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The Absence of Mutations in the Exon 2 KRAS Gene in Several Ethnic Groups in North Sumatra May Not the Main Factor for Lung Cancer

Noni Novisari Soeroso[1],
Fannie Rizki Ananda[2],
Andika Pradana[2], Setia Putra
Tarigan[1], Elisna Syahruddin[3,4],
Dimas Ramadhian Noor[4]

[1]Thoracic Oncology Division, Department of Pulmonology and Respiratory Medicine, Faculty of Medicine, Universitas Sumatera Utara, Indonesia

[2]Department of Pulmonology and Respiratory Medicine, Faculty of Medicine, Universitas Sumatera Utara, Indonesia

[3]Thoracic Oncology Division, Department of Pulmonology and Respiratory Medicine, Faculty of Medicine, Universitas Indonesia, Indonesia

[4]Human Cancer Research Center, Indonesian Medical Education and Research Institute, Universitas Indonesia, Indonesia

Corresponding author, Noni Novisari Soeroso. Department of Pulmonology and Respiratory Medicine, Faculty of Medicine, Universitas Sumatera Utara, Universitas Sumatera Utara Hospital Jl. Dr. Mansyur No. 5, Medan 20155, Sumatera Utara, Indonesia. Phone: +62 812 6018 608. E-mail: noni@usu.ac.id. ORCID ID: http//www.orcid.org/0000-0000-0000-0000.

ABSTRACT

Background: Rat Sarcoma (RAS) protein encoded Guanosine Triphosphate (GTP-ase) activity, known as a switch of cell proliferation. The mutation of this protein alters the early stage of carcinogenesis and along with the interaction with other oncogene drivers and environmental factors affect the clinical characteristics and prognosis in cancer patients, particularly lung cancer. **Objective:** This study aims to determine the Kristen Rat Sarcoma (KRAS) mutation in lung cancer patients in North Sumatera and evaluate factors that might contribute in the development of lung cancer in the absence of KRAS mutation. **Methods:** This was a retrospective cohort study enrolled 44 subjects age > 18 year with the diagnosis of lung cancer. Histopathology preparation was obtained from surgery, bronchoscopy, and percutaneus needle biopsy then formed as paraffin-block. KRAS mutation was analyzed using Polymerase Chain Reaction (PCR) method with specific primer of exon 2 for evaluating the expression of RAS protein then continued with Sanger Sequencing Method at 12th and 13th codon. **Results:** The majority of subjects were male, age > 40 years old, bataknese, heavy smoker, with Adenocarcinoma. Almost all the subjects showed the expression of exon 2 of RAS protein in PCR examinations. However, Sequencing analysis using Bioedit Software, BLASTs and Finch T showed GGT GGC as protein base 219-224 which represented 12th and 13th Codon 12 and 13. The results interpreted there was no mutations of exon 2 of KRAS in North Sumatera Population. **Conclusion:** The absence of KRAS mutation in exon 2 in several ethnics in North Sumatera populations was not the main factors of lung cancer.

Keywords: KRAS mutation, exon 2, lung cancer, Indonesian, ethnic variation.

1. BACKGROUND

Rat Sarcoma (RAS) protein has a role as a switch of cell proliferation response from the extracellular signal (1, 2). Ras signaling pathway is an upstream cell proliferative pathway that begins after binding of Epidermal Growth Factor and ErbB family receptor, particularly erBb2, and recruit Son of Sevenless (SOS) which interact with RAS protein (3). This promotes the exchange of GDP to GTP. In the active binding GTP-ase state, RAS protein will activate the various downstream signal, including RAF, RALGDS, and PI3K pathway. In the mutation of KRAS, GTP-ase activity promoted continuously followed the cell proliferation (4).

