Indonesian Genomic Landscape of Pathogenic Mutation of APC, KRAS, TP53, PIK3CA, and MLH1 in Colorectal Cancer

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Research Article

Keywords: colorectal cancer, genetic mutation, pathogenic mutation, next-generation sequencing, APC, TP53, PIK3CA, KRAS, MLH1

Posted Date: December 20th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-900277/v2

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Abstract

Background: Knowing colorectal cancer’s heterogeneity and dynamic features, recognizing its biological behaviour requires detailed identification of mutated genes involved. Colorectal cancer (CRC) requires several mutated genes to occur and those are dissimilar in each person hence essential to be discovered in specific population. Until recently, there is no known study describing genomic landscape of CRC in Indonesian population. This study aims to describe profile of pathogenic mutation of APC, TP53, PIK3CA, KRAS, and MLH1 in CRC patients treated at 3 different hospitals in Jakarta.

Methods: This is a descriptive study conducted on CRC patients who underwent neoadjuvant, surgical, and adjuvant therapy at RSCM, RSKJ, and MRCCC in 2017-2018. DNA analysis was performed using next-generation sequencing and aligned against GRCh38. Pathogenic variant was identified using ACMG classification and FATHMM score. Data related to behaviour and survival were collected from medical records.

Results: There were total 22 subjects in which APC, TP53, and PIK3CA were mutated. KRAS mutation occurred in 64%, while MLH1 in 45%. Five types of mutation were identified, including nonsense, missense, frameshift, splice-site, and silent mutation. There are 4 groups of co-occurring mutations, which are APC, TP53, PIK3CA (triple mutation/TM) alone; TM+KRAS; TM+MLH1; and TM+KRAS+MLH1, presenting different nature and survival.

Conclusion: Indonesia having various ethnicities with diverse diet and lifestyle has distinct profile of pathogenic mutation presenting mostly with locally-advanced stage with various outcome and survival rate.

Introduction

Colorectal cancer has been known as one of the most well-studied malignancy. Its dynamic and heterogeneity are characterized by many interconnecting molecular etiopathogenesis exhibiting different behavior inter and intratumor. Based on recent biomolecular study, genetic and epigenetic analysis can evaluate the nature of the tumor, hence able to predict heredity, progressivity, recurrency, response to therapy, and even survival rate. Those variables cannot be estimated by AJCC staging system alone. For this reason, precision medicine rooting to genomic profile of each individual, are starting to advance.

Colorectal malignancy, which involves at least three or four genetic mutation, are feasible for next-generation sequencing methods. Two of the three most common carcinogenic pathways are chromosomal and microsatellite instability. Five genes which frequently involved are APC, TP53, KRAS, PIK3CA, and MLH1. Different group of age, gender, and geographic location actually have different variations of mutation and genes involved so that study on specific population is important in advancing precision medicine. Until recently, there is no publication providing genomic landscape of colorectal cancer in Indonesian population. This study aims to analyze genomic profile of colorectal cancer in Indonesia.

Methods

This is a descriptive study in patients with colorectal malignancies who underwent surgery and/or chemoradiation and/or chemotherapy at RSCM, RSKJ, and MRCCC in 2017-2018 whose tumor tissue specimens were still properly stored in the form of formalin-fixed paraffin-embedded (FFPE). This study has been reported in line with STROCSS criteria.
All sequencing preparation was performed by Department of Medical Chemistry, Faculty of Medicine, Universitas Indonesia at Bioinformatics Core Facility of Indonesia Medical Education and Research Institute (IMERI).

DNA extraction was performed using QIAamp DNA FFPE Tissue Kit. The quality of extracted DNA was evaluated using an absorbance ratio of 260 nm to 280 nm ($A_{260}/A_{280}$) and 260 nm to 230 nm ($A_{260}/A_{230}$). Purity criterion for samples with the $A_{260}/A_{280}$ ratio is within the range of 1.8–2.0 and $A_{260}/A_{230}$ ratio is within the range of 2.0–2.2. After purity criterion fulfilled, sequencing was done utilizing AmpliSeq Cancer HotSpot Panel v2 for Illumina. Results in FASTQ format were quality-checked with FASTQC (v.0.9.5; http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and aligned against Genome Reference Consortium Human Reference 38 (GRCh38). Variant calling was done using LoFreq and annotated with SNPEFF and filtered with SNPSift. Annotation results were stored in variant call format (VCF) file.

