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Original Article

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High Probability of Lynch Syndrome Among Colorectal Cancer Patients Is Associated With Higher Occurrence of KRAS and PIK3CA Mutations

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Abstract

Background: In Indonesia, early-onset colorectal cancer (EOCRC) rates are higher in patients < 50 years old compared to Western populations, possibly due to a higher frequency of Lynch syndrome (LS) in CRC patients. We aimed to examine the association of KRAS and PIK3CA mutations with LS.

Methods: In this retrospective cross-sectional single-center study, the PCR-HRM-based test was used for screening of microsatellite instability (MSI) mononucleotide markers (BAT25, BAT26, BCAT25, MYB, EWSR1), MLH1 promoter methylation, and oncogene mutations of BRAF (V600E), KRAS (exon 2 and 3), and PIK3CA (exon 9 and 20) in FFPE DNA samples.

Results: All the samples (n = 244) were from Dr. Sardjito General Hospital Yogyakarta, Indonesia. KRAS and PIK3CA mutations were found in 151/244 (61.88%) and 107/244 (43.85%) of samples, respectively. KRAS and PIK3CA mutations were significantly associated with MSI status in 32/42 (76.19%) and 25/42 (59.52%) of samples, respectively. KRAS mutation was significantly associated with LS status in 26/32 (81.25%) of samples. The PIK3CA mutation was present in a higher proportion in LS samples of 19/32 (59.38%), but not statistically significant. Clinicopathology showed that KRAS mutation was significantly associated with right-sided CRC and higher histology grade in 39/151 (25.83%) and 24/151 (16.44%) samples, respectively. PIK3CA mutation was significantly associated with female sex and lower levels of tumor-infiltrating lymphocytes in 62/107 (57.94%) and 26/107 (30.23%) samples, respectively. KRAS and PIK3CA mutations did not significantly affect overall survival (120 months) in LS and non-LS patients.

Conclusions: The high probability of LS in Indonesian CRC patients is associated with KRAS and PIK3CA mutations.

Keywords: Colorectal neoplasms; Pathology; Molecular; Medical oncology; Gastrointestinal neoplasms; Neoplastic syndromes; Hereditary

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Introduction

Colorectal cancer (CRC) is the third most prevalent cancer worldwide and as well as one of the deadliest. Approximately in Indonesia, over 35,000 patients are diagnosed with CRC each year [1]. Three provinces in Indonesia have the highest incidence of CRC: Jakarta, Central Java, and Yogyakarta. Early-onset colorectal cancer (EOCRC) accounts for nearly 30% of total CRC patients, three times higher than in Europe, the UK, and the USA [2]. The epidemiological data in Indonesia showed that the proportion of CRC patients < 40 years old was more than 30% [3]. This incidence in Indonesia was higher

in males (54%) than in females (46%), with a peak age of 50 - 54 years [2]. Lynch syndrome (LS), also known as hereditary non-polyposis colon cancer (HNPCC) is a hereditary type of CRC. This syndrome is characterized by early-onset (< 50 years) [4, 5]. Our previous study introduced the higher frequency of LS cases in Yogyakarta, Indonesia linked to a high risk of EOCRC [6].

Three molecular pathways have been identified for the pathogenesis of CRC: chromosomal instability (CIN), microsatellite instability (MSI), and CpG island methylator phenotype (CIMP) [7, 8]. The CIN involves gene mutations in APC, KRAS, SMAD4, and TP53, while MSI is caused by mutations in mismatch repair (MMR) genes such as MLH1, MSH2, MSH6, and PMS2 [9]. The CIMP, which is characterized by CG dinucleotide methylation in the promoters of numerous genes, is associated with distinct clinical and pathological attributes in tumors. These subtypes, as described by Jass classification, include CIMP high/MSI high (12% of CRC), CIMP low/MSI low or microsatellite stable (20%), CIMP negative/microsatellite stable (57%), and HNPCC, with CIMP negative/MSI high and negative for BRAF mutations [7, 8, 10-12]. Testing for MSI and MMR protein deficiency (dMMR) is commonly the first step in LS diagnostics due to germline mutations of MMR genes. On the contrary, epigenetic silencing of the MLH1 and somatic mutation of BRAF are common in sporadic tumors with MSI but very rarely occur in tumors arising in LS [13, 14]. Approximately 10-15% of sporadic CRCs will also show dMMR/MSI due to somatic loss of MMR function [15]. Epigenetic silencing of the MLH1 gene is the most common cause of dMMR in sporadic tumors and very rarely occurs in LS. Thus, sporadic tumors with dMMR/MSI can be distinguished from tumors arising in LS by demonstrating methylation of the MLH1 promoter [16].

