample was added to the test tubes, followed by 3 µL of CD9, CD63, and CD81 antibodies. The mixture was thoroughly mixed and incubated at 4 °C for 30 minutes. The tube containing all three antibodies was then analyzed using the BD FACS Calibur system (BD Biosciences, San Jose, CA, USA).

ExoDNA extraction

For each test, 500 µL of the exosome solution was used. ExoDNA was extracted using the XCF™ Exosomal DNA Isolation Kit (System Biosciences, USA), including DNA binding buffer, washing buffer, and elution buffer, following the manufacturer's instructions. The purity and concentration of the isolated DNA were evaluated using Thermo Scientific™ NanoDrop™.

EGFR mutation detection

Primers for exons 18, 19, 20, and 21 were designed according to the standard criteria. The primer sequences are listed in Table 1. Nested PCR and Sanger sequencing were employed to assess the EGFR mutations in the DNA extracted from the tumoral tissue and exosomes. In summary, 10 µL of Taq DNA Polymerase Master Mix RED (Ampliqon, Demark), 0.5 µL of forward and reverse primers, 1 µL of extracted DNA, and 8 µL of double distilled water were used in a final volume of 20 µL for external nested PCR. PCR reactions were conducted in the BioRad Thermal Cycler machine using the following cycling conditions: 1 cycle at 95 °C for 15 min, followed by 25 cycles at 94 °C for 30 seconds, 58 °C for 30 seconds, and 72 °C for 30 seconds. For internal nested PCR, the protocol was the same as external nested PCR, except that we used the PCR product as a

Table 1. List and sequence of primers used in nested PCR

Primer name	Sequence	Product size (bp)
EGFR-ex18-Forward 1	5'-GGCACTGCTTTCCAGCATGG-3'	214
EGFR-ex18-Reverse 1	5'-CCAGAGGCCTGTGCCAGG-3'	
EGFR-in18-Forward 2	5'-TTGTCTCTGTGTTCTTGTCCCC-3'	176
EGFR-in18-Reverse 2	5'-CCAGAGGCCTGTGCCAGG-3'	
EGFR-ex19-Forward 1	5'-AGCATGTGGCACCATCTCAC-3'	192
EGFR-ex19-Reverse 1	5'- GCCATGGACCCCCACACAGC -3'	
EGFR-in19-Forward 2	5'- CTGTCATAGGGACTCTGGATCC-3'	231
EGFR-in19-Reverse 2	5'- CACACAGCAAAGCAGAAACTCA-3'	
EGFR-ex20-Forward 1	5'-TCTTCACCTGGAAGGGGTCCA-3'	257
EGFR-ex20-Reverse 1	5'-TGCACACACCAGTTGAGCAG-3'	
EGFR-in20-Forward 2	5'-AGCCACACTGACGTGCCTC-3'	197
EGFR-in20-Reverse 2	5'-GTTGAGCAGGTACTGGGAGCC-3'	
EGFR-ex21-Forward 1	5'-AGCCAGGAACGTACTGGTGA-3'	204
EGFR-ex21-Reverse 1	5'-CCTCCCCTGCATGTGTTAAAC-3'	
EGFR-in21-Forward 2	5'-GGTGAAAACACCGCAGCATG-3'	236
EGFR-in21-Reverse 2	5'-GGTGTCAGGAAAATGCTGGCT-3'	

Table 2. Charecteristics of parcipitants

Characteristics	No. of patients (%)
Sex	
Male	19 (63.3)
Female	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)
Stage	
II	9 (30)
IIIA	6 (25)
IIIB	10 (33.3)
IV	5 (16.6)

template, which was ran 35 cycles at 62 °C. The PCR products were electrophoresed on a 2% agarose gel. Using nested PCR before Sanger sequencing, can greatly enhance the accuracy of mutation detection by improving the sensitivity and specificity of the assay, ultimately resulting in more reliable sequencing results. Therefore, we carried out nested PCR together with Sanger sequencing. For Sanger sequencing, we submitted the products of the internal nested PCR to be sequenced using the ABI 3500 XL DNA Sequencer. The sequencing files were analyzed using CodonCode Aligner 11.0.2 software.

Statistical analysis

The free R-project software (https://www.r-project.org) was used to conduct the data analysis. The association of EGFR gene mutations with age, gender, smoking status, and disease stage was examined using Chi-square and Fisher's exact test. A statistically significant two-sided *p* value was defined as less than 0.05.