**Inclusion criteria, exclusion criteria, and identification of pathogenic mutation**

a. Retrieval of VFC files fulfilling inclusion and exclusion criteria
   - Inclusion criteria: FFPE samples fulfilling DNA purity criterion and showing PASS status in FASTQC
   - Exclusion criteria: none

b. Data filtering based on estimation of putative impact or deleteriousness showing HIGH.

c. Identification of single nucleotide variant (SNV) and synchronization with 3 databases
   - ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/)
   - COSMIC (https://cancer.sanger.ac.uk/cosmic)
   - The Ensembl project (https://asia.ensembl.org/info/index.html)

d. Identification of somatic effect based on American College of Medical Genetics (ACMG) classification and Functional Analysis through Hidden Markov Models (FATHMM) score.

e. Selection of SNVs meeting pathogenic criteria (ACMG pathogenic variant and/or FATHMM score 0.7).

f. Identification of SNVs of APC, TP53, PIK3CA, KRAS, and MLH1

g. Matching samples’ numbers and medical record data

**Results**

**Patients characteristics**

Twenty two samples were collected in accordance with sample preparation procedures mentioned above. Among these samples, 41% (9/22) were diagnosed with stage 3b of which 7 were elective cases. Fifty-nine percent (13/22) had lymphovascular invasion, of which 1 was diagnosed with stage 2A, and 12 were in stage 3B-4C.

| Table 1 Patients characteristics |
### Variables

| Variables               | Numbers | % |
|-------------------------|---------|---|
| **Age**                 |         |   |
| < 50 y.o.               | 9       | 41|
| ≥ 50 y.o.               | 13      | 59|
| **Gender**              |         |   |
| Male                    | 13      | 59|
| Female                  | 9       | 41|
| **Death**               |         |   |
| Yes                     | 11      | 50|
| No                      | 11      | 50|
| **Grade**               |         |   |
| Well                    | 13      | 59|
| Moderate                | 6       | 27|
| Poor                    | 3       | 14|
| **Stage**               |         |   |
| 1                       | 2       | 9.1|
| 2A/B/C                  | 5/1/-   | 27.3|
| 3A/B/C                  | -/9/-   | 40.9|
| 4A/B/C                  | 2/1/2   | 22.7|
| **Lymphovascular invasion** |         |   |
| Yes                     | 13      | 59|
| No                      | 9       | 41|
| **Tumor location**      |         |   |
| Group 1                 | 3       | 14|
| Group 2                 | 4       | 18|
| Group 3                 | 15      | 68|
| **Perioperative management** |     |   |
| None                    | 13      | 59|
| Neoadjuvant chemoradiation | -     | -|
| Adjuvant chemotherapy   | 7       | 32|
| Neoadjuvant chemoradiation + adjuvant chemotherapy | 2 | 9 |
| **Family history of cancer** |     |   |
| Colon cancer            | 1       | 9|
| Breast cancer           | 1       |   |

**Average interval from diagnosed to death** 259 hari (3-882)

*Group 1: Caecum to two-third proximal of transverse colon; Group 2: One third distal of transverse colon to sigmoid; Group 3: Rectum to anus.*

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**Pathogenic mutation mapping in whole chromosomes**

There were pathogenic mutation in almost all somatic chromosomes except 6, 9, 14, 16, 21, and 22 which involved 25 genes and 641 SNV (Table 2). Three types of mutation were identified i.e. synonymous (silent mutation); non-synonymous (nonsense, missense, and frameshift); and splice-site mutation.

