The Role of Liquid Biopsy for Detecting Epidermal Growth Factor Receptor (EGFR) Mutations in Patients with Advanced Non-Small Cell Lung Cancer (NSCLC) – A Preliminary Study in Indonesian Population

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INTRODUCTION

Lung cancer is the second most common cancer in the world with an incidence of 2.2 million new cases per year (11.4%) and is the leading cause of cancer-related deaths, accounting for 1.7 million fatalities or 18.0% [1]. Recent data from the World Health Organization (WHO) International Agency for Research on Cancer (IARC) 2020, showed that this disease ranked as the leading cause of death from cancer cases in Indonesia. It was also found...
to be the most frequently diagnosed cancer in males and 3rd most common generally [2]. Non-small cell lung cancer (NSCLC) constitutes approximately 85% of lung cancer cases, with adenocarcinoma emerging as the most predominant histological type, accounting for about 40% of diagnoses [3].

Early diagnosis and treatment are the most effective measures to reduce the mortality rate of patients with primary lung cancer [4]. The development of new biomarker-targeted therapies due to a better understanding of cancer biology has led to improvements in overall survival for patients with advanced or metastatic disease [5]. Although solid biopsy remains essential for clinical diagnosis to determine the molecular and histological characteristics of tumors, the procedure is not without its limitations and risks [4], specifically in cases where surgery may not be a suitable option. In these cases, obtaining sufficient tumor material for molecular testing through tissue biopsy can be challenging due to the advanced nature of the disease [6].

Liquid biopsy is a minimally invasive technique to obtain cytological and molecular insights from patients, with peripheral blood being the most commonly examined bodily fluid [4,6]. This technique can detect various biologic analytes, such as circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), exosomes, microRNAs (miRNA), peripheral blood circulating RNA, tumor-educated blood platelets (TEPs), and circulating tumor vascular endothelial cells (CTECs) [4,7]. Among these, ctDNA currently stands out as the most extensively investigated marker.

Liquid biopsy facilitates real-time monitoring of tumors and the unveiling of resistant mechanisms [4]. The initial clinical application of liquid biopsy in advanced NSCLC was for detecting epidermal growth factor receptor (EGFR) mutations, which was followed by the widespread use of high-throughput sequencing methods, such as the next-generation sequencing (NGS) [7]. Approximately, 10–30% of patients with NSCLC have mutations of the EGFR genes, with a higher incidence observed in the Asian population [8]. Therefore, this preliminary study aimed to compare the molecular profile obtained through liquid and tissue biopsy in patients with advanced NSCLC in Indonesia.

METHODS

Patient and specimen collection

This cross-sectional study was conducted on newly diagnosed NSCLC patients undergoing diagnostic procedures at Dharmais National Cancer Center Hospital from January 2018 to December 2021. The samples were recruited using the convenient sampling method, while the inclusion criteria included (I) aged 18 or older, (II) patients newly diagnosed with advanced-staged NSCLC (stage IIIB or IV), and (III) cases where tumor and plasma DNA were simultaneously analyzed. Simultaneous analysis was defined as an interval of less than 14 days between tissue and blood sampling, with no new intervention. Patients with a previous history of other malignancies and incomplete data were excluded. Tissue biopsy was performed using (I) bronchoscopy cytobrush, (II) transthoracic needle aspiration (TTNA), (III) pleural fluid, and (IV) fine needle aspiration biopsy (FNAB) cytology. EGFR mutations were considered common or classical for exon 19 deletions and L858R at exon 21 [9,10], while others were categorized as uncommon.

Tissue EGFR mutation detection and plasma NGS genotyping

For tissue biopsy, formalin-fixed paraffin-embedded (FFPE) tissue blocks were cut using a microtome into sections and placed in a microcentrifuge tube. Paraaffin removal from the sections was achieved with Citrisolv Clearing Agent and heat, resulting in tissue pellets. Subsequent steps included washing the tissue with 100% Ethanol followed by drying at room temperature. The pellet was digested through protease solution and bound using DNA Binding Buffer containing beads. The beads were washed with DNA Wash Buffer and eluted in the Elution Solution. DNA was extracted through MagMAX™ FFPE DNA/RNA Ultra Kit (Thermo Fisher Scientific) and the minimum tumor content in the specimen was 30%. Mutations in the EGFR gene were analyzed with a home-brew PCR method based on High-Resolution Melting (HRM). This approach encompassed the detection of exon 19 deletions (detects their presence but does not distinguish between the types) and exon 21 mutation. HRM data and melting curve data were also analyzed.

