Uncommon EGFR mutations in cytological specimens of 1,874 newly diagnosed Indonesian lung cancer patients

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Purpose: We aimed to evaluate the distribution of individual epidermal growth factor receptor (EGFR) mutation subtypes found in routine cytological specimens.

Patients and methods: A retrospective audit was performed on EGFR testing results of 1,874 consecutive cytological samples of newly diagnosed or treatment-naïve Indonesian lung cancer patients (years 2015–2016). Testing was performed by ISO15189 accredited central laboratory.

Results: Overall test failure rate was 5.1%, with the highest failure (7.1%) observed in pleural effusion and lowest (1.6%) in needle aspiration samples. EGFR mutation frequency was 44.4%. Tyrosine kinase inhibitor (TKI)-sensitive common EGFR mutations (ins/dels exon 19, L858R) and uncommon mutations (G719X, T790M, L861Q) contributed 57.1% and 29%, respectively. Approximately 13.9% of mutation-positive patients carried a mixture of common and uncommon mutations. Women had higher EGFR mutation rate (52.9%) vs men (39.1%; p<0.05). In contrast, uncommon mutations conferring either TKI responsive (G719X, L861Q) or TKI resistance (T790M, exon 20 insertions) were consistently more frequent in men than in women (67.3% vs 32.7% or 69.4% vs 30.6%; p<0.05). Up to 10% EGFR mutation-positive patients had baseline single mutation T790M, exon 20 insertion, or in coexistence with TKI-sensitive mutations. Up to 9% patients had complex or multiple EGFR mutations, whereby 48.7% patients harbored TKI-resistant mutations. One patient presented third-generation TKI-resistant mutation L792F simultaneously with T790M.

Conclusion: Routine diagnostic cytological techniques yielded similar success rate to detect EGFR mutations. Uncommon EGFR mutations were frequent events in Indonesian lung cancer patients.

Keywords: EGFR mutations, lung cancer, treatment naive, T790M, tyrosine kinase inhibitor, Indonesia, cytology

Introduction

Lung cancer in Indonesia ranks the fourth most common of all cancers.1 Majority of lung cancer cases are found in late stages and cytological specimens are common sources of diagnostic practices in tertiary hospitals.1 In addition to valuable diagnostic tools, cytological specimens are useful sources of epidermal growth factor receptor (EGFR) mutation testing. Specific guidelines of EGFR mutation testing in cytological specimens have been issued and adopted widely.2,3 However, there are considerable concerns regarding EGFR testing failure rates that may delay timeline of treatment decisions. Few or lack of tumor cells, improper fixation procedures, poor extracted DNA quality, and/or absence of or generation of nonspecific polymerase chain reaction (PCR) products have led to testing failures.3

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Indonesian health authority has published national formulary to reimburse expenses of tyrosine kinase inhibitors (TKI) as first-line treatment for lung cancer patients bearing EGFR mutation. In 2014, we had described common EGFR mutations associated with first-generation TKI (erlotinib and gefitinib) sensitivity mainly in exons 19 (insertions/deletions) and 21 (L858R) obtained from cytological specimens using Sanger sequencing. However, the prevalence and clinical pathology associations of rare or uncommon EGFR mutations such as G719S/A/C (collectively G719X), T790M, and L861Q had not been described extensively in Indonesia. These uncommon mutations are sensitive to second and third generations of EGFR TKI, namely afatinib and osimertinib. Specifically, T790M mutation rate is thought to be low in treatment-naïve patients, but it contributes up to 50% of patients who are resistant to first-generation TKI.

In this real-world EGFR mutation testing of treatment-naïve lung cancer patients, we had employed combination of PCR high-resolution melt (HRM) and restriction fragment length polymorphism (RFLP) to screen for common EGFR mutations (exon 19 insertions/deletions and L858R mutation in exon 21 of EGFR gene) to improve EGFR genotyping sensitivity. PCR HRM allowed rapid screening for genetic mutations due to differential melting properties of wild-type and mutant alleles PCR products. Both PCR HRM and RFLP methods have demonstrated superior sensitivity to Sanger sequencing. We also described the impact of various cytological sampling techniques to successful testing rate and evaluated frequency of individual mutation subtypes as well as their clinical pathology associations.

