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Identification of KRAS Mutations in Colorectal Carcinoma Patients at Dr. M. Djamil Hospital, West Sumatra-Indonesia

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Abstract: Kirsten rat sarcoma viral oncogene (KRAS) gene mutations lead to abnormal activation of the RAS signalling pathway and have been associated with poor prognosis and resistance to some therapeutics. This study aimed to identify mutation characteristics of the KRAS genes codon 12 and 13 in colorectal cancer patients in West Sumatra. KRAS mutations were analyzed in 20 DNA of colorectal cancer patients' tissue samples by using polymerase chain reaction (PCR) with specific primer and direct sequencing analysis. Our findings showed five samples (25%) with mutated KRAS at codons 12 and 13 (including three samples with GGT→GAT, one sample with GGT→GTT in codon 12, and one sample with GGC→GAC in codon 13). In conclusion, we found two variations of amino acid changes at codon 12 (G12D and G12V) and one at codon 13 (G13D). More research with many samples is required to obtain conclusive data on the relationship between these gene mutations and colorectal cancer response to therapy and prognosis.
Keywords: Colorectal cancer; kirsten rat sarcoma viral oncogene; mutation; polymerase chain reaction-sequencing.

INTRODUCTION

Colorectal cancer (CRC) is the third most frequent type of cancer in men and the second most common in women. It is also a primary cause of cancer-related death. Over 1.8 million new cases were diagnosed in 2018. CRC is the third most frequent type of cancer in Indonesia, following breast and cervical cancer, with age-standardized rates of 19 men and 15 women per 100,000 (Abdullah et al., 2012; Pangribowo, 2019). From 2002 to 2007, Dr. M. Djamil Hospital identified 257 colorectal cancer cases (Zahari & Sudiyatmo, 2011). In 2017, 110 people were hospitalized after a colorectal cancer diagnosis (Irfan, 2019).

The epidermal growth factor receptor (EGFR) is the molecular target for colorectal cancer treatment using anti-EGFR monoclonal antibodies. Anti-EGFR antibody treatment did not affect patients with Kirsten rat sarcoma viral oncogene (KRAS) gene mutations. KRAS is a member of the RAS family of oncogenes. KRAS mutations are a frequent occurrence in CRC. KRAS mutations lead to abnormal activation of the RAS/ RAF/MEK/ERK signalling pathway (Meng et al., 2021). A previous study has demonstrated that KRAS mutations are involved in destroying cell polarity and inhibiting apoptosis (Tsunoda et al., 2010). Charitou T et al. analyzed 4

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CRC cell lines that showed that protein synthesis and cell proliferation significantly increased in the mutated cells (Charitou et al., 2019). Around 85–90% of KRAS mutations in exon 2 (codons 12 and 13) are associated with colorectal cancer. The most prevalent KRAS mutations were G12D, G12V, and G13D (Li et al., 2019). Patients with mutant KRAS gene as a predictive negative response from anti-EGFR therapy and has a worse prognosis than those with wild-type KRAS (Van Cutsem et al., 2011).

Studies involving the relationship of KRAS mutations with CRC in some regions of Indonesia have been reported. In Bali, males were more likely to develop colorectal cancer, and the risk increased with age. KRAS mutations in exon two have been identified, with G13D being the most common, followed by G12D and G12V (Ni Nyoman et al., 2022). In West Java people, KRAS and p53 mutations are involved in carcinogenesis and have a significant association between KRAS gene expression and p53 immuno-expressions in colorectal adenocarcinoma KRAS (Rachmawati et al., 2019). Mastutik G et al. reported that the mutation KRAS gene codon 12 from adenocarcinoma patients in the Dr Soetomo Hospital obtained 33% (7/21), and there was no mutation at codon 13 (Mastutik et al., 2016). The effect of KRAS mutations on the prognosis and survival of patients with colorectal cancer (CRC) stimulates various research initiatives to develop novel medicines or targeted therapies for patients with KRAS mutant CRC. More research is needed that reveals the relationship between KRAS gene mutations and colorectal cancer response to therapy and prognosis, especially KRAS gene mutations at codons 12 and 13. This study aims to determine the status of KRAS gene mutations in codons 12 and 13 in patients with colorectal cancer at the Dr M. Djamil Hospital.

MATERIALS AND METHODS

Tissue Samples

We collected 20 tissue samples from surgical resection patients at the Dr M. Djamil Hospital in West Sumatra, Indonesia 2010. The collection of tissues was conducted prospectively and was necessary for individuals to participate in the study. The proximal tumour comprised the cecum, ascending colon, and transverse colon, while the distal site involved the splenic flexure, descending colon, and sigmoid colon. The study received approval from the Research Ethics Commission (KEP) of the Faculty of Medicine, University of Andalas.

Analysis of KRAS Mutations

DNA extraction

The Wizard Genomic DNA Purification Kit (catalogue no. A1120, Promega, USA) was used to extract genomic DNA from 30 mg of tissue, which was processed according to the manufacturer's instructions. Spectrophotometry was used to evaluate the concentration and purity of the DNA.