Table 2 Pathogenic mutation mapping
| Chr | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | Total Genes | Total SNVs |
|-----|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|-------------|------------|
| Gene | a | b | c | d | e | f | g | h | i | j | k | l | m | n | o | p | q | r | s | t | u | v | w | x | y |
| Pts | 1(1) | 2 | 2 | 1 | 1 | 2 | 2 | 1 | 4 | 5 | 1 | 10 | 1 | 1 | 1 | | | | | | | | | |
|     | 2(3) | 1 | 1 | 1 | 3 | 1 | 1 | 1 | 1 | 1 | 4 | 1 | 10 | 1 | 1 | 1 | | | | | | | | | |
|     | 3(6) | 1 | | 2 | 1 | 2 | 3 | 1 | 1 | | | 1 | 7 | | | | | | | | | | | | |
|     | 4(14) | 1 | 1 | 1 | 1 | 1 | | | 1 | 3 | 1 | | | | | | | | | | | | | | |
|     | 5(15) | 1 | 2 | 1 | 1 | 1 | 2 | 1 | | | 1 | 4 | 2 | | | | | | | | | | | | |
|     | 6(16) | | | 1 | 2 | 2 | 1 | 1 | | | | 1 | 4 | 1 | | | | | | | | | | | |
|     | 7(19+19b) | 2 | 2 | 2 | 1 | 4 | 2 | 1 | | | 1 | 2 | 4 | 1 | 14 | 2 | 1 | 1 | | | | | | |
|     | 8(20) | 1 | 2 | 1 | 1 | 2 | 1 | | 5 | 1 | 9 | 2 | 1 | | | | | | | | | | | | |
|     | 9(22) | 1 | 1 | 3 | 2 | 1 | | | | 9 | 1 | | 1 | 1 | 8 | | | | | | | | | |
|     | 10(23) | 1 | 2 | 2 | 1 | 1 | | | 4 | | 4 | 1 | 1 | 9 | | | | | | | | | |
|     | 11(29) | | | 1 | 1 | 1 | 3 | 2 | 1 | 1 | | 1 | 4 | 1 | 1 | | | | | | | | | |
|     | 12(34) | 1 | 1 | 2 | 1 | 2 | 6 | 1 | 2 | 1 | 1 | | 3 | | 10 | 1 | 1 | | | | | | | |
|     | 13(3737) | | 1 | 1 | 2 | 1 | 2 | 5 | 2 | 2 | 2 | | 1 | 3 | | 10 | 4 | 1 | 1 | | | | |
|     | 14(9) | 1 | | 1 | 1 | 3 | 1 | 1 | | 1 | 3 | 7 | | 1 | 2 | | | | | | | | |
|     | 15(11) | 1 | 1 | 1 | 1 | 3 | 6 | 1 | 2 | 1 | 1 | | 2 | 3 | 9 | 4 | 1 | | | | | | |
|     | 16(12) | 1 | 2 | 1 | 3 | 2 | 2 | 1 | 7 | 5 | 2 | 1 | | 1 | 1 | | 4 | 6 | | | | |
|     | 17(13) | | 1 | 2 | 4 | 1 | 3 | 3 | 1 | 2 | 1 | | 1 | 4 | | 17 | 4 | 1 | 1 | | | |
|     | 18(14) | | 1 | 1 | 2 | 2 | 3 | 3 | 1 | 1 | 1 | 1 | 1 | 1 | 4 | 1 | 6 | 5 | | 1 | 1 | 17 | |
|     | 19(16) | 1 | | 1 | 1 | 2 | 1 | 3 | 1 | 1 | 1 | | 2 | 3 | | 14 | 2 | | 2 | | | | |
|     | 20(17) | | 2 | | 1 | | 1 | 1 | 1 | 1 | | 1 | 4 | | 13 | 6 | | | | | | | |
|     | 21(18) | 2 | | 3 | | 1 | 4 | 2 | 1 | 1 | | 1 | 1 | 4 | 12 | | 1 | 1 | | | | |
|     | 22(19a) | 1 | | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | | 1 | 6 | 10 | 3 | 1 | 1 | | | |
| Total Patients | 10 | 5 | 9 | 2 | 17 | 10 | 22 | 3 | 15 | 22 | 16 | 15 | 4 | 5 | 9 | 4 | 6 | 14 | 21 | 5 | 22 | 18 | 12 | 10 | 5 | 641 |

Information:

A. NRAS
B. ALK
C. IDH1
D. ERBB4
E. VHL
F. MLH1
G. PIK3CA
H. CTNNB1
I. KIT
J. APC
K. BRAF
L. EGFR
M. FGFR1
N. RET
O. PTEN
P. FGFR2
Pathogenic mutation mapping of APC, TP53, PIK3CA, KRAS, and MLH1

Two types of APC pathogenic mutation occurred concurrently (nonsense and missense) in 1 patient. TP53 mutation also have 5 concurrent mutations in 1 patient (nonsense, missense, frameshift, silent, and splice-site) and only 3 of 22 patients having singular missense mutation. Only 1 type of pathogenic mutation occurring in MLH1 (nonsense) and PIK3CA (missense). Singular KRAS mutation occurred in 10 patients (8 missense and 2 silent) and multiple mutations occurred in 4 patients (Table 3).

