STUDY OF KRAS MUTANT MOLECULAR PROFILE ON INDONESIAN COLORECTAL AND LUNG CANCER PATIENTS IN KALBE GENOMICS (KALGEN)

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Abstract - Molecular profiling is the study of genetic and epigenetic characteristic of cancer biomarkers. One of the most prominent and characterized cancer biomarker is KRAS. Currently, there has been little information about KRAS molecular profile in Indonesian colorectal and lung cancer patients. In this retrospective study, 36 colorectal and 9 lung cancer DNA samples in possession of Kalbe Genomics (KalGen) were screened for KRAS mutation by the use of PCR and direct sequencing. KRAS mutation was detected on 16 colorectal and 8 lung cancer samples with the most frequent mutation type found respectively were G12D and G12C. Bioinformatics analysis was thus performed to these most frequent mutation types by Swiss Pdb Viewer software. We observe several conformational changes in KRAS mutant G12D and G12C. These changes comprised the change of Gln 61distance with γ-phosphate of GTP and the blockage of GTP from the cytoplasm by Tir32, Pro34, and the side chain of glycine (G12D) or cysteine (G12C) at codon 12. From this study, it can be concluded that the most frequent KRAS mutation type that cause conformational changes in KRAS protein collected from Indonesian colorectal and lung cancer patients respectively were G12D and G12C.

Keywords - KRAS, Cancer, Mutation, Molecular Profiling

I. INTRODUCTION

The RAS superfamily encodes 21kD proteins (p21) that act as a molecular switch in intracellular signal transduction [1][2][3]. These protein possess a crucial role in the activation of signal transduction pathway that regulate proliferation, survival, and cellular apoptosis.

RAS protein binds with GDP in their resting state and switches into GTP when activated. The active-inactive conversion of this protein is regulated by its intrinsic GTPase and several second messenger molecules such as Guanine-nucleotide Exchange Factor (GEFs) and GTP Activating Protein (GAPs). Mutation in codon 12, 13, or 61 put on hold of the protein in its active form and impair its GTPase ability[4]. Consequently, it leads to a variety of developmental defects and diseases, one of them is cancer.

There are three known RAS isoform encoded by the RAS superfamily: HRAS, KRAS, and NRAS. Out of those three, KRAS is the most frequent mutated RAS isoform found. It comprises approximately 86% of all RAS mutations [1] and about 80% of these mutations were reported to happen in codon 12[3].

In cancer treatment, patients having the same diagnosis tend to show different responses on the treatment given. This phenomenon is suspected to be caused by the diversity of personal biomarker mutations. Therefore, a study to observe the genetic and epigenetic characteristics of a person's cancer biomarker with a goal of enhancing the quality and effectiveness of cancer treatment is highly needed[5]. This specific study is known as molecular profiling.

Currently, one of the mostly used cancer biomarker is KRAS [6]. Additionally, RAS is an attractive targets for vaccine therapy[7]. Therefore, molecular profiling of KRAS is important to be studied. Molecular profiling of KRAS has been performed in many countries around the world [8][9]. However, there has only been little information about its molecular profile in Indonesian patients. On that point, the purpose of this retrospective study is to observe KRAS mutant molecular profile in two cancer with the highest incidence number in Indonesia, which are colorectal and lung cancer.

II. MATERIAL AND METHODS

A. Materials

DNA samples. The DNA samples were a possession of Kalbe Genomics (KalGen) that had been used in daily clinical tests. Based on the location, the samples were divided into two groups, which were 36 colorectal cancer and 9 lung cancer samples. In addition of the samples used, secondary data of colorectal and lung cancer patients derived from KalGen database was used for further analysis, which comprised: 193 colorectal cancer patients data from 2009 – 2011 period and 52 lung cancer patients data from 2004.

Primer - A specific custom made primer specifically designed by Stem Cell and Cancer Institute (SCI) was used in this study. This primer was modified with the addition of PNA and was used for daily clinical tests.