Patients and methods

Patients

Since the initial introduction of EGFR testing a few years ago, Indonesian clinicians and pathologists had been routinely using cytological specimens as primary testing sources as practical approach. Such routine practices cited successful EGFR testing from cytological samples by a reputable Southeast Asian laboratory. Moreover, tissue resection or surgical biopsies were not commonly performed by most clinicians (personal communications). Consequently, there were no formalin-fixed paraffin-embedded (FFPE) tissue specimens being received by our EGFR testing facility. Cytological specimens of 1,874 consecutive newly diagnosed lung cancer patients were received and tested for EGFR mutation by Kalbe Genomics Laboratory from September 2015 to April 2016. Cytological specimens along with pathology reports describing sex, age, cytopathology, and diagnostic sampling methods were received from 44 cities in Indonesia. However, fixative procedures of cytological specimens were not described in the EGFR testing request forms, except for 175 samples that arrived as FFPE blocks.

Kalbe Genomics is an ISO15189 accredited laboratory for EGFR mutation testing and has demonstrated consistent satisfactory performance in EGFR proficiency testing organized by European Molecular Genetic Quality Network and UK NEQAS annually since 2011. Ethic committees of Faculty of Medicine Universitas Airlangga, Soetomo General Hospital, Surabaya, and Persahabatan Hospital, Jakarta, approved this study. The study was performed in accordance with the 1964 Helsinki Declaration and its later amendments. Patient identities were anonymized. The approving ethics committees waived the need for informed consent because the study was based on existing administrative records and clinical data.

EGFR mutation screening program in Indonesia

During the study period, Astra Zeneca Indonesia (AZI) and Roche Indonesia (RI) invited physicians to test for EGFR mutation in newly diagnosed lung cancer patients and covered the costs for any patients diagnosed with lung adenocarcinoma or any non-small-lung cancers, respectively. The program stipulated no obligation in any kind of prescription in compliance with both AZI and RI ethical code of conduct. Test results were sent directly from the laboratory to the physicians.

DNA extraction

Genomic DNA was isolated from tumors using the QIAamp DNA Micro kit (Qiagen, Hilden, Germany) following the manufacturer’s protocol. DNA from each sample was eluted in 50 µL of AE buffer (included in the kit).

Mutation analysis

EGFR exons 19 and 21 mutation screenings were performed using PCR HRM analysis. PCR cycling and HRM analyses were performed on Rotor-Gene Q (Qiagen) using intercalating dye SYTO9 (Thermo Fisher Scientific Inc., Waltham, MA, USA) as described. Samples were denatured with an initial hold of 95°C for 30 s and a melting profile from 79°C to 90°C rising at 0.2°C. HRM data were presented as derivative graph to observed “split peak” indicating presence of mutated alleles (Figure 1) using Rotor-Gene Q software (Qiagen). Split peak pattern was observable in low percentage of mutant alleles, that is, less than 25%, a percentage that was usually not detect-
able using direct sequencing method. PCR RFLP method was then used to follow up suspected mutation suggested by presence of “split peak” pattern. Genotyping of EGFR L858R and L861Q hotspot mutations in exon 21 was performed using PCR RFLP that had been shown to detect 1% mutant allele.10

Mutations in EGFR exons 18 (namely to detect G719X) and 20 (T790M and insertions) were analyzed using Sanger sequencing. Primer pairs for EGFR exon 18 were designed with primer-BLAST software and for EGFR exon 20 was described previously.14 Some samples carrying mutation in exon 20 T790M were retested using Therascreen EGFR RGQ PCR kit (Qiagen, Manchester, UK) and/or AmoyDx EGFR 29 mutations detection kit (AmoyDx, Xiamen, China), according to the manufacturer’s instructions.

Direct sequencing
PCR amplification products were purified using the ExoSAP-IT PCR Product Cleanup (Affymetrix/USB, Santa Clara, CA, USA) according to the manufacturer’s protocol. Sequencing analysis was performed on an Applied Biosystem 3500 Genetic Analyzer. Nucleotide sequences of primers are available upon requests.

Statistical analysis
Fisher’s exact test was used to analyze associations between the presence of EGFR mutations and clinical pathology characteristics. Significance was set at \( p < 0.05 \) (two-sided).

Results
Utility of melting peak PCR HRM to screen EGFR mutations
Direct sequencing method generally could not detect the presence of mutations when there were less than 25% as shown in left panels of Figure 1A (exon 19 mutations) and 1B (exon 21 mutations). To improve our capability to detect EGFR mutations, we employed PCR HRM to screen for the presence of EGFR mutations in exons 19 (Figure 1A right panel) and 21 (Figure 1B right panel) and observed different forms of melt pattern between PCR amplicons containing normal/wild-type EGFR and mutant EGFR samples when...
presented in derivative graph mode (Figure 1). As shown in Figure 1A, melt pattern (pointed by arrow) of wild-type exon 19 PCR amplicon (green trace line) showed single peak, while samples (red and blue trace lines) with PCR amplicons bearing mutational exon 19 insertions or deletions (ins/dels) demonstrated split peaks.