KRAS mutations, the most common RAS family mutation, affect cell proliferation, differentiation, senescence, and apoptosis in 40% of sporadic CRC. These mutations increase CRC tumor aggressiveness, reduce survival rates, and promote treatment resistance [17]. Anti-epidermal growth factor receptor (EGFR) monoclonal antibodies, such as cetuximab and panitumumab, are ineffective in CRC patients with KRAS codon 12, 13, or 14 mutations. Hence, these agents are only effective in RAS wild-type tumors [18]. BRAF mediates RAS-RAF-MAP kinase growth signal responses. BRAF mutations are found in 4% of MSI-low tumors and 40% of MSI-high tumors [19]. The most frequent of these mutations are $BRAF^{V600E}$ (Val600Glu). These $BRAF^{V600E}$ mutations help to differentiate between familial and sporadic CRC and are associated with poorer prognosis. Generally, BRAF mutations are confined to tumors without KRAS exon 2 mutations. BRAF is downstream of activated KRAS in the EGFR pathway, making cetuximab or panitumumab ineffective for inhibiting EGFR, unless given BRAF inhibitor [10, 20].

Phosphatidylinositol-3-kinase (PI3K) is a heterodimeric lipid kinase involved in cell signaling and cell membrane function. Mutations in PIK3CA have been reported in 10-20% of CRC cases [21]. PIK3CA mutations are associated with worse clinical outcomes and a negative predictor of response to anti-EGFR targeted therapy [22]. It has been shown that RAS mutations are negative predictors of anti-EGFR mAb response and survival benefit [22]. Over 80% of PIK3CA mutations are found in two hotspots: the helicase domain of exon 9 and the kinase domain in exon 20. Several studies have analyzed PIK3CA mutation in these hotspots for the discrepancy of the predictive values of PIK3CA as a biomarker for anti-EGFR [22, 23]. The PI3K pathway's downstream effectors include AKT and mTOR, which increase cell cycle regulator mRNA translation [24]. The decreased expression of tumor suppressor gene PTEN, a direct antagonist, has been shown to be correlated with poor outcomes in CRC [25].

The possibility of LS can be inferred if a tumor is shown to be dMMR or shown to have MSI, but a definitive diagnosis of LS can only be made by demonstration of a germline mutation in MMR gene. KRAS mutations tend to occur in the context of CIN, which is characteristic of MSS tumors [26, 27]. Meanwhile, PIK3CA mutations have been found to be more prevalent in MSI tumors [28]. In Indonesia, there has been limited investigation into the genetic mutation profiles of CRC patients, particularly those with LS. As reported in our previous study and others, several clinical features are associated with this syndrome, including tumor location of which 60-70% were found in the right-sided (proximal) colon [6, 10]. In this study, we examine the association of oncogenic mutations of KRAS and PIK3CA with MSI, MLH1 promoter methylation and LS as well as the demography and clinicopathology profile of CRC patients in Yogyakarta, Indonesia.

Materials and Methods

Ethical statements

This study was approved by the Medical and Health Research Ethics Committee (MHREC) Faculty of Medicine, Public Health, and Nursing of Universitas Gadjah Mada, Yogyakarta (Ethical Approval Number KE/FK/0837/EC/2022). Informed consent has been obtained for the use of tumor samples, clinical data and any other relevant data in the research for all subjects.