Table 3 Pathogenic mutation mapping of 5 genes

| Gene | n = 22 |
|------|--------|
| MLH1 |        |
| PIK3CA |       |
| APC  |        |
| KRAS |        |
| TP53 |        |

| Mutation Type |
|---------------|
| Nonsense      |
| Missense      |
| Frameshift    |
| Splice-site   |
| Silent        |
| None          |
Co-occurring mutation in more than 3 genes were presented in all subjects. Combination of triple mutation (APC, TP53, PIK3CA) occurred in 4 of 22 patients. Combination of quintuple mutation (APC, TP53, PIK3CA, KRAS, MLH1) occurred in 6 of 22 patients. (Table 4)

**Table 4 Subjects with co-occurring mutation**

| Co-occurring mutation | Number of subjects |
|-----------------------|--------------------|
| APC + TP53 + PIK3CA + KRAS + MLH1 | 6 |
| APC + TP53 + PIK3CA + KRAS | 8 |
| APC + TP53 + PIK3CA + MLH1 | 4 |
| APC + TP53 + PIK3CA | 4 |
| **Total** | **22** |

**APC mutation**

Gene mutation occurred in 100% subjects with 17 SNVs (16 missense and 1 nonsense). Mutation cluster region (MCR) were located in exon 14-17. Median of SNV frequency was 4 (range 1-10). Most frequently occurred SNV was Q879* (Table 5).

**Table 5 APC Mutation**

| Nonsense mutation SNV (n=16) | Missense mutation SNV (n=1) |
|-----------------------------|-----------------------------|
| Nucleotide change | Codon | Number (n=22) | Nucleotide change | Codon | Number (n=22) |
| C>T | Q879* | 10 | C>T | T1493M | 1 |
| Q1123* | 8 |
| R876* | 6 |
| R1114* | 6 |
| Q1367* | 6 |
| Q1517* | 5 |
| Q1095* | 4 |
| Q1303* | 4 |
| Q1096* | 4 |
| Q1378* | 4 |
| Q1291* | 2 |
| Q1294* | 2 |
| Q1429* | 2 |
| Q1444* | 1 |
| R1450* | 1 |
| Q1469* | 1 |

**KRAS mutation**

In this study, KRAS mutation occurred in 14 of 22 patients (63,6%). Nine SNVs were identified in 3 types of mutations i.e. missense, nonsense, and silent. Nonsense mutation cause termination of codon 22, missenses occurred in 6 codons, and silent in 2 codons. Most frequently occurred SNVs are T20= in 4 subjects, A146T and P34L in 3 subjects (Table 6).

**Table 6 KRAS mutation**
### TP53 mutation

TP53 mutation also occurred in 100% subjects in with 65 SNVs categorized into 5 types of mutations i.e. (1) missense, (2) nonsense; (3) frameshift; (4) silent; (5) splice-site. In missense mutation, 2 most frequent SNVs are M237I and C238Y. In nonsense mutation, 2 most frequent SNVs are R342* and R213* (6 of 22 patients) (Table 7-8).