Similar split peak patterns were also observed in L858R sample (orange trace line, Figure 1B), while normal or wild-type samples showed single peak (green and brown trace lines). Unlike exon 19, however, split peak patterns shown in samples having mutations in exon 21 were due to single base substitution instead of multi PCR amplicons. Agarose electrophoreses of exon 21 PCR amplicon also confirmed the presence of single PCR product amplicon (Figure 1B upper panel). Therefore, split peak pattern strongly suggested the presence of mutant alleles.

To determine the analytical sensitivity of HRM approach, we tested using serial dilution of artificial DNA method. As shown in right panels of Figure 1A and 1B, threshold of detection limit indicated by the presence of split peak corresponded to 12% and 6% of mutant alleles in exons 19 and 21, respectively.

**Impact of cytological techniques to EGFR testing**

Clinical pathology characteristics of consecutive 1,874 newly diagnosed lung cancer patients drawn from 44 Indonesian cities are described in Table 1.

Male patients (61%) were more frequent than female patients (39%). Median age was 57 years with a range between 19 and 92 years.

Most cytological specimens were adenocarcinoma (94%), obtained as malignant pleural effusion (MPE, 26%), as well as from fine needle aspirations (FNA, 20%), bronchoscopies (17%), and transthoracic needle biopsies (20%).

Out of the 1,874 consecutive samples, 95 (5.1%) samples failed EGFR testing. Failures were divided into preanalytical and analytical failures. Seventy-four (3.9%) samples were rejected outright (preanalytical failure), because the numbers of tumor cells were too few (<100 cells) or absent altogether.

MPE specimens showed the highest preanalytical failure rate (6.6%) and FNA specimens demonstrated the least (1.6%) (Table 2). Upon passing preanalytical step, there were only 11 samples that failed to generate specific amplicons after repeated PCR attempts at least twice. Out of the 11 samples, 7 were formalin-fixed while 4 were direct smear samples. Therefore, formalin fixation had higher frequency of PCR failure (2.4%; 4 out of 164 FFPE samples) than direct smear preparations (0.4% or 7 out of 1,626 samples; *p*=0.0019).

**EGFR mutation frequency and clinical pathology associations**

Overall EGFR mutation frequency was 44.5% (95% CI: 42.09–46.71). Approximately 57.1% and 29% of EGFR mutation–positive patients had common TKI-sensitive mutations (exon 19 ins/dels and L858R) and uncommon mutations (G719X, T790M, exon 20 insertions, and L861Q), respectively. The remaining 29% of patients harbored mixture of common and uncommon EGFR mutations (G719X, T790M, and L861Q) (Table 3).

Most patients harbored single mutations (80.5%). However, 19.5% of patients had multiple or complex mutations involving more than one mutation subtypes. Furthermore, first-generation TKI-resistant T790M mutations were found as single (3.4%) and complex (4.2%) TKI-sensitive mutations. The proportion of T790M in complex mutations (48.7%) was higher than in single mutations (9.6%, Table 3). Moreover, complex mutation cases of T790M/L858R (30%)
Uncommon EGFR mutations in Indonesian lung cancer patients were found more frequently than T790M/exon 19 ins/del (9%).

When stratified according to gender, EGFR mutations were higher in women (52.9%) than in men (39.1%, p<0.05). Furthermore, adenocarcinoma patients had higher rate of EGFR mutations (45.1%) than nonadenocarcinoma (34.3%, p=0.028) (Table 4).

Common mutations (exon 19 ins/dels, L858R) conferring sensitivity to TKI were more prevalent in female (54.9%) than in male patients (45.1%). In contrast, uncommon EGFR mutations conferring either sensitivity (G719X, L861Q) or resistance to TKI (T790M, exon 20 insertions) were more frequent in male (64.9%) than in female patients (35.1%) (Table 4).

**EGFR T790M mutation**

Using Sanger sequencing, we found that majority of T790M mutations were heterozygous as shown in a typical sequencing result (Figure 2A upper panel), which we confirmed using real-time PCR (Figure 2B). Examination of T790M-positive cases revealed that up to 16% showed mutant allele specific imbalances (MASI) due to overrepresentation of mutant alleles (Figure 2A lower panel). To detect probable presence of germline mutation, DNA was isolated from adjacent
normal cells. In 1 out of 5 randomly selected MASI cases, a heterozygous T790M mutation was detected (Figure 2C) in normal cells indicating germline mutation. Confirmation in peripheral blood was not done because the patient died before the initiation of this study. In the other 4 cases, T790M mutations were absent in normal cells supporting the relatively