For liquid biopsy, plasma samples were obtained from 5 mL of blood anticoagulated with EDTA. DNA was extracted using MagMAX™ Cell-Free Total Nucleic Acid Isolation Kit (Thermo Fisher Scientific), while sequencing for ctDNA was performed with NGS. The kit used was Oncomine Lung cfTNA Research Assay (Thermo Fisher Scientific), designed to detect single nucleotide variations and short insertions/deletions in genes such as ALK, BRAF, EGFR, ERBB2, KRAS, MAP2K1, MET, NRAS, PIK3CA, ROS1, and TP53. The assay also covered gene fusions in ALK, RET, and ROS1, as well as copy number variations in MET. Sequencing data analysis was carried out with the Ion ReporterTM 5.10.1.0 software directly integrated with Torrent SuiteTM 5.10.1 software (Thermo Fisher Scientific). This was followed by manual inspection and further analysis with the commercial software Sequence Pilot version 4.3.0 (JSI Medical Systems). All laboratory analyses were conducted at KALGen Innolab, Jakarta.

Data analysis

Sequencing results from both assays were descriptively compared for each patient. Statistical analysis was conducted using a statistical package for social sciences
The normality was assessed with the Shapiro-Wilk test, while the baseline patient demographic and clinical characteristics were summarized using standard descriptive statistics.

RESULTS

A total of 22 subjects consisting of 10 males and 12 females participated in this study as samples, with a mean age of 59 years. Almost all subjects (21 out of 22) had a primary diagnosis of lung adenocarcinoma, while one was diagnosed with lung squamous cell carcinoma. Eighteen subjects were classified as stage IV NSCLC (81.8%), with 8 (36.3%) having distant metastases. Smoking history was found in 5 subjects (22.7%). Tissue samples for biopsy were obtained using different methods including bronchoscopy, TTNA, FNAB, and pleural fluid for 9, 8, 4, and 1 sample respectively. The mutational characteristics are depicted in Table 1.

Evaluation of EGFR mutations from tissue biopsy was depicted in Figure 1 with wild-type EGFR as the most common type, followed by exon 19 and 21 (L861Q and L858R). The results from liquid biopsy found EGFR exon 19 deletion as the most frequent type (Figure 2, Figure 3). A total of 13 subjects (50%) had common mutations. Among these, 3 had single common types, while 10 had two or more mutations in combination with uncommon or common types. EGFR in T790M exon 20 was found in 5 subjects and was categorized as pre-treatment or de novo mutation.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Diagnosis</th>
<th>Staging</th>
<th>Tissue Sampling Method</th>
<th>EGFR Tissue</th>
<th>NGS liquid biopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Adenocarcinoma</td>
<td>IVA</td>
<td>Bronchoscopy</td>
<td>Wild Type</td>
<td>EGFR del 19, L858R; KRAS G12C</td>
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<td>Ex 19 Del</td>
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<td>IVA</td>
<td>Bronchoscopy</td>
<td>Wild Type</td>
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<td>Pleural fluid</td>
<td>Ex 21 L858R, Ex 21 L861Q</td>
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<td>EGFR G719S, L858R</td>
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<td>IVA</td>
<td>TTNA</td>
<td>Wild Type</td>
<td>Wild Type</td>
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</tbody>
</table>

BRAF, B-Raf proto-oncogene; EGFR, epidermal growth factor receptor; FNAB, fine needle aspiration biopsy; KRAS, Kirsten rat sarcoma virus; MET, mesenchymal epithelial transition proto-oncogene; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; ROS1, ROS proto-oncogene 1; TP53, tumor protein p53; TTNA, transthoracic needle aspiration
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EGFR Mutations in Non-Small Cell Lung Cancer

Figure 1. Mutation distribution in tissue samples of 22 NSCLC patients at diagnosis. EGFR, epidermal growth factor receptor; Ex, exon.