B. Methods

DNA Amplification. Colorectal and lung cancer DNA samples were amplified by the use of PCR. A standard PCR reaction from HotStarTaq DNA Polymerase (Qiagen) kit was used. The amplification product were then tested qualitatively using electrophoresis and preserved at 4°C. Cycle and Direct Sequencing. Suspected mutant samples were purified using High Pure PCR Product Purification Kit (Roche). Standard reaction from BigDye Direct Cycle Sequencing Kit (Applied Biosystem) was used to perform the cycle sequencing. Twenty µl of the amplicons derived from this process were purified with the addition of 2 µl ammonium acetate 7.5 M and 60 µl absolute ethanol. The solution was thus centrifuged at 12000 rpm for 15 minutes and supernatant was discarded. Two hundreds µl of 70% ethanol was added into the pellet. The solution was centrifuged again at 12000rpm for 5 minutes. Supernatant was discarded once again and the pellets were dried out. Completely dried pellets was then mixed thoroughly with 20 µl HiDi formamide before going through direct sequencing. Sequences derived from these processes were further analyzed with bioedit software. Bioinformatics analysis of KRAS Mutants. RAS protein models with PDB ID 4EFL, 4EFM, 4L9W, 1AGP, 40BE, and 2PMX were downloaded from Protein Data Bank (www.rcsb.org). Bioinformatics analysis of KRAS mutants was performed using Swiss PDB Viewer software. The parameters observed were GTP relative position at the protein surface, GTP-binding-pocket conformational changes, and intrinsic GTPase (Lys16 and Gln61) distance towards GTP. When you submit your final version, after your paper has been accepted, prepare it in two-column format, including figures and tables.

III. RESULT AND DISCUSSION

KRAS mutant screening was performed on 36 colorectal and 9 lung cancer DNA samples collected from Indonesian patients who met the criteria for inclusion in this study. The initial screening was performed using qPCR that has been modified with the addition of Peptide Nucleic Acid (PNA). The amplicons were then tested qualitatively by electrophoresis (Fig 1).

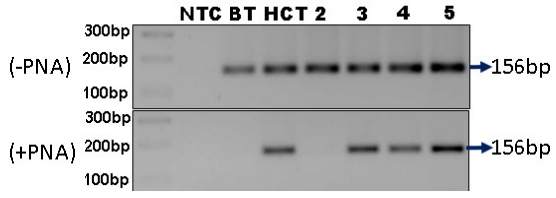


Fig 1 Electrophoresis of control amplicon (above) and amplicon from PCR that has been added with PNA (bottom). NTC: negative control, BT: wild type control, and HCT: mutant control. Number 2, 3, 4, and 5: KalGen colorectal and lung cancer DNA samples.

Based on the initial screening, we obtained 2 false negative, 18 wild type, and 16 suspected KRAS mutant samples on colorectal cancer patients. On the other hand, we found 1 false negative and 8 suspected KRAS mutant samples on lung cancer patients. Samples that were suspected to contain mutants were anaylyzed further by direct sequencing to identify the type of KRAS mutation (Fig 2). From this method, we found 15 samples that contain KRAS heterozygote mutant and 1 wild type in colorectal cancer samples. In lung cancer samples, we found 7 KRAS heterozygote KRAS mutant (Table 1).

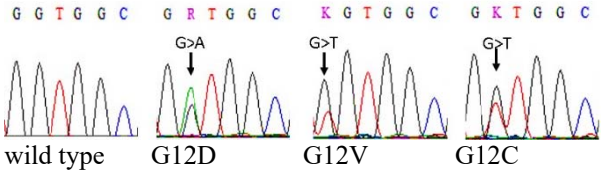


Fig 2. Detection of KRAS mutant in colorectal and lung cancer DNA samples using direct sequencing. (left to right: KRAS wild type, mutant G12D, G12V, and G12C)

Samples number		Sequence			
Colorectal cancer	Lung cancer	Codon 12	Codon 13	Amino Acid	Mutation Type
		GGT	GGC	Glycin	-
9	1	GAT	-	Aspartic Acid	G12D
3	2	GTT	-	Valin	G12V
2	3	TGT		Cystein	G12C
1	-	-	GAC	Aspartic Acid	G13D
-	1	GCT	-	Alanine	G12A

Table 1. Identification of KRAS mutant on Indonesian colorectal and lung cancer patients

Further analysis of KRAS molecular profile on Indonesian patient was performed based on the data collected. To compare the prevalence of KRAS mutation in Indonesian patients with other countries, an addition of 193 colorectal cancer patients data (2009 – 2011 period) and 52 lung cancer patients data (2004 period) was used. These data were taken from other Indonesian patients and were a possession of Kalbe Genomics. Consequently, the total data used in this study were 229 for colorectal cancer and 61 for lung cancer.