The development of cancer is a long process that involved the interaction of several oncogenic drivers, tumor suppressor gene mutation and environmental factors (5). The mutation of KRAS or other oncogenic drivers played role in the early of tumorigenesis. This mutation promotes tumor development then epigenetic mechanism takes a role to determine the carcinogenesis (6). The complex process of tumorigenesis caused several factors that contribute to develop lung cancer and predict the prognosis of the patient, including the response of therapy, site of metastasis, the histopathology grade, and overall survival.

Apart from relating the nature of disease and prognosis of lung cancer, KRAS mutation exhibit an association with several clinical and demographic factors, including ethnic variability and environmental alteration (5–8).

Several studies showed that the Asian population has lower KRAS mutation incidence compare with the Western and Black populations (7). In the Chinese population, the prevalence of KRAS mutation was 13.1% in lung adenocarcinoma (9), wherein Indonesian population, KRAS mutation was about 7% in all types of lung cancer (10).

2. OBJECTIVE

This study was the first study in North Sumatera province and the second study in Indonesia to determine the KRAS mutation in non-small cell lung cancer patients. This study aimed to determine the mutation of KRAS and evaluate factors that might contribute to the development of lung cancer in a negative mutation of KRAS.

3. MATERIAL AND METHODS

Study Design

This is a descriptive retrospective study held in the Department of Pulmonology and Respiratory Medicine, Faculty of Medicine, Universitas Sumatera Utara in June 2020 until January 2021. All the procedure was conducted according to the Declaration of Helsinki and approved by Ethics Committee of Faculty of Medicine, Universitas Sumatera Utara, Indonesia. This study had gotten the permission from Department of Pathology at each collection site to genetically analyze their paraffin block. Written informed consent was obtained from all the patients or the family. The experimental protocol of this study was performed strictly following the guidelines.

Patients and samples

Patients were selected at several hospitals collaborate with the Department of Pulmonology and Respiratory Medicine, Faculty of Medicine, Universitas Sumatera Utara. The inclusion criteria were patients who had been diagnosed with lung cancer based on clinical, radiological, and confirmed by histopathology, age > 18 years old, and had complete medical records. Histopathology preparation was obtained from surgery, bronchoscopy, and percutaneus needle biopsy and all tumor samples were reviewed by several pathologists on site. The exclusion criteria were subject with incomplete medical records, tumor cell consentration < 50 cells in paraffin block, and not willing to participate in this study.

Samples DNA Extractions

DNA was extracted from Formalin-Fixed Paraffin-Embedded (FFPE) using Quick-DNA FFPE mini prep (Zymo Research, USA) according to manufacturer protocols as follows. Approximately FFPE were sliced using microtome in 1-2 slices and 4 μm thickness each. Slices were transferred to 1.5 ml centrifuge tubes, deparaffinization, tissue digestion, and DNA purifications were carried out according to manufacturer protocols. The DNA was eluted in 50 μl of DNA elution buffer. DNA concentrations and purity were measured using Biodrop Nanospetrophotometer.

KRAS Amplifications and DNA Sequencing

The DNA was amplified using KRAS Exon 2 primers forward 5′-AAGGCCTGCTGAAAATGACTG-3′ and reverse 5′-CAAAGAATGGTCCTGCACCAG-3′ according to Zinsky et al. The PCR were performed on using MyTaq HS Red Mix (Bioline, UK). Conditions as follows: one cycle of initial denaturation at 95 °C for 1 minute. The step was continued by 40 cycles of PCR steps, including denaturation in 95 °C for 15 s, annealing in 55 °C for 15 s. and elongations in 72 °C for the 30s. The final elongation step was performed at 72 °C for 10 minutes. Non-template control was using PCR primer mix without any DNA to detect unwanted DNA contaminations within PCR mix.

The sample was then run in 2 % of agarose electrophoresis in Tris Buffer EDTA pH 8.0. The amplicon was then compared with 100 bp, Amplicon size was 173 bp. DNA sequencing was performed using DNA sequencing plus services of FirstBase Asia. However since, the reverse primers better from optimizations, we focused on sequence using reverse primers only to captured corresponds codons 12-13.