#### Table 7 TP53 mutation

| Nucleotide change | Codon | Number (n=22) | Nucleotide change | Codon | Number (n=22) |
|-------------------|-------|---------------|-------------------|-------|---------------|
| G>A               | M237I | 8             | A>G               | M237V | 2             |
| C>T               | C238Y | 7             | H142R             | H214R | 2             |
|                   | R248Q | 6             | K132E             | K132E | 1             |
|                   | C277Y | 6             | Q192R             | Q192R | 1             |
|                   | G245S | 6             | N235D             | N235D | 1             |
|                   | G245D | 5             | Y236C             | Y236C | 1             |
|                   | G244D | 4             | C>T               | S127F | 6             |
|                   | V197M | 4             | R248W             | R248W | 6             |
|                   | R213Q | 4             | R282W             | R282W | 5             |
|                   | R175H | 4             | T256I             | T256I | 4             |
|                   | E258K | 4             | A138V             | A138V | 4             |
|                   | R273H | 4             | P152L             | P152L | 4             |
|                   | R196Q | 3             | L194F             | L194F | 4             |
|                   | C135Y | 3             | P250L             | P250L | 3             |
|                   | G154S | 3             | R273C             | R273C | 3             |
|                   | R280K | 3             | P152S             | P152S | 2             |
|                   | R267Q | 2             | T155L             | T155L | 2             |
|                   | E285K | 2             | P278L             | P278L | 2             |
|                   | E286K | 1             | R175C             | R175C | 1             |
|                   | R290H | 1             | G>C               | V272L | 1             |
|                   | C275Y | 1             | T>C               | L755S | 1             |
|                   | G266E | 1             | F134L             | F134L | 1             |
|                   | R249K | 1             | C238R             | C238R | 1             |
|                   | R156H | 1             | L252P             | L252P | 1             |
|                   | R158H | 1             |                   |       |               |

#### Table 8 TP53 mutation (cont.)
Nonsense mutation
SNV (n=6)

| Nucleotide change | Codon | Number (n=22) | Nucleotide change | Codon | Number (n=22) |
|-------------------|-------|---------------|-------------------|-------|---------------|
| C>T               | R342* | 6             | G>A               | M1?   | 4             |
| R213*             | 6     |               | c.919+1G>A        | p.?   | 4             |
| R196*             | 5     |               | c.673-1G>A        | p.?   | 3             |
| Q136*             | 4     |               | c.560-1G>A        | p.?   | 3             |
| Q165*             | 1     |               | c.994-1G>A        | p.?   | 2             |
| G>A               | W91*  | 2             | c.559+2T>C        | p.?   | 1             |

Frameshift mutation
SNV (n=1)

| Nucleotide change | Codon | Number (n=22) | Nucleotide change | Codon | Number (n=22) |
|-------------------|-------|---------------|-------------------|-------|---------------|
| G>A               | M1?   | 4             | c.919+1G>A        | p.?   | 4             |

Splice-site mutation
SNV (n=6)

| Nucleotide change | Codon | Number (n=22) | Nucleotide change | Codon | Number (n=22) |
|-------------------|-------|---------------|-------------------|-------|---------------|
| c.919+1G>A        | p.?   | 4             | c.673-1G>A        | p.?   | 3             |
| c.560-1G>A        | p.?   | 3             | c.994-1G>A        | p.?   | 2             |
| c.559+2T>C        | p.?   | 1             |

Mutasi silent
SNV (n=3)

| Nucleotide change | Codon | Number (n=22) | Nucleotide change | Codon | Number (n=22) |
|-------------------|-------|---------------|-------------------|-------|---------------|
| G>A               | M1?   | 4             | c.919+1G>A        | p.?   | 4             |

**PIK3CA mutation**

Mutation of PIK3CA occurred in exon 2, 5, 7, 8, 10, 19, and 21. In this study PIK3CA missense mutation were identified in all subjects. Median of SNV frequency was 4 (range 1-16). Most frequently occurred SNV was G914R (Table 9).

**MLH1 Mutation**

MLH1 mutation occurred in 10 of 22 (45,45%) subjects. Nonsense mutation occurred in exon 9-13 causing termination in 4 codons. Most frequently occurred SNV was Q391* (Table 10).
Biological behavior of malignancy with co-occurring mutation (Table 6)

Co-occurring mutation of APC, TP53, PIK3CA, and KRAS were identified in 8 patients with average age of 48.5 years old, with locally-advanced stage (n=5), located in rectum (n=6), well-differentiated (n=6), and positive lymphovascular invasion (n=5).

Co-occurring mutation of APC, TP53, PIK3CA, and MLH1 were identified in 4 patients, with average age of 52.3 years old, with locally advanced stage (n=3), located in rectum (n=3), without lymphovascular invasion.

Quintuple mutation were identified in 6 patients, dominated by older age, locally-advanced stage, well-differentiated, positive lymphovascular invasion, and located in rectum or left colon.