Indonesian KRAS mutation prevalence. Point mutation of KRAS has been detected in many types of cancers. In colorectal cancer, the prevalence of KRAS mutation is reported as much as 20-60% [1]. Our result showed a prevalence of 34.06% of KRAS mutation in Indonesian colorectal cancer patients. This finding was similar with several epidemiological studies that had been conducted in other countries such as Hongkong (44.5%) [10], France (38%) [11], and Danish [12].In contrast with colorectal cancer, we found 11.48% of KRAS mutation prevalence in Indonesian lung cancer.

This prevalence is low compared to other western countries such as Europe and USA, but coherent with the result generated from Asian countries. Asian patients were reported to show a lower tendency of KRAS mutation in lung cancer (about 5-20%) than western patients (about 25-50%) [13][14][15].

Indonesian KRAS mutation spectrum. Of all the DNA samples screened, the transition of guanine to adenine (G>A) in the second nitrogen base of codon 12 and 13 was the most frequent mutation found in colorectal cancer patients, with a percentage of 55.13% (Table 2). This mutation led to a change from glycine (GGT or GGC) into aspartic acid (GAT or GAC). Our result was coherent with several studies that had been conducted in countries all over the world, some of them were Switzerland, USA, Norway, Mexico, and Netherlands. All of the studies indicated G>A transition in codon 12 and 13 as the dominating mutation in colorectal cancer[16]. Similar result was reported on a comprehensive study on RAS gene mutation in a variety of cancers, that reported G>A transition in codon 12 and 13 of KRAS gene to be as much as 43% [3].

Another KRAS mutation pattern that was greatly observed on Indonesian colorectal cancer patients was G>T transversion on codon 12 (Table 2). This mutation pattern was also found dominating in lung cancer (85.71%). This pattern especially cause glycine (GGT) substitution into valine (GTT) or cysteine (TGT), and therefore generated mutant G12V and G12C. The tendency of KRAS mutational pattern found in Indonesian patients was similar to a study conducted by ZInsky [17].

Cancer Type	Mutation Pattern	N (%)
Colorectal (N=229)	Mutation pattern	
	Transition G>A	43 (55.13)
	Transversion G>T ; G>C	35 (44.87)
Lung (N=61)	Mutation pattern	
	Transition G>A	1 (14.29)
	Transversion G>T ; G>C	6 (85.71)

Table 2. KRAS mutation pattern in Indonesian colorectal and lung cancer patients

With reference to this result, G12D was the most frequent mutation type found in Indonesian colorectal cancer patients (Fig 3). This mutation type tendency showed a similarity with studies conducted in Jordan[9], Brazil[18], and Nedherlands[16]. Additionally, we also found the same point mutation on codon 13 (G13D). Mutation in this codon was reported to be found specifically in colorectal cancer [19].

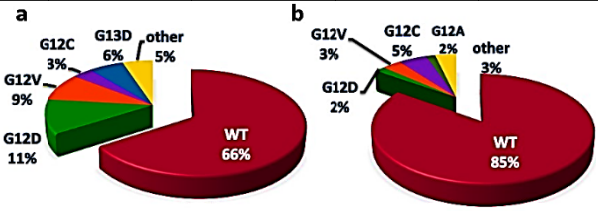


Fig 3. KRAS mutation spectrum in Indonesian (a) colorectal cancer and (b) lung cancer patients. Wild type is abbreviated into WT.

Different with our finding in colorectal cancer, we found the change from glycine into cysteine in codon 12 (G12C) of KRAS as the most frequent mutation in Indonesian lung cancer patients. The frequency of this type was followed by the change from glycine into valine in the same codon (G12V), as can be seen on Figure 3. This result was coherent with the studies from Japanese[20] and American[21] patients.