Sequence analysis

Sequencing results were imported, converted to reverse complement, and analyzed using ClustalW in BioEdit Sequence Alignment Editor 7.2.5 editions. The reverse sequence was also analyzed to human reference genome NG_007524.2 (LRG_433) using nBLAST NCBI using standard preferences. The BLASTn results were also aligned with corresponding Coding Sequences/ (CDS). Lastly, the sequence was analyzed using FinchTv 1.4 Software by using reverse complement views.

4. RESULTS

Demographic characteristics

A total sample enrolled in this study was 100 subjects but after evaluating the tumor concentration, only 44 subjects analysed in this study with adequate tumor sample and complete medical records. The majority of subjects were male, bataknese, and heavy smokers. Bataknese is the dominant ethnic in this study with adenocarcinoma type of lung cancer (Table 1).

KRAS Exon 2 Amplification

The amplification of KRAS was performed and amplicon

No.	Characteristics	N	%
1	Sex		
	Female	11	25
	Male	23	75
2	Smoking Status		
	Never smoker	7	15.9
	Light smoker	17	38.6
	Heavy Smoker	20	45.4
3	Age		
	<40 years old	3	6.8
	40-60 years old	21	47.7
	>60 years old	20	45.5
4	Histopathology type		
	Adenocarcinoma	33	75
	Squamous Cell	9	20.5
	Adenosquamous	2	4.5
5	Ethnic		
	Bataknese	28	63.4
	Javanese	12	27.3
	Malay	3	6.8
	Acehnese	1	2.2
6	Staging		
	IIIA	5	11.4
	IIIB	5	11.4
	IVA	31	70.4
	IVB	3	6.8

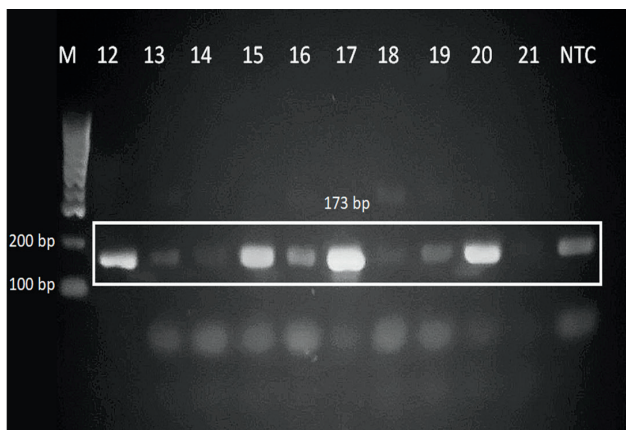Table 1. General Characteristics of subjects

Figure 1. PCR results of KRAS Exon 2. M was 100 bp DNA ladder (Bioline, UK), NTC was Non-template Control and Samples number as listed in picture. Samples were amplified at the correct sizes.

size was approximately 173 bp according to NCBI primer BLAST results. Representative results were shown in Figure 1. Almost all the subjects represents the presence of RAS protein which showed as the white bar in PCR ladder.

Sequence Analysis using Bioedit Software

Sequence Analysis using bio edit of reverse sequence in reverse complement mode, shown that no mutations were observed within samples. The codon 12 and 13 were shown at base positions 219 to 224 were GGT GGC sequence of KRAS Exon 2 (Figure 2) interpreted as no mutation of KRAS mutation detected.

Sequence Analysis using BLASTn and FinchTV

The sequence analysis was then confirmed using BLASTn and FinchTV to confirm the electropherogram results of the DNA sequence (Figure 3 and Figure 4). Same as Bioedit software, this sequence analyze in 12th and 13th codon which showed GGT GGC sequence of KRAS exon 2. There was no mutation detected in total 44 samples.