Table 11. Clinical Manifestation of each combination of co-occurring mutations

| Combination | Age (mean) (range) | Stage | Lymphovascular invasion | Tumor location | Grade | Mortality |
|-------------|--------------------|-------|-------------------------|----------------|------|-----------|
| APC + TP53 + PIK3CA + KRAS (Cluster 1) | Mean 48,516 (27-75) | 50 y.o. | Early | Group 1 | Well | Yes | 4 |
| | | | | | | No | 3 |
| | | | | | | | 0 |
| APC + TP53 + PIK3CA + MLH1 (Cluster 2) | Mean 52,319 (27-67) | 50 y.o. | Locally-advanced | Group 2 | Moderate | Yes | 5 |
| | | | | | | No | 3 |
| | | | | | | | 0 |
| APC + TP53 + PIK3CA + KRAS + MLH1 (Cluster 3) | Mean 58,713 (40-74) | 50 y.o. | Advanced | Group 3 | Poor | Yes | 6 |
| | | | | | | No | 3 |
| | | | | | | | 3 |
| APC + TP53 + PIK3CA (Cluster 4) | Mean 56,322 (39-87) | 50 y.o. | Early | Group 1 | Well | No | 4 |
| | | | | | | | 2 |
| | | | | | | | 0 |

Survival

Patients with co-occurring mutation of APC, TP53, PIK3CA, and MLH1 (cluster 3) had the longest median life expectancy (1197 days) compared to cluster 2 with shortest median life expectancy (577 days) (Table 4, Figure 1).

Fifty percent of subjects of cluster 1 and 2 were deceased in less than 6 months after therapy, in cluster 4, 50% of subjects were deceased before month 15. All patients of cluster 3 can survive up to 30 months after therapy and only 1 patient deceased afterwards. Cluster 2 and 4 shows highest mortality rate with highest number of deceased patients in shortest period compared to other clusters (Figure 2).

Other findings
Early recurrence (<5 years) occurred in 2 patients of cluster 4, of which 1 patient underwent neoadjuvant chemoradiation and adjuvant chemotherapy (MFOLFOX6) and another was given XELOX after surgery. Both patients have disease-free interval of 15 months.

One patient was given anti-EGFR therapy (cetuximab) + MFOLFOX6. Patient's PCR result for KRAS was wild-type. There is no data of therapeutic response due to patient’s death during midcycle (127 days after surgery). This patient was included in cluster 2 (with KRAS mutation) and also had EGFR mutation (rs121913467).

One patient was given anti-VEGF therapy (bevacizumab) + MFOLFOX6 after diagnosed with local recurrence after 1-year of oral capecitabine. This patient was included in cluster 3 with noted BRAF mutation as well (rs121913353). Patients was known to have complete response to bevacizumab.

Two of 22 patients had family history of malignancy (Table 1). In patient with family history of colon cancer, there was 1 germline mutation identified in STK11, meanwhile in patient with family history of breast cancer, 2 germline TP53 mutations were identified.

**Discussion**

Colorectal cancer (CRC) patients in Indonesia are dominated by male (59%), more than 50 years old (59%), with well-differentiated (59%), stage 3B (40.9%), located in rectum (68%). Recently, incidence of CRC in young adults increased by 1.4% per year influenced by obesity and sedentary life style. High percentage of locally-advanced stage on hospital admission can be caused by low educational level about CRC risk factors and importance of screening especially in individuals with family history of malignancy. Intricate system of national health insurance also has role in slacking patients with unspecific complaints to see doctors before having clear disorder and getting worse. These are several of many reasons that cause delay in diagnosis and management of CRC.

The heterogeneous and dynamic nature of the CRC are related with its overlapping pathways of carcinogenesis. There are 4 principles of neoplasia in CRC, (1) colorectal tumors arise due to the activation of proto-oncogene mutations into oncogenes and inactivation of tumor suppressor genes; (2) at least mutations in 4-5 genes are required for malignant formation; (3) accumulation of numbers is more important than sequence of mutations in determining tumor biologic behavior; (4) the mutated tumor suppressor gene continues to express the phenotype without loss of heterozygosity.

The theory of colorectal neoplasia, namely adenoma-carcinoma sequence (ACS) states that the formation of colorectal carcinoma must be preceded by the presence of an adenoma. Changes in the normal intestinal mucosal epithelium to adenoma are triggered by mutations in the tumor suppressor gene, that is APC. APC can be detected in the aberrant crypt foci (ACF), which is a precursor lesion that occurred early in the beginning of the formation of adenomatous polyps and can only appear in dysplastic lesions.