G>A transition in human cancer was assumed to be caused by high consumption of linoleic acid and polysaturated fatty acids (PUFA). ω - 6 PUFA peroxidation can resulted in the accumulation of several byproducts such as malondialdehide (MDA) and 4-hydroxynonenal (40HNE). MDA had been proved to be mutanogenic towards mammalian and bacterial cells and carsinogenic on rat's cell. Furthermore, the products of MDA and 4-NHE cellular metabolism could generate secondary adduct molecules pirimidopurinone and deoxyguanocine. These adduct molecules could potentially damage DNA and became mutational precursor [22].

In western countries, high consumption of linoleic acid and PUFA was deducted to come from red meat [23]. However, Indonesia diet did not include as much red meat as western diet. Therefore, we suspected fried protein, offal, margarine, and overly cooked coconut milk to be the source of high level of linoleic acid and PUFA in Indonesian patients [24]. This suspicion was supported by Sartika[24], who reported that the ultimate fat source in Indonesian diet comes from fried foods.

In contrast to G>A transition, G>T transversion in codon 12 of lung cancer patients could be caused by polycyclic aromatic hydrocarbons and heterocyclic amines that were abundant in tobacco or cigarette smoke. We suspected this due to a high number of Indonesian smoker, which is about 61.4 million smokers (31.6%)[25]. The relation between G>T transversion and cigarette smoke was supported by Riely [26], who suggested G>T transversion in lung cancer as cigarette smoke molecular signature. Consequently, this deduction was also supported by epidemiological data that had been done on lung cancer with smoking history. Based on that study, G>T

transversion was the most prevalent mutation pattern found on patients with smoking history. Those who did not have smoking history, on the other hand, were found to possess G>A transition rather than G>T transversion [26][27].

Bioinformatics analysis of KRAS mutant protein. The G-domain of RAS protein shares 70-80% of sequence identity with its three isoform. This region consist of amino acids 1-165 and is important for the signaling function of the protein. Structurally, the G-domain of KRAS is composed of three different region, each of which undergo significant conformational changes as the proteins become activated[27] (Fig 4). These regions are known as p-loop (codon 10-16), switch I (codon 30–38), and switch II (codon 59-67). Along within these regions lies GTP binding pocket, where codon 10-16 and 56-59 of the p-loop interact with β-phosphate and γ-phosphate of GTP and residue 116-119 and 152-165 interact with the guanine base. Binding of regulators and effectors happens in switch I and II regions of RAS protein. These region is essential for the intrinsic GTPase activity of RAS protein and interactions between GAPs and other downstream effectors [28].

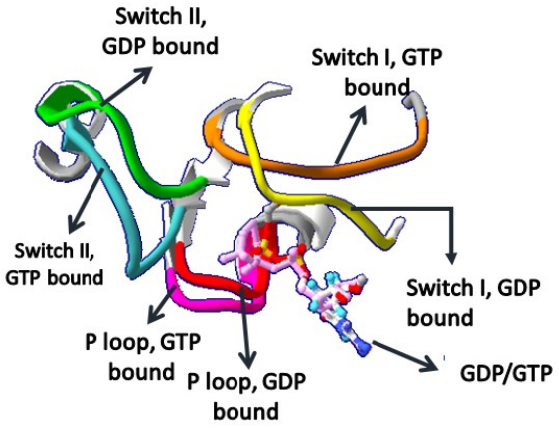


Fig 4. GTP conformational alteration of KRAS wild type bound with GDP (switch I: yellow; switch II: green, and p-loop: red) and bound with GTP (switch I: orange; switch II: blue, and p-loop: pink)

Normally, codon 12 and 13 of KRAS protein are filled with amino acid glycine that has no side chain. Mutation that substitutes glycine with any other amino acid that contain side chain can potentially cause conformational changes in the structure of KRAS protein due to the interaction between the newly added side chain with the surrounding amino acids [29]. These conformational changes impaired KRAS intrinsic GTPase activity and hold the protein in its active form, resulting in continuous proliferation and downstream pathway activation, therefore promoting tumors and cancers.

We analyzed codon-12 KRAS mutant found in Indonesian colorectal and lung cancer patients bioinformatically with the assumption that there were no other mutation in other part of KRAS. The analysis was conducted with Swiss PDB Viewer software. The parameters observed were the conformational alteration of GTP binding pocket, GTP relative position in the surface of KRAS protein, and the distance changes of KRAS intrinsic GTPase amino acids towards GTP.