CRC clinical samples

For this observational cross-sectional study, we performed retrospective consecutive sampling. A total of 288 formalin-fixed paraffin-embedded (FFPE) CRC samples from the primary tumors, with no data about simultaneous or metachronous metastasis, were collected from the Department of Anatomical Pathology at Dr. Sardjito General Hospital Yogyakarta, Indonesia between 2016 and 2021. The samples were limited due to the hospital being the national tertiary referral hospital, where many of the patients received treatment in our center (Dr. Sardjito General Hospital) following resection procedures that had been conducted in secondary/regional hospitals elsewhere. Of these, 244 CRC samples were eligible for mutation detection. The patient data acquired for each case included sociodemographic (age and sex), tumor pathology (location/site, staging

Table 1. KRAS and PIK3CA With MSI Mutational Status

Characteristic	MSI			
	Overall, N = 244[a]	MSI, N = 42[a]	MSS, N = 202[a]	P-value[b]
KRAS				0.038*
Mutant	151 (61.88%)	32 (76.19%)	119 (58.91%)	
Wild-type	93 (38.11%)	10 (23.81%)	83 (41.09%)	
KRAS CO BRAF				0.7
Yes	14 (5.73%)	3 (7.14%)	11 (5.45%)	
No	230 (94.26%)	39 (92.86%)	191 (94.55%)	
PIK3CA				0.027*
Mutant	107 (43.85%)	25 (59.52%)	82 (40.59%)	
Wild-type	137 (56.14%)	17 (40.48%)	120 (59.41%)	

[a]n (%). [b]Fisher's exact test. *P-value < 0.05. MSI: microsatellite instability.

analysis by TNM staging system, histologic grade, lymphovascular invasion status, morphology, tumor-infiltrating lymphocytes (TILs), and various clinical parameters (hemoglobin (Hb), Eastern Cooperative Oncology Group (ECOG) scale, and body mass index (BMI)).

DNA extraction

Paraffin blocks from CRC patients were cut into six pieces with 5 µm thickness. Only one piece in one slide continued with DNA extraction. Genomic DNA was extracted using the QIAamp DNA FFPE tissue kit (Qiagen, USA) according to the manufacturer's protocol. DNA samples were quantified using a NanoDrop™ spectrophotometer (Thermo Scientific, Waltham, MA, USA). Samples of sufficient concentration and quality were adjusted to a concentration of 20 ng/µL for PCR applications.

Detection of MSI, BRAF, MLH1 and oncogenes mutation

All CRC biomarkers were detected using an IVD kit called BioColomelt-Dx manufactured by Biofarma Ltd, Indonesia. BioColomelt-Dx is a PCR-HRM molecular diagnostics kit for screening MSI, MLH1 promoter methylation, and important oncogene mutations of KRAS (exon 2 and 3), BRAF (V600E), PIK3CA (exon 9 and 20) in FFPE DNA samples. MSI, BRAF, and MLH1 methylation promoter detections were previously described as N-LyST panel [29]. The N_LyST panel is a detection method for five mononucleotide microsatellite repeats, $BRAF^{V600E}$ mutations, and MLH1 region C promoter methylation status. For MSI analysis, samples were regarded as MSI if > 2 markers (40%) showed instability; otherwise, they were regarded as MSS tumors. Samples showing MSI, BRAF wildtype, and MLH1 promoter methylation (unmethylated) were classified as "probable Lynch". Out of the total samples, 244 were successfully determined for oncogene mutation detection, while 223 were suitable for probable Lynch determination.

Statistical analysis

Correlation between variables was calculated using two-sided Fisher's exact test; the test is considered significant if the P-value < 0.05. The overall survival analysis was conducted using a log-rank test to compare between groups and visualized with Kaplan-Meier curves. All analyses were performed using R software version 4.3.2.

Results

Association between KRAS and PIK3CA mutation with MSI status

The analysis of KRAS and PIK3CA with MSI status is shown in Table 1. There was a significant association between mutant KRAS and MSI vs. MSS (76.19% vs. 58.91%; P-value = 0.038); and mutant PIK3CA with MSI vs. MSS (59.52% vs. 40.59%; P-value = 0.027). There were 14/244 samples that had the concomitant mutation of KRAS and BRAF. There was no significant association between KRAS and BRAF concomitant mutation with MSI status (7.14% vs. 5.45%; P-value = 0.7).

Association between KRAS and PIK3CA mutation with probable Lynch status

The association of KRAS, PIK3CA and Lynch status is shown in Table 2. There was a significant association between mutant KRAS and probable Lynch status (81.25% vs. 58.64%; P-value = 0.018). However, there was no significant association between PIK3CA and probable Lynch status.