RESULTS

Patients' characteristics

After examining the 110 NSCLC patients, 30 cases with stage II-IV of NSCLC and confirmation of EGFR mutations were selected for further investigation of the EGFR mutations on plasma exoDNA. The average age of the patients was 56.5, with 27 having adenocarcinoma and 3 having squamous cell carcinoma. There were 19 women and 11 men, with 8 having a history of smoking. Nine individuals were in stage II, while 21 were in stages III-IV. Furthermore, 21 and 8 patients had exon 19 and 21 mutations, respectively, and only one patient exhibited mutation in exon 20. The characteristics of the patients are shown in Table 2. Characterization of plasma-derived exosomes plasma-isolated exosomes were evaluated by TEM, DLS, and flow cytometry for morphology, size distribution, and specific immunological markers. TEM revealed the exosomes as cup-shaped morphologically (Fig. 1A). Flow cytometry was positive for CD9, CD63, and CD81 in exosomes (Fig. 1B). The size of the exosomes as per DLS was 67.1 nm in diameter (Fig. 1C).

EGFR mutations on plasma exoDNA

Following the extraction of exoDNA and its amplification through nested PCR, the resulting PCR products were subjected to electrophoresis on a 2% agarose gel and then submitted for further analysis via Sanger sequencing. Figure 2 shows the results of electrophoresis and sequencing of selected samples. Among the 30 patients with confirmed EGFR mutations in DNA extracted from tissue biopsy, 23 (76.6%) patients exhibited detectable mutations in their plasma ExoDNA. The mutation was detectable in plasma exoDNA of 71.43% of patients with exon 19 deletion and 87.5% of patients with the L858R mutation. However, there was no significant association between the type of mutation and the probability of mutation detection in plasma exoDNA ($p = 0.63$). The results indicated that EGFR mutations were detectable in the exoDNA of 66.67% in the patients with squamous cell carcinoma and 74% with adenocarcinoma. Statistical analysis revealed no significant association between tumor histology and the likelihood of mutation detection in plasma exoDNA ($p > 0.67$). Regarding cancer stage, 66% of patients with stage II tumor and 80% of stage III- IV, exhibited detectable mutations in exoDNA. The statistical analysis displayed that the tumor stage has no significant association with the likelihood of the EGFR mutations finding in plasma exoDNA ($p = 0.64$). Our data showed that 72.7% of non-smoking patients exhibited EGFR mutations in exoDNA, while 75% of smokers demonstrated the same mutations. We observed no significant association between smoking status and the likelihood of detecting mutations in plasma exoDNA ($p > 0.99$). Detectable EGFR mutations were found in 73.7% of females and 82.7% of males. The likelihood of finding common EGFR gene mutations in exoDNA was not significantly associated with gender ($p = 0.37$). Data analysis indicated that 82% of individuals above 50 and 62.5% below 50 exhibited detectable mutations in their exoDNA. Statistical analysis did not show a significant association between patients' age and the likelihood of identifying common EGFR mutations in their exoDNA ($p = 0.34$).