We observed several conformational alteration in G12D and G12C KRAS mutant. However, we did not find any alteration in G12V mutant (Fig 5). We suspected this phenomenon to be caused by the orientation of valine side chain towards the cytoplasm, producing no interaction between this side chain with another codons. Our hypothesis is coherent with a study by Futatsugi & Tsuda[30], that explained the perpendicular orientation of valine side chain in G12V towards the main structure or pointing towards the cytoplasm.

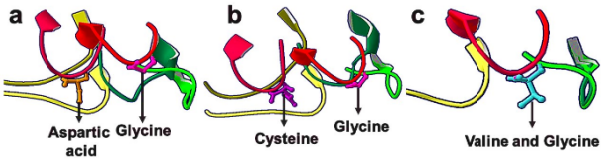


Fig 5. Conformational changes of p-loop (red), switch I (green), and switch II (yellow) in mutant KRAS: (a) G12D, (b), G12C, and (c) G12V.

Intrinsic GTPase activity of KRAS protein is mainly affected by two amino acids, Lys16 on switch I and Gln 61 on switch II[30][31]. Lysine plays an important role in nucleophilic attack of water molecules towards γ–phosphate of GTP in KRAS intrinsic GTP hydrolysis[30]. We suspected Gln61 to have the same role. However, this residue was reported to have bigger role on GAPs facilitated GTP hydrolysis than in the intrinsic GTP hydrolysis[31].

The observation of Lys16 distance alteration towards GTP γ-phosphate in KRAS mutant using static model could not prove a significant comparison between KRAS wild type (6.09 Å) and mutants, such as G12D (5.75 Å), G12C (6.14 Å), and G12V (6.07 Å). Due to this reason, a molecular dynamic simulation needs to be conducted to explore this effect further, as had also been stated by Futatsugi & Tsuda[30], who discovered that the distance between Lys16 and γ-phosphate of GTP in KRAS wild type and G12V mutant, which was observed at ± 3 Å, was indifferent.

In GAP facilitated GTP hydrolysis, Gln61 reorientation allows the formation of hydrogen bonds between amine groups of Gln61 in KRAS with carboxyl group of Arg789 in GAP. This reorientation thus becomes the precursor of nucleophilic attack of water molecule towards γ–phosphate of GTP, which

then initiate GTP hydrolysis. Mutation in codon-61 of KRAS reduce the rate of GAP facilitated GTP hydrolysis as much as 10^6 fold[32]. On that point, we conducted a measurement of Gln61 distance towards γ–phosphate of GTP statically (Fig 7).

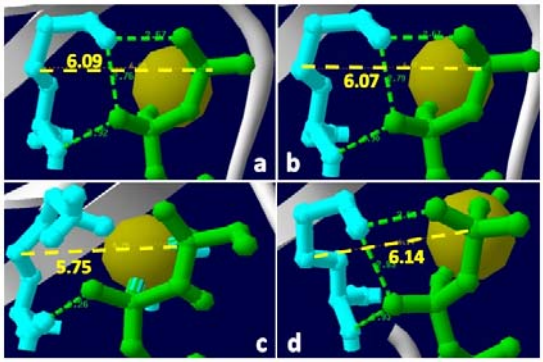


Fig 6. Lys16 (light blue) distance towards γ-phosphate of GTP (green) in: (1) wild type KRAS, (b) G12V, (c) G12D, and (d) G12C. The distance were shown in yellow discontinued lines

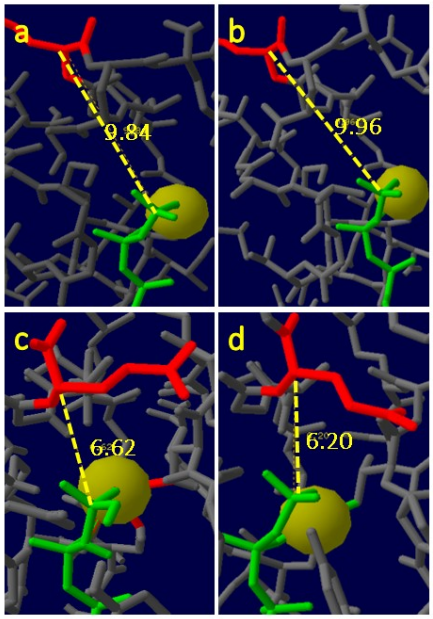


Fig 7. Gln61 (red) distance towards GTP γ-phosphate (green) in (a) KRAS wild type, (b) G12V, (c) G12D, and (d) G12C. The distance are shown in discontinued yellow lines.