KRAS PCR Amplification

Exon, two of the KRAS gene, was amplified using the following primers (Macrogen, Korea): forward, 5'-ACTGGATATAAACTTGTGGTAGTTGGACCT-3' and reverse primer 5'- TAATATGTCGACTAAAACAAGATTTACCTC- 3' (Miyakura et al., 2002). Amplification was carried out in a total volume of 25 µL containing 3 µl DNA genomic, 12,5 µl Go Tag Green Master Mix (Promega, USA), 1 µl for each forward and reverse primer either KRAS gene (10 µM) and 9,5 µl ddH2O Water PCR Grade. PCR (T100 Thermal Cycler- BioRad) program or KRAS was carried out at 96°C for 5 min and followed by 40 cycles of denaturation at 96°C for 30s, annealing at 55°C for 60 s and extension at 72°C for 30s and a final elongation step at 72°C for 5 min. The

length of the amplicon for KRAS was 135 bp. The PCR product was applied into 2% gel agarose dissolved in 1X TBE buffer. Electrophoresis was performed at 60 volts for 1.5 hours.

KRAS Direct Sequencing

Direct sequencing was used to identify KRAS mutations in exon two. The purified PCR products were sequenced with the same two primers used for KRAS PCR amplification. The BigDye Terminator Cycle Sequencing Kit v.3.1 was used for the sequencing (Applied Biosystems, Foster City, CA). Macrogen, Inc. (Seoul, Korea) resolved the sequencing products using an Applied Biosystems model 3730XL Automated DNA Sequencing System.

RESULTS AND DISCUSSION

The 20 samples were seven males (35%) and thirteen females (65%), with a mean age of 52 years (ranging from 30 to 82 years). The tumour was found in the cecum colon, descending colon, sigmoid colon, and rectum (Table 1). Previous research in Indonesia found that the incidence of colorectal cancer was higher in people over 50 (Indrayani M & Sriwidyani, 2017; Ni Nyoman et al., 2022; Saleh et al., 2019). Molenaar et al. reported that 90% of CRC occurs at ages above 50. It may be due to the accumulation of DNA mutations in colonic wall cells, as well as a deterioration of the body's immune system, as evidenced by decreased immunoglobulin production, lymphocyte configuration and responses in the fight against infection, and a decreased ability of the body's immune system to recognize foreign objects that enter the body (Molenaar et al., 2017). The rectum had the highest frequency of colorectal carcinoma, with ten samples (50 %). This is linked to a more rectal function of stool and defecation, where food is one of the environmental factors that can cause cancer. Consuming foods high in pure carbohydrates may increase the risk of developing colorectal cancer and cause changes in faecal flora, bile salt degradation, and protein and fat breakdown, which are carcinogenic. A low-fibre diet also causes faeces to be concentrated and stool transit to be increased. Furthermore, carcinogenic substances have a longer time to get into the colon mucosa and rectum (Nistal et al., 2015).

Table 1. Subject Characteristics

Subject characteristics	n=20	(%)
Age (years)		
≤40	4	20
41-49	5	25
≥ 50	11	55
Sex		
Male	7	35
Female	13	65
Tumour Location		
Colon	10	50
Male	4	20
Female	6	30
Rectum	10	50
Male	3	15
Female	7	15

This study used direct sequencing to analyze KRAS exon two codons 12 and 13 mutations. Direct sequencing results show that 20% (4/20) harboured mutations at

codon 12, and 5% (1/20) had mutations at codon 13. There were 3 of 20 (15.0%) samples with mutations from GGT (Gly) to GAT (Asp) (G→A; G12D) (Figure 1b) and 1 of 20 (5%) samples with mutations from GGT (Gly) to GTT (Val) (G→T; G12V) at codon 12 (Figure 1c). Meanwhile, there are 1 of 20 (5%) samples with heterozygous mutations from GGC (Gly) to GAC (Asp) (G→A; G13D) at codon 13 (Figure 1c**.**

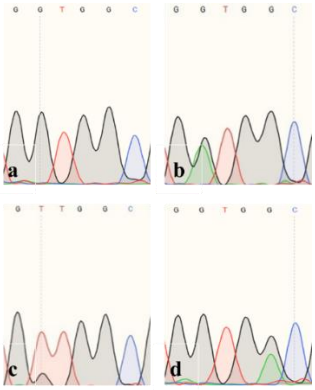

Figure 1. Sanger sequencing electropherogram of KRAS *gene* at exon two codon 12 and 13 (GGT and GGC). The electropherograms display wild-type KRAS codon 12 (GGT), codon 13 (GGC) (a) and mutations of *KRAS* G12D (G→A) (a), KRAS G12V (G→T) (b), and KRAS G13D (G→A) (c).

The distribution of codon 12 and 13 polymorphism genotype and allele type mutations in the KRAS gene in 20 CRC patients is shown in Table 2. We detected Ga (7.5%) and Gt (2.5%) heterozygous mutant genotypes in codon 12 and Ga (2.5%) in codon 13. Saleh M et al. found that of 30 CRC patients, 15 (50%) were polymorphic for KRAS codon 12, 12 (40%) were heterozygous mutant genotype (Gg), and 3 (10%) were homozygous mutant genotype (Saleh et al., 2019). We found no homozygous mutant genotype (gg) in codon 12 or 13.