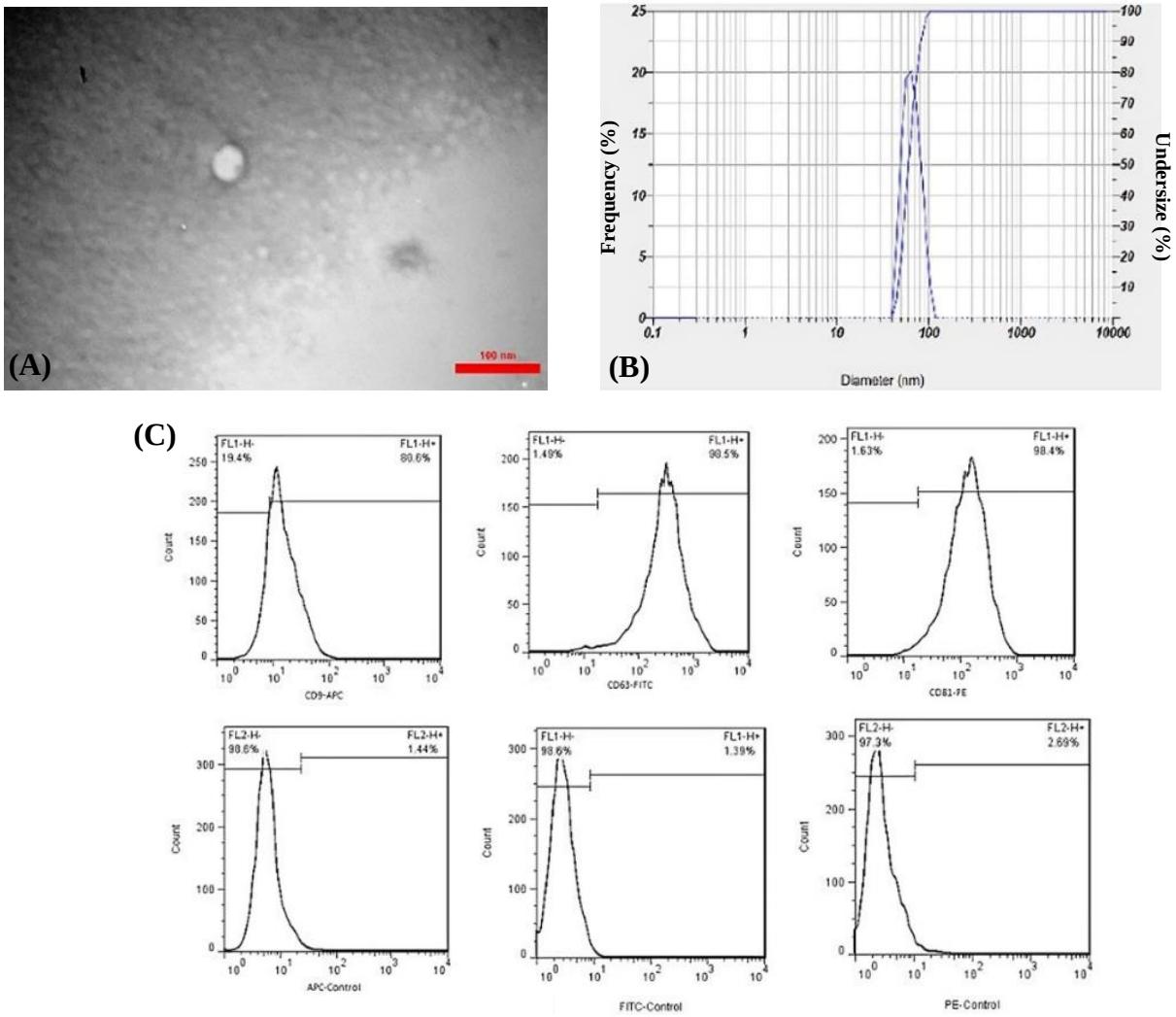

Fig. 1. Characterization of the plasma-isolated exosomes. (A) Morphology of exosomes detected by TEM; (B) particle size distribution by DLS; (C) expression of exosome markers (CD9, CD63, and CD81) by flow cytometry.

DISCUSSION

The ability of exosomes to incorporate and deliver genetic materials, such as DNA, makes them potentially valuable biomarkers in cancer liquid biopsy. ExoDNA, in particular, captures the genetic profile of the source cells and reveals the tumor genotype[11]. Therefore, the exoDNA assay could serve as a useful tool for the early detection of EGFR mutations in the high risk populations, such as smokers and those with a family history of lung cancer, enabling timely and precise treatment for improved outcomes[12,13]. This study was designed and conducted to offer a noninvasive method for the minimally invasive diagnosis of targetable mutations in the EGFR gene, facilitating the identification of the most effective drugs for targeted therapy. Thirty out of 110 patients with stage II-IV NSCLC, who referred to Masih Deneshvari Hospital, were included in the study based on the presence of EGFR mutations in tissue biopsy. Our findings showed that the diagnostic sensitivity of exoDNA was 76.6% compared to tissue biopsy results. In addition, factors such as tumor staging, tumor histology, type of mutation, smoking status, patient gender, and age did not significantly influence the probability of mutation detection.