Figure 2. Mutation distribution in plasma samples of 22 NSCLC patients at diagnosis. BRAF, B-Raf proto-oncogene; EGFR, epidermal growth factor receptor; KRAS, Kirsten rat sarcoma virus; MET, mesenchymal epithelial transition; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; ROS1, ROS proto-oncogene 1; TP53, tumor protein p53.

Figure 3. Mutation distribution of EGFR mutations from liquid biopsy. EGFR, epidermal growth factor receptor; Ex, exon.
Approximately 50% (10 subjects) had a single exon mutation, while others were diagnosed with multiple cases.

Liquid biopsy using the NGS approach also found KRAS, TP53, ROS-1, BRAF, and MET mutations with the most common being KRAS and TP53. Specifically, KRAS mutations included KRAS G12c in 2 subjects, as well as G12D, and G13C in 1 each. TP53 was found in 3 subjects including G245D, R158L, and C277F, while BRAF, MET, and ROS1 mutations were detected in 1 subject each.

**DISCUSSION**

Based on current clinical guidelines, the testing of molecular mutations is considered mandatory for treatment [11,12]. Tumor tissue genotyping remains the ‘gold standard’ for lung cancer diagnosis due to its high sensitivity and specificity [11-13]. Both the National Comprehensive Cancer Network (NCCN) and the European Society for Medical Oncology (ESMO) emphasize tissue biopsy as the primary diagnosis method. Liquid/plasma biopsy cannot replace tissue biopsy for diagnosis and treatment initiation [11,12]. This is due to the low sensitivity of ctDNA testing, which can yield up to 30% false negative rate despite its high specificity [12].

For tissue biopsy, approximately 40% of patients need more than one procedure to achieve a definitive diagnosis of lung cancer due to the scarcity of tumor tissue [7]. The failure rate of adequate molecular characterization is higher in needle biopsies, ranging from 20.5% to 30.8%. Meanwhile, larger surgical resection specimens exhibit lower failure, between 0% to 4.21%, and cell block preparations show intermediate rates of 5.47% to 8.21% [14]. This theory was reflected in this study which found a higher frequency of wild-type EGFR in tissue biopsy (81.1%) compared to liquid biopsy (1 sample). This was due to the limitation of the home-brew-based HRM PCR which detected mutations only in exon 19 and 21. Mutations in other exons were analyzed as wild type using the same method. At Dharmais National Cancer Center Hospital, reflex testing for NSCLC is only performed to detect mutations in exon 19 and 21, while liquid biopsy with NGS is suggested in patients with wild-type results.

Differences in samples (plasma vs. tissue) and methods (HRM PCR vs. NGS) also influenced mutation detection sensitivity. The concordance between tissue and plasma in detecting EGFR mutations was wide, ranging from 27.5 to 100% [15]. Among the various methods, NGS demonstrated greater sensitivity, specifically in detecting complex mutations. PCR identified only 48.6% of exon 20 EGFR mutations compared to NGS in a previous study [16].

A report from the International Association for the Study of Lung Cancer (IASLC) global survey on molecular testing in lung cancer showed that the main causes of failures include insufficient tumor cell quantity (83%), inadequate tissue quality (55%), assay sensitivity or usage issues (18%), and limited technical expertise in the laboratory (10%) [17]. For patients with advanced NSCLC, invasive biopsies are often associated with risks, such as bleeding and pneumothorax. The incidence of pneumothorax in patients who underwent transthoracic needle biopsy ranged from 9–54% [18]. Therefore, adequate tissue sampling is not always feasible in advanced NSCLC.

Liquid biopsy was introduced as a new technique for screening, diagnosing, and treating lung cancer, specifically when tissue samples cannot be obtained. It facilitates the assessment of the molecular, genetic, and epigenetic profile of cancer cells, as well as the detection of drug-resistant clones from previous therapies [4]. Therefore, liquid biopsy can be used to monitor the efficacy of targeted treatment, evaluate minimal residual disease with resistance mechanisms, and guide medical decisions to further chemotherapy cycles or change treatment strategy [4,13]. This technique also presents certain advantages over tissue biopsy, such as faster turnaround time, repeatability, and minimally invasive [7,13]. Various methodologies for ctDNA analysis are employed in clinical settings, including next-generation sequencing (NGS), and non-NGS-based techniques. NGS techniques provide broad genomic detection of not only common mutations but also rare or unknown variants [13].