| Parameters       | N  | EGFR Normal Mutation p-value | Common mutations TKI sensitive Uncommon mutations TKI sensitive TKI resistant |
|------------------|----|-----------------------------|---------------------------------|---------------------------------|
| Overall          | 1,779 | 988 791                      | 452 191 148                     |
| Gender           |     |                             |                                 |                                 |
| Female           | 696  | 328 47.1 368 52.9 0.0001     | 248 54.9 67 35.1 53 35.8        |
| Male             | 1,083 | 660 60.9 423 39.1            | 204 45.1 124 64.9 95 64.2       |
| Age              |     |                             |                                 |                                 |
| >57 years        | 872  | 469 53.8 403 46.2            | 235 52.0 88 46.1 80 54.1        |
| ≤57 years        | 894  | 510 57.0 384 43.0 0.18       | 214 47.3 102 53.4 68 45.9       |
| Unspecified      | 13   | 9 4                          | 3                               |
| Cytopathology    |     |                             |                                 |                                 |
| Adenocarcinoma   | 1,671 | 917 54.9 754 45.1 0.0282     | 427 94.5 186 97.4 142 95.9      |
| Nonadenocarcinoma| 108  | 71 65.7 37 34.3              | 25 5.5 5 2.6 6 4.1             |
| Squamous         | 18   | 12 66.7 6 33.3               | 3 0.7 0 0.0 0 0.0              |
| Adenosquamous    | 38   | 24 63.2 14 36.8              | 11 2.4 1 0.5 0 0.0             |
| Bronchioalveolar | 9    | 4 44.4 5 55.6               | 2 0.4 2 1.0 0 0.0              |
| Not specified NSCLC | 43 | 31 12                          | 2                                 |

Abbreviations: NSCLC, non small cell lung carcinoma; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor.

Figure 2 Representative T790M detection in cytological samples using direct sequencing. Notes: (A) Upper panel shows the case of heterozygous T790M mutation and lower panel shows the case of homozygous T790M mutation indicating mutant allele-specific imbalance (MASI). (B) Real-time PCR-independent confirmation of T790M detection. Red arrow points to T790M control positive specimen. Blue arrow points to specimens showing positive T790M signal. (C) T790M MASI in DNA of tumor cells and heterozygous T790M in DNA of normal cells scraped from the same cytological smears. (D) Complex mutation of T790M and L792F in treatment-naive patient. Red arrow points to mutations. Abbreviation: PCR, polymerase chain reaction.
rare frequency of T790M germline mutation. Last, we found that 1 out of 136 patients had concomitant T790M and L792F mutations, a putative resistance marker to second- and third-generation TKI (Figure 2D).

**Discussion**

We reported that the overall failure rate of EGFR mutation testing in real-world population was 5.1%, with MPE showing the highest (7.6%) and FNA the lowest (1.6%). MPE failure rates were combination of rejection during preanalytical step (6.6%) and PCR failures (1%) during analytical step. In addition, improper formalin fixation protocol performed at the referring hospitals to our laboratory might contribute to PCR failure. For instance, fixation time may affect the integrity of pre-PCR DNA template. However, an FFPE specimen failure rate of 2.4% obtained in our cohort was still lower than that of 11.4% obtained by the recent RING diagnostic trial involving 13 laboratories. Taken as a whole, our experience in receiving cytological specimens had similar success rate (94.9%) to previous descriptive review analyzing 19 publications of EGFR mutation testing in cytology samples stating an overall success rate of ~95%.

The rate of total EGFR mutation in our population (44.5%) using cytological specimens was also similar to what laboratories in neighboring Southeast Asian countries had reported using either cytological or tissue specimens. Notably, the EGFR mutation rate was higher than our previous study (29%) when Sanger sequencing was used. The current study used PCR HRM and RFLP that had higher analytical sensitivity than Sanger sequencing and covered 4 exons (18–21) instead of just 2 exons (19 and 21). In addition, others and we had described the utility of split peak pattern of PCR HRM previously to screen for RAS and EGFR mutations rapidly.

Most EGFR mutations in our current cohort were also detected as single mutations and the remaining 19.5% was complex mutations (containing more than one mutation). Our complex mutation rate was slightly higher than that demonstrated by other studies varying between 7% and 14%. Using massive parallel sequencing, the complex mutation rate is increased to 26% and has been associated with poor prognosis.