Several studies have been conducted to identify EGFR gene mutations in the serum and plasma of NSCLC patients as a less invasive approach. The findings indicate that the accuracy of the test varies depending on the methods employed. In 2013, Kim et al. attempted to evaluate the sensitivity of peptide nucleic acid-mediated PCR clamping to identify EGFR mutation on ctDNA of NSCLC patients. They detected EGFR in 17% of plasma samples, with 70% showing exon 19 deletions and 30% showing exon 21 point mutations[14].

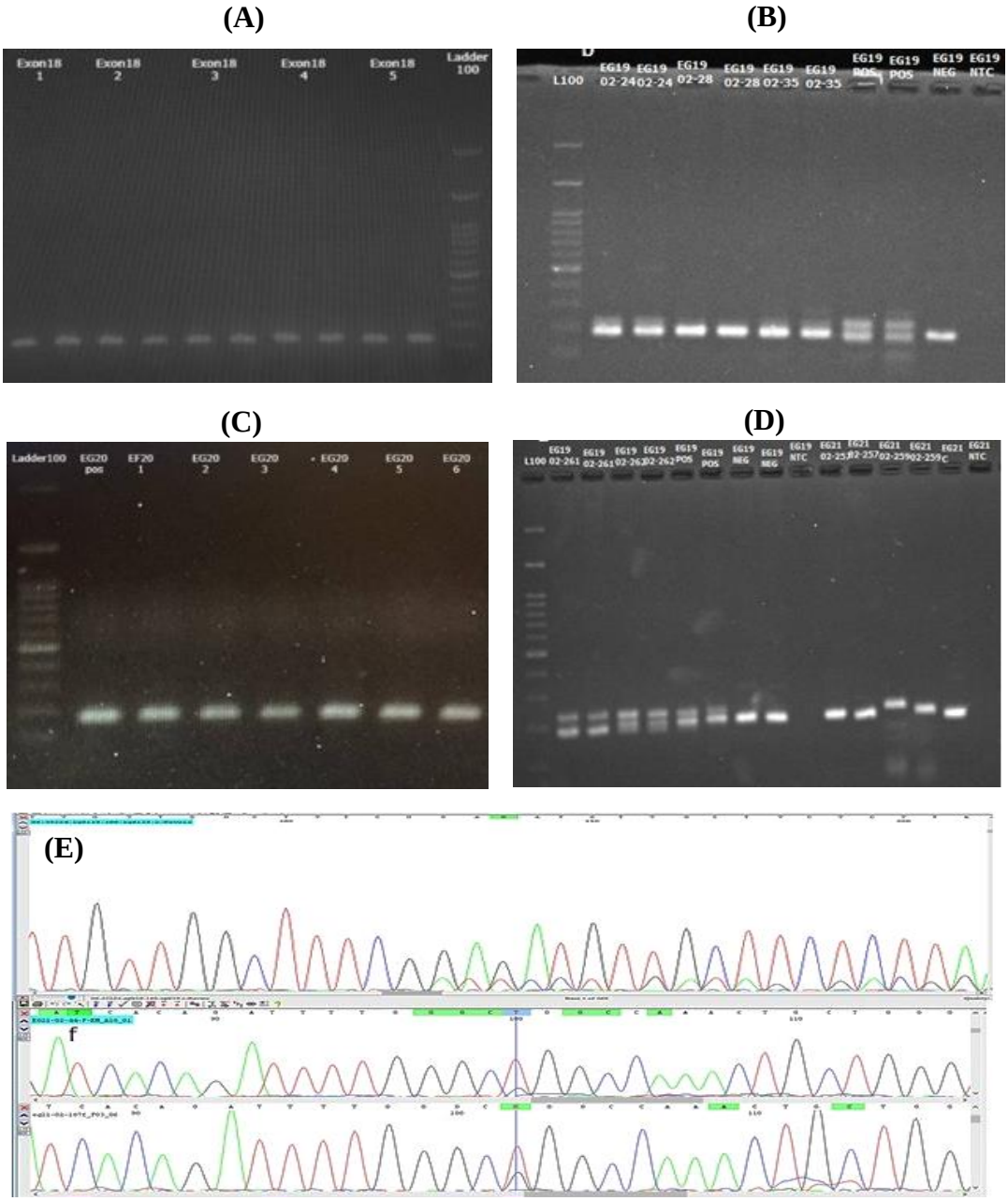

Fig. 2. Gel electrophoresis and sanger sequencing results of selected samples, gel electrophoresis results of (A) exon 18, (B) exon 19, (C) exon 20, and (D) exon 21; sequencing results of (E) exon 19 deletion and (F) exon21 point mutation.