5. DISCUSSION

Carcinogenesis is a complex multistep process involved genetic alteration, epigenetic, and environmental factors (6). Genetic alteration including mutation of several oncogenic drivers and tumor suppressor genes demonstrates complex interaction with demographic characteristics (11). According to the Cancer Genomic Atlas (TCGA), lung cancer is one of the highest somatic mutations in both histopathology type including adenocarcinoma (ADC) and squamous cell carcinoma (SqCC). Few studies have been conducted in several sites to assess the genomic atlas in lung cancer in American and and

Asian studies (12). In East Asian, mutation rate of lung SqCC was similar to American population (13) while mutation rate of ADC was lower compare with American Population (14). In case of ADC, EGFR and KRAS mutations are the most common mutations and affect lung carcinogenesis through receptor kinase/RAS/RAF pathway (15). Other oncogenic driver mutations also occurred although in very small amout (less than 10%) such as BRAF, MEK, MET, BRAF, MAP2K1, HRAS, NRAS, ROS1, ALK, and ERBB2 (12). Further, KRAS mutation was more common in American population and found less common in Asian Population (12, 14).

A recent study showed that KRAS mutation is mostly found in males, smokers, and the western population (16, 17). But in another study showed that KRAS tend to more common in women never smokers (18). Variations of KRAS mutation might affect this. G12C and G12V mutation are more common in smoker patients with more complex interaction with the p53 compare with G12V which more common in never smokers (19). Both G12C and G12D were mostly detected in exon 2 at codon 12 and codon 13 (17). Apart from exon 2, less than 1% KRAS mutation occurs in exon 3 codon 61 (20). In this study, we performed sequencing analysis at the 12th and 13th codon location however there was no KRAS mutation found. So, other factors may dominantly affect the carcinogenesis in the Indonesian population.
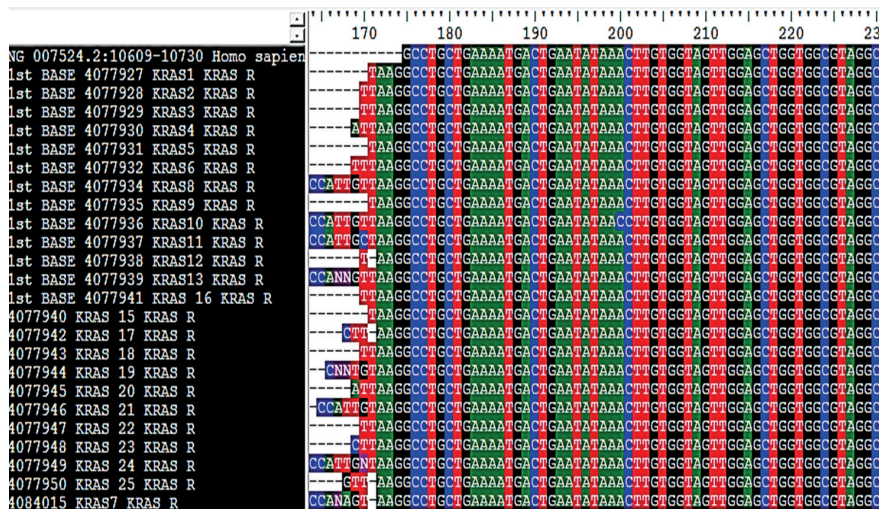

Figure 2. Sequence Alignment of Samples to Exon 2 KRAS in Human RefSeqGene (LRG_344) using Bioedit Alignment Software. Codon 12 and 13 were GGT GGC shown from base 219-224 and marked by blue bars.

