Impact of smoking on frequency and spectrum of K-RAS and EGFR mutations in treatment naive Indonesian lung cancer patients

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Background: Indonesia has the highest cigarette consumption in the world. We explored the clinical impact of smoking on the prevalence of EGFR and K-RAS mutations and survival in this prospective study.

Methods: 143 treatment naive lung cancer patients were recruited from Persahabatan Hospital, a national tertiary hospital. DNA from cytological specimens had been extracted and genotyped for both EGFR and K-RAS mutations using a combination of PCR high resolution melting, restriction fragment length polymorphism (RFLP) and direct DNA sequencing.

Results: EGFR mutation frequency in never smokers (NS) and ever smokers (ES) were 75% and 56% (p = 0.0401), respectively. In this cohort, the overall K-RAS mutation rate was 7%. Neither gender nor smoking history were associated with K-RAS mutation significantly. However, K-RAS transversion mutations were more common in male ES than transition mutations. Smoking history did not affect EGFR and K-RAS mutation frequencies in women. Concurrent EGFR/K-RAS mutation rate was 2.8% (4 of 143 patients). Four out of 91 EGFR mutation positive patients (4.4%) had simultaneous K-RAS mutation.

Conclusions: In region where cigarette consumption is prevalent, smoking history affected frequencies of EGFR and K-RAS mutations, mainly in males.

Keywords: lung cancer, Indonesia, K-RAS mutation, smoking

Introduction

Lung cancer is the most common and deadly cancer, contributing to 11.6% of total cancer and 18.4% of total cancer-related mortality. WHO estimates the incident and mortality rate of lung cancer in Indonesia is 12.4 and 10.9 per 100,000, respectively. In males, lung cancer shows higher incidence and mortality (19.4 and 17.4 per 100,000, respectively) than females (6.0 and 5.1 per 100,000, respectively).1

Epidermal growth factor receptor (EGFR) mutation is an important predictive biomarker in lung cancer targeted therapy. Common mutations such as deletion (del) of exon 19 and L858 substitution mutations in exon 21 predict tumor sensitivity to first-generation tyrosine kinase inhibitors (TKI) such as gefitinib and erlotinib.2,3 There are also rare/uncommon mutations such as G719X and L861Q that confer variable therapeutic responses to TKI treatment.4-6 On the other hand, there are some oncogenic mutations, such as EGFR T790M, insertions of exon 20 of the EGFR gene, and K-RAS, that contribute to primary and/or acquired resistance to TKI.7,8 Moreover, baseline K-RAS

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mutation either alone or together with EGFR mutation may have negative or neutral outcomes to chemotherapy.

Gender, ethnicity, histology and smoking history are known factors affecting prevalent of EGFR and K-RAS mutations. EGFR mutations generally occur in nonsmoker, female, East Asian, and adenocarcinoma patients. On the other hand, K-RAS mutations were observed mainly in western or European patients and may be associated with smoking history. Moreover, K-RAS mutation is typically showing transversion purine to pyrimidine substitution subtypes a signature of smoking history. EGFR and K-RAS mutations are thought to be mutually exclusive although there are reports showing some cases of simultaneous mutations both in European and Asian patients albeit with various rates (0.4–1.1%).

We have recently reported the frequency of EGFR mutations (44%) in a large retrospective study of newly diagnosed lung cancer patients using cytological specimens. Smoking is highly prevalent among male Indonesians and has contributed to a major proportion of lung cancer incidence. However, the impacts of smoking to the prevalence of EGFR and K-RAS mutations in Indonesian lung cancer patients have not been analyzed.

Recent meta-analyses evaluating different histopathology, gender and ethnicities have described the likelihood of ALK-EML4 translocations, and EGFR and KRAS mutations among lung cancer patients with or without smoking history. Never smoker (NS) patients tend to have higher rates of EGFR mutations and ALK-EML4 translocations than ever smoker (ES) patients. On the other hand, NS patients are less likely to bear KRAS mutations than ES patients. Other factors, such as ethnicities, gender, and histopathology are also associated with key driver mutations in lung cancer.

We aimed to evaluate the impact of smoking on the incidence and spectrum of EGFR and K-RAS gene mutations in lung cancer patients referred to Jakarta tertiary hospital.