Zaini and his colleagues conducted a study using high resolution melt analysis, restriction fragment length polymorphism, and direct sequencing techniques to identify the common EGFR gene mutations in the tumoral tissue and plasma of NSCLC patients. Their study showed that the detection sensitivity was different and varied from 9. 1% to 39% depending on the technique employed[15]. In 2019, Castellanos-Rizaldos and his team designed a study to identify the activating and drug-resistance mutations in the EGFR gene. Their study analyzed exosomal RNA/DNA and ctDNA from 110 patients with NSCLC using a quantitative PCR-based test. The results demonstrated a sensitivity of 90% for the L858R mutation, 83% for T790M mutation, and 73% for exon 19 indels[16]. In another study, Steendam et al. examined 36 patients with NSCLC whom EGFR mutations were identified through tissue biopsy. They investigated the EGFR mutations in patients' ctDNA using digital droplet PCR and NGS methods. The results indicated that the detection sensitivity varied depending on the method employed, with 69% for digital droplet PCR and 83% for NGS[17]. In 2016, Thompson et al.

applied the NGS method to identify targetable mutations in tumor tissues and ctDNA of patients with NSCLC. Their results showed an 84% concordance in the mutations observed in both tissue and plasma samples[18]. In a study conducted by Kim et al. in 2019, exoDNA and RNA and ctDNA from 47 plasma samples were analyzed for EGFR gene mutations. The sensitivity of short-length exoTNA (76.5%) was found to be higher than that of ctDNA (64.7%) for detecting EGFR mutations in NSCLC patients. It was noted that the target nucleic acids and size distribution may play a crucial role in determining the appropriate extraction methods and detection assay protocols[19]. In a recent study, pyrosequencing was used to detect EGFR mutations in tissue biopsy and exosome samples from 28 NSCLC patients. The findings indicated that exosomes are valuable tools for monitoring EGFR mutation status, and could serve as a noninvasive method for assessing tumor mutation status, thereby facilitating personalized treatment for NSCLC patients[20].

Liquid biopsy-based cancer detection is a promising tool not only for early detection of NSCLC but also for selecting patients for targeted therapy and monitoring residual tumors. Various sources, such as plasma, serum, pleural effusion samples, and even sputum samples, have been utilized for minimally invasive access to tumor DNA. Different molecular methods have also been used to investigate EGFR gene mutations, which are the most common and significant mutations for targeted therapy. According to the results of previous studies, each of the mentioned methods exhibited varying sensitivity and specificity. Our findings demonstrated that using nested PCR followed by direct sequencing could be used as an acceptable molecular method with sufficient sensitivity for investigation of common EGFR mutations in the plasma ExoDNA of NSCLC patients. This minimally invasive detection method could facilitate timely targeted treatment of patients and reduce the need for tissue biopsy and its associated complications.

CONCLUSION

The use of liquid biopsy for diagnosing EGFR mutations represents a significant advancement in the field of personalized oncology. This noninvasive yet efficient alternative to traditional tissue biopsies enhances the effeciency and timeliness of patient management. Hence, future research should concentrate on validating the results obtained from liquid biopsy procedures and their integration into clinical practice to fully realize the potential benefits of this practice for cancer-related outcomes.

DECLARATIONS

Acknowledgments

We sincerely thank the patients who participated in the present study. No artificial intelligence was used in this study.

Ethical approval

All the experimental procedures in this study were conducted in accordance with the guidelines of Helsinki and approved by the Research Ethics Committee of Pasteur Institute of Iran, Tehran, Iran (ethical code: IR.PII.REC.1401.038).

Consent to participate

All the patients agree to voluntarily participate in this research. All the participants have provided written informed consents.

Consent for publication

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

Authors' contributions

MJ: data curation, formal analysis, investigation, and writing–original draft preparation; PM: conceptualization, formal analysis, funding acquisition, supervision, validation, visualization, and writing–review and editing; MDO: conceptualization, funding acquisition, and supervision; AKH: resources; AD: methodology, software, and validation; SAM: project administration and validation; MZ: investigation and software; MM: methodology and resources; SS: visualization and resources; BS and PR: resources.

Data availability

All relevant data can be found within the manuscript.

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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