All subjects (100%) in this study had nonsynonymous mutations in APC. Only 2 patients had adenomas on colonoscopy. One of those had tubulous adenomas with mild dysplasia on colonoscopy and first-degree relative with CRC. Nonsense mutated APC was found at codons 879, 1095, 1123, which completely stopped glutamine production (Q). Meanwhile, in another patient with villous adenomas and well-differentiated adenocarcinoma, nonsense mutations were found at codons 876, 879, 1096, 1291, 1294 and 1517 that stopped the production of the amino acids glutamine (Q) and arginine (R). Mutations in APC are known to have high-penetrance that can reach 100% for FAP and CRC. In contrast to the Japanese population, whose APC mutations scattered at codons 142 – 1513, subjects in this study had APC mutations occur at codons 876 – 1517 with mutation cluster region (MCR) in exons 14 – 17.

After the normal mucosal epithelium turned into an early adenoma, KRAS mutation occurred subsequently triggering the change of early into intermediate adenoma. However, in contrast to APC, KRAS can act on nondysplastic ACF precursor lesions.
In this study, mutations in the KRAS gene occurred in 14 of 22 samples (63.6%) at 9 codons and were most commonly found in older age group, locally-advanced stage, well-differentiated/low grade, with positive lymphovascular invasion, and located at the rectum. There were differences of codon location in missense mutation between Jakarta (Indonesia) and United States population, i.e. codons 13, 14, 34, 58, 59, 146 VS 12, 13, 61, 146.22 In addition, nonsense mutations were also found at codon 22 which only occurred in 1 patient. This patient diagnosed with stage 2A (pT3N0M0) undergoing elective curative resection and was given 8 cycles of capecitabine adjuvant chemotherapy with complete response. Mutation located in codon 12 has more aggressive behavior than codon 13, because patients were commonly presented in advanced stage.22 Nevertheless, number of cases with metastases involving KRAS mutation in this study was found in 3 of 5 samples without involvement of codon 12.

KRAS mutation can occur concomitantly with APC mutation leading to increased accumulation of b-catenin in the cytoplasm, by destroying its binding to E-cadherin, which has actually been increased due to loss of mutated APC degradation function. This causes the Wnt signal to become more active so that motility and cell invasion are more aggressive.15,18,21,23–26 In CRC, the combination of APC and KRAS mutations (co-occurring mutations) can occur up to 80%, whereas in this study only occurred in 63.6% of subjects.27

In this study, patients with APC, TP53 and KRAS mutations were predominantly 50 years old, with locally-advanced stage and positive lymphovascular invasion. Two shortest median life expectancy were found in patients with KRAS mutation (Figure 1), in addition 50% of patients died within 6 months after therapy (Figure 2).

Before turning into carcinoma, intermediate adenomas differentiate into late adenomas triggered by mutations in the SMAD4, CDC4, and DCC genes.2,7 In this study, we found SMAD4 nonsense and missense mutations in 18 of 22 patients (82%).

In ACS theory, late adenomas who developed into carcinomas have mutations in TP53, TGFBR2, BAX, and IGF2R. Mutated TP53 was found in all subjects in this study in the form of nonsense, missense, frameshift, splice-site, and silent mutation. Five most frequently occurred codon locations in this study were 237, 238, 127, G245S, and R248Q. Those are different compared to world database in The Cancer Genome Atlas Program (TCGA) portal which stated that the five codon positions with the highest frequency were 175, 282, 248, R273H, and R273C.28

In contrast to the UK population, in 64% (14 out of 22) subjects, TP53 and KRAS mutations co-occurred.18,21 In Indian population, these two combinations were only found in 13 of 112 cases, whereas the study by Timar can occur in up to ~40%,27,29 TP53 and KRAS activate different carcinogenesis pathways so that they rarely coexist.30

Similar to APC and TP53, PIK3CA mutations were found in all subjects (100%) with 9 SNVs. PIK3CA has no role in the aggressive behavior of CRC.31,32 Even so, when it occurs concurrently with KRAS mutations, it will show evident aggressive behavior, especially when occurs in exons 9 and 20. While in this study mutations occurred in exons 2, 3, and 4, aggressive behavior presenting as locally-advanced stage and positive lymphovascular invasion can be found.