**Methods**

**Patients**

A total of 143 newly diagnosed non-consecutive lung cancer patients with known EGFR mutation status were enrolled to participate in prospective disease monitoring study. DNA was also genotyped for baseline K-RAS mutations. Patients’ age ranged from 26 to 84 years, with median of 55 years and average of 53.7 years. Ethical Committee of Faculty of Medicine Universitas Indonesia, Jakarta (396/UN2.F1/ETIK/2016) approved this study. The study was performed in accordance with the 1964 Helsinki declaration and its later Amendments. All patients had signed informed consent.

**DNA isolation**

Cytological specimens were obtained as malignant pleural effusion as well as from fine needle aspirations, bronchoscopy, and transthoracic needle biopsies. Pathologists had marked areas with enriched tumor cells in the cytological specimens. Marked areas were then subjected to DNA isolation using QIAamp DNA Micro (Qiagen NV, Venlo, the Netherlands) according to the kit protocol.

**EGFR mutation detection**

The method used for mutation detection is PCR high resolution melting (PCR-HRM), restriction fragment length polymorphism (RFLP), and sequencing as described. Briefly, PCR-HRM was used to screen for mutations in exon 18, 19, and 21. Suspected specimens showing mutation specific melting profiles were subjected to genotyping using direct sequencing (exon 18), fragment sizing (exon 19) and RFLP (exon 21 L858R and L861Q). Mutation detection in exon 20 was performed using direct sequencing.

**PCR HRM of EGFR of exons 18, 19, and 21**

PCR-HRM was performed in 25 μL reaction volume, containing 200 nM of each forward in reverse primer, 200 μM dNTP, 1 × buffer, 2.5 mM MgCl₂, 1.25 U of HotStarTaq (Qiagen) polymerase, 1 μL of template, 5 μM Syto-9 (Invitrogen) and PCR grade water. PCR-HRM analysis was performed on Rotor gene 6000™ in the following conditions: 95°C (15 min), followed by 10 cycles of 95°C (10 s), 65°C (10 s) with touchdown for (1 cycle/1°C), 72°C (30 s), 40 cycles of 95°C (10 s), 55°C (10 s), 72°C (30 s), one cycle of 97°C (1 s). The HRM condition was: melt from 80°C to 90°C, rising 0.1°C per second.

**PCR and RFLP of EGFR exon 21**

RFLP was performed for exon 21, to detect point mutation in codon 858 and 861. The products of PCR-HRM were digested using MSC I and PVU II enzyme, to detect mutation on codon 858 and 861. To detect the mutation in codon 858, the reaction performed in 25 μL of reaction, consists of: 10 μL of PCR product, 5 U of MSC (NEB), 1 × buffer, and ddH₂O. Another reaction was performed to detect the mutation in codon 861 using 10 U of PVU II (NEB), 1 × buffer, 10 μL PCR product and then ddH₂O.
added until the reaction volume reached 25 μL. RFLP was performed on 37°C for 3–16 h.

PCR and sequencing of exons 18 and 20
For exon 18, the product of PCR-HRM was purified using Exo Sap IT. Direct sequencing was performed using Applied Biosystems 3,500 Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA).

PCR of exon 20 was performed in 25 μL reaction volume, containing: 1 × buffer, 1.5 mM MgCl₂, 500 nM of forward primer and 500 nM reverse primer, 200 μM of dNTPs, 1.25 U of HotStar Taq polymerase (Qiagen), 1 μL of DNA template and PCR grade water. The PCR was performed using Veriti Thermal Cycler (Thermo Fisher Scientific) in the following conditions: 95°C (15 min), followed by 40 cycles of 95°C (30 s), 57°C (30 s), 72°C (30 s), one cycle of 72°C (7 min).

K-RAS mutation detection
PCR and direct sequencing of K-RAS mutations were performed as described. Briefly, RAS SplitSCAN (KalgenDNA, Jakarta, Indonesia) HRM primers were used to screen a hotspot mutation of exon 2 of the K-RAS gene. Samples were run using Rotor-Gene 6000TM (Corbett Life Science, Mortlake, Australia) or Rotor-gene Q (Qiagen). Melting curves were generated and scanned for the presence of split peaks predicting the presence of mutations. Samples showing putative mutated split peak patterns were then genotyped using direct DNA sequencing.