Most EGFR mutation studies to date reported major tyrosine kinase sensitizing mutations such as exon 19 insertions/deletions and L858R substitution mutations in exon 21. These common mutations generally comprised 80%–90% of total EGFR mutations, while the remaining mutations or uncommon mutations contributed 10%–20%. However, our population had high rates of uncommon mutations (composed of G719X, exon 20 insertions, T790M, and L861Q) contributing up to 43% of total EGFR mutations. High proportion of uncommon EGFR mutations to total EGFR mutations has been reported in European population (50%) and Chinese regions of Yunnan Province (70%).

The reasons of variations in EGFR mutation subtypes may be attributed to geographical differences, ethnic backgrounds, and environmental exposures (coal burning, wooden smoke, cigarette smoking). Although information about smoking history was not available within our cohort, Indonesian lung cancer smoking attributable fractions in males is as high as 87% and in females 12%. This is consistent with the high prevalence of smoking among Indonesian males (65%), which ranks third in the world. Our data showed that male patients (65%; p<0.05) had higher rate of EGFR uncommon mutations than female patients (35%), which may be partly explained by recent descriptive studies suggesting putative association between uncommon mutations (G719X and L861Q) and smoking history. Therefore, future studies are needed to clarify definite association between EGFR uncommon mutations and smoking history in Indonesian lung cancer patients.

Among uncommon EGFR mutation types, T790M mutation has generated diagnostic as well as clinical interests. Up to 50% of common EGFR mutant patients would develop resistance to first-generation TKI due to acquired T790M mutations. TKI-naïve patients are not expected to harbor T790M mutations as confirmed by insensitive detection method such as Sanger sequencing with frequency typically less than 5%. Using Sanger we did find significant portion of T790M mutations in treatment-naïve patients (7.6%) having slightly higher frequency than what other studies have reported. T790M prevalence in complex mutation contributed up to 48.7% and mostly coexisted with L858R that is consistent with recent meta-analysis study of baseline T790M.

Although Sanger sequencing is not as sensitive as amplification-refractory mutation system PCR, it may yield certain advantages. We were able to discern the extent of MASI by comparing the relative sequencing tracer heights of mutant allele vis-a-vis normal allele. We found that the rate of MASI in T790M mutations was similar to other mutations such as L858R and exon 19 insertions/deletions (26%–37%).

Clinical significance of baseline T790M with or without MASI was not clear. However, presence of baseline T790M has been correlated with good prognosis, while others have demonstrated shorter progression free survival (PFS) and median overall survival. Nevertheless, current clinical
practice does not exclude prescription of first-generation TKI to patients harboring concurrent T790M and TKI sensitive mutations. Interestingly, recent clinical trial subjecting 60 EGFR mutated patients (5 of whom harbored T790M mutations) to osimertinib as first-line treatment demonstrated an objective response rate of 77% and a PFS of 19.3 months, a significant extension of historical PFS of 10–13 months. Therefore, population with significant numbers of baseline T790M mutations like ours may benefit from using osimertinib as first-line treatment.

Furthermore, we had explored the potential presence of T790M germline mutations in some specimens. Out of the 5 specimens showing T790M MASI, we found 1 specimen having heterozygous T790M mutation in normal cells from the same slide. Unfortunately, we were not able to confirm mutation in the blood because the patient died at the time of our study. To our knowledge, this was the first study to demonstrate the utility of cytological specimens to screen for potential germline mutation. Germline mutation T790M has been an interest due to associated risk to develop lung cancer in family members having inherited the identical mutant alleles. Due to relatively rare mutation of T790M, the extent of germline T790M mutation may be as high as 50% of patients with somatic mutations. However, in general population, prevalence of germline T790M mutation has been estimated to be 1 in 7,500. We were not able to compare the proportion of germline T790M within our cohort, because majority of cytological slides whose tumor cells had been scraped during routine EGFR mutation testing were not available. Lastly, we also found one specimen having L792F mutation in complex with T790M mutation. L792F mutation has been proposed as putative resistant marker to second-generation (afatinib) and third-generation (osimertinib) TKI. L792F-acquired mutations had been shown in plasma of 3 patients who were resistant to osimertinib but not in pretreatment samples. Therefore, we found evidence that resistance marker to third-generation TKI may exist prior treatment albeit with extremely low frequency.

Conclusion
We used PCR HRM “split peak” melt pattern to screen and analyze EGFR mutation in real-world testing of Indonesian lung cancer samples obtained from major cities using routine cytological specimens. We found high rates of uncommon EGFR mutations (G719X, L861Q) in Indonesian male lung cancer patients, potential germline T790M mutation, and L792F next-generation TKI resistance EGFR mutation in cytological samples of untreated patients. These patients may benefit from first-line treatment using second- and third-generation TKIs.

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Disclosure
The authors report no conflicts of interest in this work.

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