Mutations in MLH1 can also occur in non-hereditary/sporadic CRC. The existence of microsatellite instability due to mutations in genes that play roles in the MMR system such as MLH1 actually provides a good prognosis with a higher survival rate.33 In this study, the group of cases with MLH1 mutations alone, had the highest median life expectancy and had a 30-month survival rate of up to 100%.

Referring to the colorectal neoplasia principle mentioned above, all subjects in this study indeed involved activation of oncogenes (PIK3CA and KRAS) and inactivation of tumor suppressor genes (APC, TP53 and MLH1) and also involved a range of 8-19 mutated genes per person. In this study, mutated APC and KRAS, which supposed to be occurred on early sequence of ACS, supports what Fearon stated about unimportance of mutational sequence in determining tumor biologic behavior.1,2

We are strongly aware of our study's limitation. Limited number of samples is the lack of this study. Further research is truly required to accomplish complete mapping of Indonesian profile, especially in investigating our unique findings in each of genes described.
Nevertheless, this is the first study fully describes the profile of pathogenic mutations of CRC in Indonesian population with its unique characteristics, compiled of various ethnicities with diverse diet and lifestyle which may have roles in contributing natures of Indonesian version of CRC presenting in locally-advanced stage with large tumor size and moderate-severe malnutrition status. This study is also the first in the world to examine the co-occurring mutations of APC, TP53, PIK3CA, KRAS, and MLH1.

**Conclusions**

1. Different profile of pathogenic mutation in colorectal cancer patients is found in Indonesia’s population
2. Mutated APC, TP53, and PIK3CA occurred in 100% subjects, while KRAS and MLH1 occurred in 63.6% and 45.4% subjects
3. The longest median life expectancy occurred in the group of patients with mutations APC, TP53, PIK3CA, and MLH1 with a 30-month postoperative survival of 100%.
4. The shortest median life expectancy occurred in the group of patients with APC, TP53, PIK3CA, and KRAS mutations with a 50% life expectancy <6 months post-treatment.

**Abbreviations**

ACF : aberrant crypt foci
ACMG : American College of Medical Genetics
ACS : adenoma-carcinoma sequence
AJCC : American Joint Committee on Cancer
APC : Adenomatous Polyposis Coli
BAX : Bcl-2 Associated X protein
CDC4 : Cell division control protein 4
CRC : colorectal cancer
DCC : deleted in colorectal cancer
DNA : deoxyribonucleic acid
EGFR : Epidermal Growth Factor Receptor
FAP : Familial Adenomatous Polyposis
FATHMM : Functional Analysis through Hidden Markov Models
FFPE : Formalin-fixed Paraffin-Embedded
GRCh38 : Genome Reference Consortium Human Reference 38
IGF2R : Insulin Like Growth Factor 2 Receptor
KRAS : Kirsten rat sarcoma viral oncogene
MFOLFOX6 : modified Folinic Acid + 5-Fluorouracil + Oxaliplatin
MLH1 : MutL Homolog 1
Declarations

Ethical Approval

The Ethics Committee of the Faculty of Medicine, Universitas Indonesia – RSUPN Cipto Mangunkusumo with regards of the Protection of human rights and welfare in medical research, has carefully reviewed the research with registry number: KET-445/UN2.F1/ETIK/PPM.00.02/2021. All procedures of Ethical Approval are performed in accordance with ICH-GCP standard procedure.

Declarations

Authors declare no conflict of interest. Provenance and peer review. Not commissioned, externally peer-reviewed.

Availability of data and materials

The datasets generated and/or analysed during the current study are not publicly available due to presence of individuals’ personal details but are available from the corresponding author on reasonable request.

Authors’ Contribution

VMGM analysed and interpreted data related to colorectal cancer, gathered information in medical records, and synchronized patients’ data with VCF data files, and was a major contributor in writing the manuscript.

LE carried out the molecular genetics study and participated in sequence alignment.

TJML participated in its design and coordination and helped to draft the manuscript.

All authors read and approved the final manuscript.

Consent for publication

Consent was not required in this study because no details on individuals reported within the manuscript. This was already reviewed by the Ethics Committee mentioned above.

Acknowledgment
Not applicable

**Competing Interest**

Not applicable

**Funding**

No funding was obtained in this study.

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Figure 1

Median life expectancy in days
Figure 2

Survival rate based on co-occurring mutation