Figure 3. The figure shows the alignment results of the sample (Querry) with the Subject (Human RefSeqGene reference sample (LRG_344). The sequence in the blue box shows the location of the 12th codon and 13th exon of the KRAS gene.
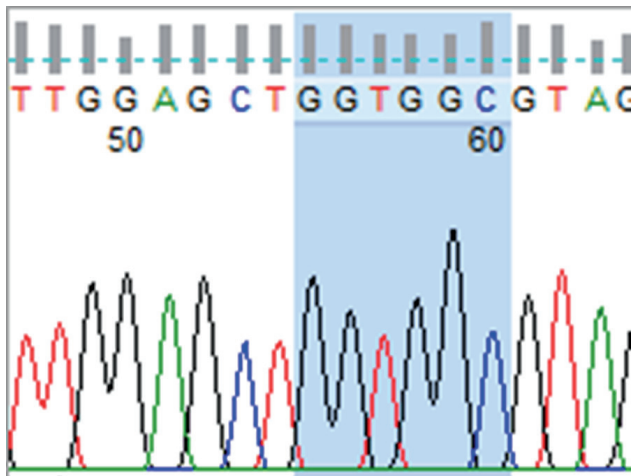
Figure 4. The electropherogram results view using FinchTV shown the sequence in the blue box showing the location of the 12th codon and exon 2 of KRAS genes.

The epidemiological data of the distribution of KRAS mutation may vary from several studies. From Asian population, Chinese and Japanese population was the most provided data in KRAS population with approximately 10-15% cases (9, 18, 19). Whereas, there is a lower incidence of KRAS mutation in the Indonesian population compare with average Asian data (21). While differentiation of country also plays role in the percentage of KRAS mutation, the ethnic differences might also affect the expression of KRAS. Indonesia is a country with a wide variation of ethnic. In North Sumatera, there were several ethnics with different habits including smoking, eating behavior, and traditional rite which might affect the lung carcinogenesis out of oncogenes factors (22). Previous studies in Indonesia showed that Bataknese have polymorphisn of CYP2A6 which is the main enzymes for nicotine metabolism and it was associated with dependence rate of nicotine consumption (23) eventhough it was not significant related to the incidence of lung cancer (22). Bataknese, as the most common ethnic in North Sumatera, tends to have a lower prevalence of oncogene driver. Along with local data in our region, Bataknese has lower EGFR expression compare with the average percentage in a recent study (24). A further larger scale of study was needed to evaluate and compare the expression of several oncogenic drivers including EGFR, KRAS, ALK, BRAF, ROS-1, and their consequences in clinical manifestation and prognosis in lung cancer in several ethnic in Indonesia.

KRAS mutation is a hereditary mutation but its expression may associate with smoking dose (25). Cigarette smoking habit promotes not only the mutation of KRAS as the oncogene, but also p53, tumor suppressor gene. Perri's study concluded that the mutation of p53 has occurred in 50% of tumors, particularly solid tumors. p53 can interact with oncogenes and accelerate carcinogenesis (26). Fortunately, there was no data about the mutation of p53 in Indonesia, so the role of p53 in developing lung cancer remains unclear. However, the probabilities of p53 as the more significant factors to promote lung cancer compare with KRAS mutation cannot be ruled out.

Since KRAS mutations detections were not detected and most of the samples were wild-type cancer in non-small

lung cancer, tumor cells that carried KRAS mutations should be considered while detected KRAS to increase sensitivity to detect mutations. However, in samples with the majority of tumor cells (<90%) no mutations were observed, meaning that sequencing using sanger methods was still reliable to detect patients' status, in particular in Indonesia (27).

Limitation of this study

The limitation of the study was the small number of samples and a small amount of DNA extraction. From almost 100 paraffin-block collected in this study, more than half were considered not significant for genotype analysis because of the lack of cell tumors. In this study, all of our samples were obtained from the bronchoscopy procedure. The majority of histopathology preparation was performed by forceps biopsy and transbronchial lung biopsy that provide a small size of histopathology preparation. Lack of cryotherapy and thermal ablation facilities in our area caused the increased risk of massive bleeding when obtaining more size of tumor from bronchoscopy.

6. CONCLUSION

There was no mutation of KRAS detected in our study. Interaction of several factors including ethic variation, life habituation, and mutation of other oncogene or tumor suppressor genes might contribute to promote lung cancer in the North Sumatera population.

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