PCR HRM of KRAS exon 2
PCR HRM was performed in 20 μL reaction volume containing 200 nM of each forward in reverse primer, 200 μM dNTP, 1 × buffer, 2.5 mM MgCl₂, 1 U of HotStarTaq (Qiagen) polymerase, 1 μL of template, 5 μM Syto-9 and PCR grade water. HRM analysis was performed on the Rotor-Gene 6000™ in the following conditions: 95°C (15 min), followed by 50 cycles of 95°C (10 s), 68°C (10 s), 72°C (20 s), one cycle of 95°C (1 s), one cycle of 95°C (1 s), melt from 72°C to 90°C, rising 0.1°C per second.

PCR and sequencing of KRAS exon 2
Conditions for first PCR reaction were 95°C (4 mins), followed by 25 cycles of 95°C (30 s), 50°C (30 s), 72°C (30 s), and 1 cycle of 72°C (7 min). PCR reaction performed in 25 μL reaction volume, containing: 1 × buffer, 1.5 mM MgCl₂, 500 nM of forward primer and 500 nM reverse primer, 200 μM of dNTPs, 1.25 U of Faststart Taq (Roche) polymerase, 1 μL of DNA template and PCR grade water. First PCR product diluted to 1:10, and 1 μL of the diluted product used as template for nested PCR. Nested PCR was performed in the following conditions: 95°C (4 min), followed by 35 cycles of 95°C (30 s), 55°C (30 s), 72°C (30 s), and 1 cycle of 72°C (7 min). PCR reaction performed in 25 μL reaction volume, containing 1 × buffer, 1.5 mM MgCl₂, 300 nM of forward primer and 300 nM reverse primer, 200 μM of dNTPs, 1.25 U of Faststart Taq (Roche) polymerase, DNA template and PCR grade water.

Statistical analysis
Categorical variables were summarized by frequency and percentage. Pearson’s chi-squared test (or Fisher’s exact test if cell frequencies less than 5 were expected) was used to test for associations between patient characteristics and mutation type and smoking status. A two-sided p-value of less than 0.05 was taken as statistically significant.

Results
Prevalence of EGFR and K-RAS mutations
A total of 143 newly diagnosed non-consecutive patients with adenocarcinoma histology had been genotyped for EGFR and K-RAS mutations. Most patients were males (71%), and the majority (64%) was smokers (Table 1). Common EGFR mutations (exon 19 indels and L858R) were the major (67%) subtypes, followed by uncommon mutations (19%, G719X, T790M, and L861Q), and mix or compound (14%) mutation subtypes. K-RAS mutation frequency was 7%, and K-RAS transversion mutation (60%) was slightly more common than transition mutation (40%, p > 0.05). Moreover, K-RAS mutation rate was slightly higher in females, 3 of 39 (7.6%, p = 1.0), than males, 6 of 101 (5.9%). The rate of concomitant or simultaneous EGFR and K-RAS mutations in the same individuals was 2.8% (or 4 out of 143 patients).

Impact of smoking history on EGFR mutation rate
When stratified according to smoking history, EGFR mutation was higher in NS (75%, p = 0.0465) than ES (57%) (Table 2). Furthermore, young NS had also more frequent EGFR mutation rate than ES patients (81% vs 53%, p = 0.0343). The trend toward higher EGFR mutation rate was also observed in NS males than ES males (82% vs 57%, p = 0.06), but did not reach statistical significance (see Figure 1). Similar EGFR mutation
frequency was also observed in females (71% in NS females vs 60% in ES females, \( p = 0.63 \) see Figure 1). Mix or multiple EGFR mutations containing both common and uncommon EGFR mutants in the same individuals seemed to be more prevalent in smoker (20%) vs nonsmoker (5%, \( p = 0.0632 \)) patients (Table 2).

### Impact of smoking history on K-RAS mutation rate

When stratified according to smoking history, K-RAS mutation was slightly higher in ES (7%, \( p = 1.0 \)) than NS (6%) (Table 2). ES male patients had a tendency of a higher rate of K-RAS mutation (7.5%) than NS male patients (0%, \( p = 0.56 \)) (Table 3, Figure 1). A signature of smoke-associated mutation, K-RAS mutation transversion type (mainly K-RAS G12C) was also consistently more frequent in ES than NS patients. On the other hand, K-RAS mutation rate and patterns (transversion or transition) among females were independent of smoking history (Table 3).