Table 2. KRAS Codon 12 and 13 Polymorphism Genotype and Allele Type Distribution

Characteristic	N	%
Codon 12		
Genotype		
Wild type (GG)	16	80
Heterozygous mutant (Ga)	3	15
Heterozygous mutant (Gt)	1	5
Homozygous mutant (gg)	-	-
Allotype		
Wild type (G)	36	90
Polymorphic (a)	3	7.5
Polymorphic (t)	1	2.5
Codon 13		
Genotype		
Wild type (G.G.)	19	95
Heterozygous mutant (Ga)	1	5
Homozygous mutant (gg)	-	-
Allotype		
Wild type (G)	39	97.5
Polymorphic (a)	1	2.5

This study found a polymorphic KRAS codon 12 genotype frequency in the lower 40 years (10%) and more than 50 years (10 %). In addition, codon 13 has been present for more than 50 years. Females have a higher frequency of polymorphic KRAS codons 12 and 13; the location was in the colon's rectum (Table 3). Roa. I et al. reported that KRAS mutations are identified in 46 (42.2%) of 106 CRC cases. The most common point mutations are found at codons 12 (80.4 %), G12D (39.1 %), G12V (24.2 %), and 19.6 % at codon 13, the G13D. Patients with G12D and G12V mutations have a worse prognosis (Roa et al., 2013).

Table 3. Associations of Age, Sex and Tumour Location with the Mutational Status of KRAS in CRC

Characteristics	KRAS codon 12				KRAS codon 13			
	Wild Type		Polymorphic		Wild Type		Polymorphic	
	n	%	n	%	n	%	n	%
Age (years)								
≤40	2	10	2	10	2	10	-	-
41-49	5	25	-	-	5	25	-	-
≥ 50	9	45	2	10	8	40	1	5
Sex								
Male	6	30	1	5	7	35	-	-
Female	10	50	3	15	12	60	1	5
Tumour Location								
Colon	10	50	-		9	45	1	5
Rectum	6	30	4	20	10	50	-	-

Some studies in Indonesia showed the prevalence of KRAS mutation was 22.2% in 27 cases (two samples mutation in KRAS G12D, one sample mutation in KRAS G12V, and three samples mutation in KRAS G13D) (Ni Nyoman et al., 2022),12,3% (G12D) and 4.9% (G13D) in 121 cases (Levi et al., 2017), 56,65 % (9 samples mutation in KRAS codon 12, 4 samples mutation in KRAS codon 13) in 23 cases (Indrayani M & Sriwidyani, 2017), 30% (7 samples mutation in KRAS codon 12) in 21 cases (Mastutik et al., 2016) and 50% (15 samples mutation in KRAS codon 12) in 30 cases. Jones et al. also reported that mutations in codon 12 were found at the most, as many as 34.6% of cases (Jones et al., 2017). Some studies have suggested that codon 12 mutations are associated with poor overall survival and progression-free survival, particularly for the mutations G12D and G12V. No study has been reported between codon 13 mutations and prognosis (Li et al., 2019). The meta-analysis included 275 studies with 77,104 mCRC patients and discovered that females had significantly more KRAS mutations than males. Patients with KRAS mutations had significantly lower overall survival than those with W.T. tumours. Patients with KRAS mutations had significantly lower progression-free survival (Levin-Sparenberg et al., 2020). Geographic, environmental, lifestyle, and ethnic factors all influence the distribution of the KRAS polymorphism, which may influence the epigenetic regulation of the KRAS oncogene. A low-folate, high-fat diet, smoking, and alcohol consumption may all be linked to KRAS oncogene activation in the development of CRC (Ahmad Kendong et al., 2021; Baskin et al., 2014).

Studies involving the relationship of KRAS mutations with CRC in some regions of Indonesia have been reported. In Bali, males were more likely to develop colorectal cancer, and the risk increased with age. KRAS mutations in exon two have been identified, with G13D being the most common, followed by G12D and G12V (Ni

Nyoman et al., 2022). In West Java people, KRAS and p53 mutations are involved in carcinogenesis. The limitation of this study is that the sample collection was small, and Our findings show limited data on the prevalence and characteristics of KRAS mutation in colorectal cancer patients in West Sumatra. In the future, the study needs a larger sample size.

CONCLUSION

This study concludes that the KRAS gene mutation from surgical resection of the colon and rectum adenocarcinoma patients at the Dr M. Djamil Hospital, West Sumatra, obtained 20% (4/20) harboured mutations at codon 12 (G12D and G12V), and 5% (1/20) had mutations at codon 13 (G13D). The results of our study confirm the significance of performing analyses to identify KRAS mutations, specifically in codons 12 and 13 in cases of colon cancer. These findings offer valuable prognostic information.

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CONFLICT OF INTEREST

All authors have no conflict of interest.

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