We observed a greater distance between Gln61 and GTP γ-phosphate in KRAS G12V mutant (9.96 Å) compared to the wild type (9.84 Å). We suspected this 0.12 Å gap to inhibit the right orientation between carboxyl group of Arg789 in GAP molecule with amine group of Gln61 in KRAS. As a consequence of this gap, arginine finger could not form and Gln61 could not perform nucleophilic attack towards γ-phosphate of GTP. This could lead to a decrease in GAP catalyzed GTP hydrolysis and therefore was known as KRAS GAP resistance. A study by Khrenova[33] supported our result. The G12V mutation in KRAS protein widen up the distance of Gln61 side chain from GTP and could cut the hydrogen bond between Gln61 with γ-phosphate of GTP. With regards to the following study and our result, we suspected the gap created by G12V mutation to decease Gln61 catalytic ability to initiate GAP facilitated GTP hydrolysis in KRAS protein.

Unlike the greater distance of Gln61 in G12V mutant, we found a shorter distance changes in G12D and G12C mutant (6.62 Å and 6.20 Å) compared to KRAS wild type (9.96 Å). This shorter distance allowed G12D and G12C mutant to have a more rapid GTP hydrolysis than wild type and G12V. However in actual fact, these mutants show an impairment in GTP hydrolysis. We suspected GTP relative position in KRAS protein surface that was blocked by amino acids on switch I to be the cause for this (Fig 8).

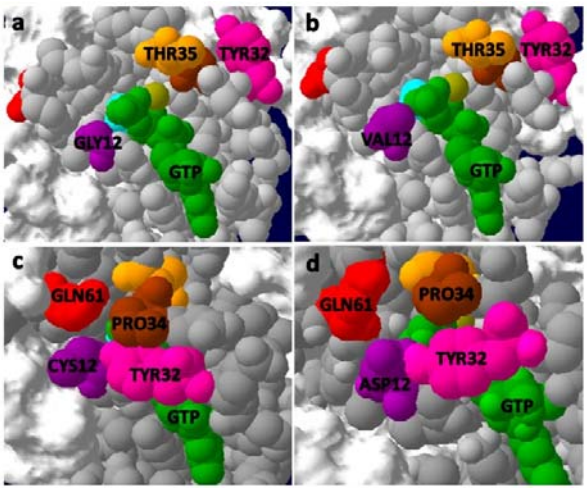


Fig 8. GTP relative position analysis on KRAS protein surface. On KRAS wild type (a) and G12V mutant (b), GTP was exposed completely towards the cytoplasm whereas at G12C (c) and G12D (d) mutant, GTP was blocked by tyr32 and pro34.

The blockage of GTP by amino acids residue in switch I such as Tyr32, Pro34, and Thr35 can be caused by the interaction of cysteine or aspartic acid side chain in codon 12 with the surrounding residues. This blockage inhibits GTP interaction with Gln61 from KRAS protein with Arg789 from GAP (arginine finger) thus implicates in the inability of GAP catalyzed GTP hydrolysis. Similar results were explained by Rajaligam[29], who reported that the appearance of any side chain in residue 12 and 13 position in KRAS protein could inhibit the interaction between KRAS protein with GAP and ended up in the reduction of GTP hydrolysis rate.

IV. CONCLUSION

The prevalence of KRAS mutation in Indonesian colorectal and lung cancer patients are 34.06% and 11.48% respectively. The transition of G>A (leading to G12D mutant) and transversion of G>T (resulting in G12V mutant) in codon 12 of KRAS protein were

found equally frequent in colorectal cancer patients. In lung cancer patients, on the other hand, the transversion of G>T which leads to G12C and G12V was found dominating. Bioinformatically, we observed several conformational changes in the G12D and G12C mutant. The changes include the blockage of GTP from cytoplasm by side chain in switch I and the alteration of Gln61 distance towards γ–fosfat of GTP.

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