Circulating tumor DNA (ctDNA) is a fraction of cell-free DNA (cfDNA) that originates from a tumor or metastatic cell and exists in plasma or serum [19,20]. Several challenges are associated with ctDNA-based assays. The fraction derived from tumors in cancer patients ranges from <0.1 to >30% of the total DNA depending on the tumor burden, stage, cellular turnover, accessibility to circulation, and factors affecting blood volume [20]. According to a previous study, low tumor burden or the stage of cancer contributes to false-negative results in blood-based assays [21]. False positives may also arise from DNA shedding caused by non-cancerous sources, including germline or noncancerous somatic variants. Therefore, mutations identified in plasma may not accurately reflect the true tumor genotype [20,21].

In a study conducted by Uchida, et al, the sensitivity of ctDNA in detecting EGFR mutations using deep sequencing plasma DNA was significantly higher in stages IIB-IV (72.2%, 95%CI 60.9%–82.1%) compared to stages IA-IIIA (22.2%, 11.5%–38.3%) [22]. This supported the data from a previous report that showed EGFR mutations in 81.8% of subjects with stage IIB and stage IV NSCLC using the liquid biopsy method. Another study in metastatic NSCLC also found that liquid biopsy identified biomarkers in 76.5% of patients, surpassing the 54.9% detection rate of the tissue biopsy approach [23]. Therefore, liquid biopsy is not suitable for screening in...
the early stages of cancer due to the low tumor DNA burden which may reduce the sensitivity of the test. It is recommended for advanced-stage or metastatic disease where the tumor burden is high, culminating in enhanced sensitivity and specificity.

One of the most studied genes in lung cancer is EGFR which serves as an efficacy marker of target therapies. The first clinical application of liquid biopsy involves testing for EGFR mutations in plasma at baseline when tissue analysis is not feasible. This approach has been accepted for selecting patients for first-line treatment with EGFR tyrosine kinase inhibitors (TKIs) [7]. The latest NCCN guideline suggests using liquid biopsy, when (I) the patient is unfit for invasive tissue sampling, (II) there is insufficient material for molecular analysis, and (III) initial tissue-based testing does not cover all recommended biomarkers [12]. Meanwhile, the International Association for the Study of Lung Cancer (IASLC) recommends considering plasma ctDNA as a valid tool for genotyping newly diagnosed patients with advanced NSCLC [13].

EGFR mutations considered ‘common’ or ‘classical’ encompass exon 19 deletions and L858R at exon 21, accounting for approximately 85% of all cases [9,10]. The classical mutations have been associated with good responses to TKI and improved overall outcomes [24]. Based on the results, the most frequent mutation was the EGFR L858R, while classical exon 19 deletions were only found in two subjects with NGS examination. The tissue examination showed exon 19 deletions in three, L858R in one, and wild type in the majority of the subjects (81.8%). This value was higher than those of previous studies in Indonesia reporting EGFR mutations in 36% [25] and 44.5% [26] of patients with lung cancer. This might be due to the limited sample size in this study, or the patient selection criteria which focused on advanced-stage (IIIB or IV) NSCLC [10]. In this study, 59% of the subjects (81,8%). This value was higher than those of previous studies in Indonesia reporting EGFR mutations in 36% [25] and 44.5% [26] of patients with lung cancer. This might be due to the limited sample size in this study, or the patient selection criteria which focused on advanced-stage (IIIB or IV) NSCLC. Previous studies also found EGFR exon 19 and 21 L858R mutations as the most common types [25,26].

Uncommon EGFR mutations encompass all cases excluding exon 19 deletion and 21 L858R, as well as complex variants. These rare mutations exhibit variable responses to TKI and generally result in shorter survival rates [10,27]. A systematic review of 10 studies found uncommon mutations in 1.0–18.2% of all EGFR in NSCLC stage III-IV [10]. In this study, 59% of the subjects presented cases of uncommon mutations, which was significantly higher compared to previous reports. A high proportion reaching 42.9% was also discovered among 1, 874 newly diagnosed lung cancer patients, with 13.9% being a mixture of common and uncommon mutations [26]. The results showed that EGFR L861Q, T790M, and E746_A750del were the most frequent uncommon mutations. Meanwhile, previous studies reported G719X, exon 20 insertions, L861X, and S768I as the most prevalent uncommon mutations [10,26]. These findings suggest that the majority of patients in this study could experience poorer outcomes and limited responses to standard TKIs.