### Double mutation of EGFR and K-RAS genes

Of the 143 patients, 4 (4.4%) had simultaneous EGFR and K-RAS gene mutations (Table 4, Figure 1). K-RAS mutation transversion types (G12C) were found with EGFR L858R mutation. Within our cohort, EGFR exon 19 deletion was never found together with K-RAS mutations. Although not statistically significant, frequency of

| Table 1 Demography |
|---------------------|
| Characteristics     | N (143) | Percent |
| Gender              |         |         |
| Male                | 102     | 71%     |
| Female              | 41      | 29%     |
| Age                 |         |         |
| Median              | 55      |         |
| Average             | 53.7    |         |
| Range               | 26–84   |         |
| Smoking History     |         |         |
| Ever smokers (ES)   | 89      | 62%     |
| Never smokers (NS)  | 51      | 36%     |
| Unknown             | 3       |         |
| All adenocarcinoma  |         |         |
| EGFR Genotypes      |         |         |
| Wild type (normal)  | 52      | 36%     |
| Mutations           | 91      | 64%     |
| Common Mutations (Exon 19 Dels, L858R) | 61 | 67% |
| Uncommon mutations (G719X, L861Q, T790M) | 17 | 19% |
| Mix mutations       | 13      | 14%     |
| K-RAS Genotypes     |         |         |
| Wild type           | 133     | 93%     |
| Mutations           | 10      | 7.0%    |
| Transversion        | 6       | 60%     |
| G12C                | 4       |         |
| G12A                | 1       |         |
| G12R                | 1       |         |
| Transition          | 4       | 40%     |
| G12D                | 3       |         |
| G12S                | 1       |         |
| Mix EGFR and K-RAS concomitant mutations | 4 | 2.8% |
| K-RAS mutations in 91 EGFR Mutant patients | 4 | 4.4% |
| EGFR mutations in 10 K-RAS mutant patients | 4 | 40% |
Table 2 Impact of smoking history to prevalence and spectrum of EGFR mutation

| Characteristics          | EGFR genotypes | p-value | EGFR mutation types | p-value |
|--------------------------|----------------|---------|---------------------|---------|
|                          | Wild types     | Mutants | Common              | Uncommon | Mix |
|                          | Ever smokers   |         |                      |          |     |
|                          | 89             | 38      | 43%                  |          |     |
|                          | 51             | 38      | 75%                  |          |     |
|                          | Never smokers  | 13      | 25%                  |          |     |
|                          | 51             | 38      | 75%                  |          |     |
|                          | Unknown        | 3       | 13%                  |          |     |
| Age 55 years old and younger | Ever smokers   | 24      | 47%                  |          |     |
|                          | 27             | 53%     |                      |          |     |
|                          | Never smokers  | 4       | 19%                  |          |     |
|                          | 17             | 81%     |                      |          |     |
|                          | Unknown        | 1       | 33%                  |          |     |
|                          | 1              | 33%     |                      |          |     |
| Age older than 55 years old | Ever smokers   | 14      | 37%                  |          |     |
|                          | 24             | 63%     |                      |          |     |
|                          | Never smokers  | 9       | 30%                  |          |     |
|                          | 21             | 70%     |                      |          |     |
|                          | Unknown        | 3       | 18%                  |          |     |
|                          | 14             | 82%     |                      |          |     |
| Male                     | Ever smokers   | 36      | 43%                  |          |     |
|                          | 48             | 57%     |                      |          |     |
|                          | Never smokers  | 3       | 18%                  |          |     |
|                          | 14             | 82%     |                      |          |     |
| Female                   | Ever smokers   | 2       | 40%                  |          |     |
|                          | 3              | 60%     |                      |          |     |
|                          | Never smokers  | 10      | 29%                  |          |     |
|                          | 24             | 71%     |                      |          |     |

Notes: *p*-value <0.05 were statistically significant.
double $EGFR$ and $K-RAS$ mutations was higher in young (3 of 52 or 6%) than old patients (1 of 91 or 1%, $p > 0.05$). Patients having uncommon or mix $EGFR$ mutations also tend to harbor additional $K-RAS$ gene mutation (3 of 30 or 10%) than those having $EGFR$ common mutations only (1 of 61 or 1.6%, $p > 0.05$). Lastly, NS patients tend to have a higher frequency of concomitant $EGFR$ and $K-RAS$ mutations (2 of 51, 3.9%) than ES patients (1 of 89 or 1.1%, $p > 0.05$).