Studies regarding the efficacy of EGFR-TKIs for uncommon mutations remain a subject of debate. NSCLC patients with G719X, S768I, L861Q, and certain complex mutations are sensitive to TKIs, although their responses are generally less favorable than those of Ex19del and L858R [9,10,24]. Compared with other uncommon mutations, patients with exon 20 insertion have the poorest response to EGFR-TKIs and may require alternative treatment strategies [10].

For the detection of acquired resistance after TKI therapy, such as the T790M mutation, plasma genotyping can be considered before tissue biopsy, [11] while another guideline [13] suggests the use of ctDNA. T790M mutation is the most common acquired resistance mechanism to first and second-generation EGFR TKIs including erlotinib, gefitinib, and afatinib [7,11]. In this study, pre-treatment or ‘de novo’ mutations were identified in 5 subjects (22.7%). Meanwhile, a meta-analysis of 22 studies found that the rate was between 22–28%. Patients with T790M mutations exhibited poorer outcomes than others without the condition [28]. Clinically, this underscores the importance of new-generation T790M-positive EGFR-TKIs [22], specifically, osimertinib which has been approved for NSCLC patients with promising outcomes [29,30]. Proper monitoring is also essential to predict the presence of acquired mutations [11,22].

The NGS analysis results also found other mutations, such as TP53, ROS, BRAF, and MET. Among these, MET amplification is the most common alternative pathway for resistance to TKI and accounts for approximately 5–10% of cases. BRAF, HER2 amplification, and PIK3CA mutations were also implicated in increased resistance mechanisms to TKI [29].

In this study, KRAS mutations were identified as the second most common after EGFR, while Cascetta et al., reported a fair rate of approximately 30% across NSCLC cases [31]. These mutations often appear with other molecular alterations and also have clinical implications in treatment efficacy and outcomes. In NSCLC, KRAS mutations have a distinct biologic subtype with favorable responses to immune checkpoint blockade therapy [32]. Sotorasib and adagrasib are novel inhibitors approved for treatment in patients with NSCLC [31,32].

There are a few limitations associated with liquid biopsy, including the lack of standardized methods, low accessibility, high costs, and uncertainty regarding cutoff levels [4]. Other related issues are the short half-life of cfDNA, uncertainty about the amount of tumor DNA, and very low concentrations of ctDNA extracted [7,13]. Consequently, the use of liquid biopsy is recommended for patients with wild-type results, disease monitoring, and molecular diagnosis in those with high tumor burden.
to enhance sensitivity. This technique is also suggested for patients with progressive disease wherein tissue biopsy cannot be performed.

A notable strength of this study is its preliminary nature, which underscores the importance of using liquid biopsy as a viable alternative for detecting mutations in NSCLC patients. Meanwhile, the several limitations include (i) the limited number of patients, and (ii) the comparison of different methods using distinct samples (liquid biopsy using plasma vs. PCR HRM with tissue sample). PCR HRM EGFR detection was only performed to identify del19 and LQ858R mutations in exon 21 (common mutation), hence, other EGFR mutations could not be detected.

CONCLUSIONS

The availability of liquid biopsy has provided opportunities for clinicians to fill the gap in diagnostic testing and disease monitoring. Plasma ctDNA analysis and tissue genotyping are complementary tools in therapeutic decision-making for advanced NSCLC. Furthermore, liquid biopsy is beneficial for patients who cannot undergo tissue biopsy, or possess inadequate tissue for molecular profiling. The combination with NGS is also recommended in those with wild-type results to detect other mutations not covered by the PCR HRM method.

Discrepancies between tissue and liquid biopsy molecular result highlights the importance of conducting broad genomic examinations. This becomes more critical in the era of novel targeted treatments and immunotherapy for NSCLC, including cases with uncommon mutations. Further large-scale studies are needed to expand the molecular profile of NSCLC patients in Indonesia.

DECLARATIONS

Competing interest

The authors declare no competing interest in this study.

Ethics approval and consent to participate

Informed consent was obtained from all patients in the study and the procedure was conducted according to the Declaration of Helsinki. This study was approved by the ethical committee of Dharmais National Cancer Centre, Indonesia (no. 110/KEPK/V/2022).

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