**Discussion**

A meta-analysis by Dearden et al showed that $EGFR$ mutations occurred more frequently in Asian than Western patients (47.9% versus 19.2%), while $K-RAS$ mutations are more frequent in Western than Asian patients (KRAS, 26.1% versus 11.2%).$^{26}$ The overall rates of $EGFR$ and $K-RAS$ mutations in this study confirmed the Asian profile. Patients with smoking history had 56% $EGFR$ mutation rate, similar to other Asian studies,$^{27,28}$ confirming the importance of $EGFR$ testing regardless of smoking history. In the current study, we also found a consistent trend of higher $EGFR$ mutation in NS patients compared to ES patients (see Figure 1). However, the difference in $EGFR$ mutation rates in NS vs ES female patients was not statistically significant. It is probably related to low numbers of female ES patients enrolled in this study (only 5 ES females out of a total of 39 patients). Nevertheless, a recent study enrolling a large number of ES female patients from seven Asian regions indeed demonstrated more frequent $EGFR$ mutations in NS females (62%, N=358) than ES females (51%).$^{29}$ Another study by Hsiao et al also showed higher rates of $EGFR$ mutations in NS females compared to ES females (60% vs 29%, N=426)$^{30}$

Within our cohort, there was a trend toward more frequent uncommon $EGFR$ mutations among ES patients, although statistically not significant, which is consistent with other Asian studies.$^{3,31}$ On the other hand, a significant association of $EGFR$ uncommon mutations with smoking has been described amongst European lung cancer patients.$^{32}$

Overall $K-RAS$ mutation rate in this study (7%) was also similar to other Asian studies (around 8–10%), which is generally lower than Western patients (25–30%).$^{26,33}$ Our results also showed a consistent tendency of a high frequency of $K-RAS$ mutations among smokers. Smoking history association with $K-RAS$ mutations has been shown in many studies,$^{33}$ with G12C or transversion mutation type being most common in smokers. We also observed that $K-RAS$ mutation G12C (transversion) types were frequent among male ES. However, our study and others have also shown that the association between smoking history and $K-RAS$ mutation may not necessarily be strict.$^{34}$ For instance, we
Table 3 Impact of smoking history to prevalence and spectrum of K-RAS mutation

|                  | Total | K-RAS WT | K-RAS mutation | *p-value | Transversion | Transitions |
|------------------|-------|----------|----------------|----------|--------------|-------------|
|                  |       | n       | Rate           | n        | Rate         | n           | Rate       |
|                  | 140   | 131     | 94%            | 9        | 6%           |             |            |
| Smoking history  |       |         |                |          |              |             |            |
| Ever smokers     | 89    | 83      | 93%            | 6        | 7%           | 4           | 67%        | 2           | 33%        |
| Never smokers    | 51    | 48      | 94%            | 3        | 6%           | 1           | 33%        | 2           | 67%        |
| Unknown          | 3     |          |                |          |              |             |            |
| 55 years and under | 55    | 47      | 92%            | 4        | 8%           | 2           | 50%        | 2           | 50%        |
| Ever smokers     | 51    | 47      | 92%            | 4        | 8%           | 2           | 50%        | 2           | 50%        |
| Never smokers    | 21    | 19      | 90%            | 2        | 10%          | 0           | 0%         | 2           | 100%       |
| Older than 55 years | 30    | 29      | 97%            | 1        | 3%           | 1           | 100%       | 0           | 0%         |
| Ever smokers     | 30    | 29      | 97%            | 1        | 3%           | 1           | 100%       | 0           | 0%         |
| Never smokers    | 17    | 17      | 100%           | 0        | 0%           | 0           | 0%         | 0           | 0%         |
| Male             | 129   | 112     | 95%            | 6        | 6%           | 4           | 67%        | 2           | 33%        |
| Ever smokers     | 84    | 78      | 93%            | 6        | 7%           | 4           | 67%        | 2           | 33%        |
| No smokers       | 17    | 17      | 100%           | 0        | 0%           | 0           | 0%         | 0           | 0%         |
| Female           | 39    | 36      | 93%            | 3        | 7%           | 0           | 0%         | 0           | 0%         |
| Ever smokers     | 5     | 5       | 100%           | 0        | 0%           | 0           | 0%         | 0           | 0%         |
| Never smokers    | 34    | 31      | 91%            | 3        | 9%           | 1           | 33%        | 2           | 67%        |
did find some K-RAS mutations among NS patients having concurrent EGFR and K-RAS mutations (Figure 1).

The significance of K-RAS mutations in women with smoking history has been described. The study suggests that women are more susceptible to smoking-related lung cancer because they have higher rates of K-RAS G12C mutations occurring in a younger age of diagnosis and with fewer pack-years of smoking than men with the same mutations. However, in our cohort, we did not find any K-RAS mutations in ES females. Instead, K-RAS G12D types mutations were found in NS females. Therefore, future studies may characterize driver mutations in Indonesian ES females.

Recent review shows a higher tendency of K-RAS mutation frequencies in smokers (25%) and male patients (22%) than nonsmokers (6%) and female patients (20%). In our study, the mutation frequency among ES patients and male gender in this study was modest (7%). Asian studies in Japan, Korea, and China show that the rates of K-RAS mutations among patients are 14%, 23%, and 33%, respectively. This relatively low frequency of K-RAS mutations in male and ES patients was unexpected, given the high prevalence of cigarettes consumptions among Indonesian men. Between 85% and 90% of all cigarettes smoked in Indonesia are kretes, a type of clove cigarette with high tar content.

In addition, we speculate that the extensive use of woods as solid fuels for cooking that are prevalent among up to 50% of the Indonesian population may affect the rate of K-RAS mutation as described in a Mexican study. Lung cancer patients exposed to wood smokes have a low frequency of K-RAS mutations regardless of cigarettes smoking history. Therefore, future studies may explore lifestyle and environmental related K-RAS gene and possibly other oncogenic driver mutations in Indonesia.

EGFR and K-RAS mutations are thought to be mutually exclusive. However, in our cohort, EGFR and K-RAS co-mutation frequency was 2.8% overall or 4.6% (of EGFR mutation positive cases). In Asia, the rate is 0.25% (6 K-RAS mutation cases of 2,387 EGFR mutation positive) or 1.5% (6 EGFR mutations of 398 K-RAS mutation positive), 1.5% (29 of 1,854 EGFR mutation cases) or 6.7% (29 of 429 cases).

In Taipei, K-RAS mutation rate was 8.3%, while concomitant K-RAS and EGFR mutation rate was 1.4% (1 out of 69 EGFR mutation positive). In a Chinese study, 1 K-RAS (0.3%) mutation has been found in 320 EGFR mutation positive patients. In Western patients overlapping EGFR and K-RAS mutations represented 6.8% (3 of 44) and 3.2% (3 of 92) of the population with single EGFR or K-RAS mutations, respectively. Among Asians, our rate of concurrent K-RAS and EGFR gene mutations seemed to be the highest. Moreover, other studies show that Del19 EGFR mutations occur together with KRAS mutations, which are not observed in our cohort. In a recent large study, concurrent mutation is associated with non-smoking patients and may affect progression-free survival, but not overall survival. These accumulated data may reconsider a given dogma of EGFR and K-RAS mutually exclusive mutations in lung cancer. Furthermore, conflicting prognostic and/or predictive utility of baseline K-RAS mutations to chemotherapy may question the clinical utility of K-RAS genotyping in lung cancer. Interestingly, a recent commentary on promising results of selumetinib, a potent inhibitor of mitogen-activated protein kinase 1 (MEKI) and MEK2 has pointed out an association of K-RAS G12C mutations and good response rate. Therefore, future studies may clarify the roles of K-RAS mutation in the routine management of lung cancer patients.

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Disclosure

The authors report no conflicts of interest in this work.

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