Clinicopathology association with oncogene status

As shown in Table 3, PIK3CA gene mutation frequency was higher in female patients compared to the male patients

Table 2. KRAS, PIK3CA and Probable Lynch Status

Characteristic	Probable Lynch			
	Overall, N = 223[a]	Yes, N = 32[a]	No, N = 191[a]	P-value[b]
KRAS				0.018*
Mutant	138 (61.88%)	26 (81.25%)	112 (58.64%)	
Wild-type	85 (38.12%)	6 (18.75%)	79 (41.36%)	
PIK3CA				0.13
Mutant	103 (46.19%)	19 (59.38%)	84 (43.98%)	
Wild-type	120 (53.81%)	13 (40.63%)	107 (56.02%)	

[a]n (%). [b]Fisher's exact test. *P-value < 0.05.

(57.94% vs. 42.06%; P-value = 0.040). The lower level of TILs was found in mutant PIK3CA (30.23% vs. 16.24%; P-value = 0.021). The mutation rate of KRAS in the right sided was higher and statistically significant than the left sided (25.83% vs. 13.04%; P-value = 0.022). Mutant KRAS was significantly higher in histology grade 3 compared to wild-type KRAS (16.44% vs. 7.53%; P-value = 0.038). There was no significant association between other clinicopathology parameters with KRAS oncogene status.

Overall survival on KRAS and PIK3CA mutation stratified by LS status

We analyzed the overall survival based on KRAS and PIK3CA mutations stratified by LS status. The numbers of samples that met the criteria for this analysis on PIK3CA and KRAS mutation are 220 and 222, respectively. There were no statistically significant differences of overall survival (follow-up period of 120 month) based on KRAS and PIK3CA mutation with probable Lynch and non-Lynch status patient (Fig. 1). Additionally, we conducted an overall survival analysis considering PIK3CA and KRAS mutations stratified by MSI status (Supplementary Material 1, www.wjon.org), comparing PIK3CA and KRAS mutant versus wild-type cases (Supplementary Material 2, www.wjon.org), and differentiating probable Lynch from sporadic cases (Supplementary Material 3, www.wjon.org). None of these analyses yielded statistically significant results.

Discussion

As molecular testing for CRC is not routinely performed in clinical settings, currently there are only limited data on molecular landscape of CRC among the Indonesian patients. We previously reported higher incidence of EOCRC and probable Lynch in our patient cohort from Yogyakarta, Indonesia [6]. In this study, we sought to examine the association of oncogenic mutations of KRAS and PIK3CA, LS status and the clinicopathological features. As a brief description, Indonesia has a total population of approximately 238 million, with 131.48 million females compared to 132.68 million males [30, 31]. The country has a predominantly young age population, particularly from 20 to 40 years old [32]. The agricultural sector plays a significant role in socioeconomic development [33]. Yogyakarta is one of the most populated provinces in Indonesia. As of 2023, the total population accounted for 3.7 million people, mainly between 20 and 24 years of age [34]. The sex ratio between male and female population is 0.98. Yogyakarta is the region with the highest incidence of cancer in Indonesia, hence, highlighting the importance of this study [35].

The frequency of KRAS mutations (exon 2 and exon 3) in our cohort reached 61.88%. This was much higher than what was reported in the Asia-Pacific (37-52%) and Western populations (32-49%) [36-49]. Nevertheless, our data are in line with a previous study in Indonesia, showing that KRAS mutation was found in 71.8% of serrated adenocarcinoma (SA), which is intriguingly higher than the generally reported incidence of 40% [50-52]. Another study, based on next-generation sequencing (NGS) analysis, showed that KRAS mutation occurred in 63.6% of 22 Indonesian patients with mostly advanced CRC [53]. Distinct mutations located in codons 13, 14, 34, 58, 59, and 146 were found as opposed to more commonly reported mutations in codons 12, 13, 61, 146 [53-55]. A recent study in China has also reported that KRAS mutation was found in 69.4% of the early lesions [56, 57].

Furthermore, in this study, we found that the KRAS mutation was enriched in MSI compared to MSS cases (76.19% and 58.91%, respectively). We previously reported a lower frequency of BRAF mutations (20.45%) in this MSI-CRC cohort [6], similar to what has been reported in China [58-60]. This is unlike a common dogma in which KRAS mutation is more associated with MSS while BRAF mutation is associated with MSI [61-63]. The frequency of KRAS mutations in MSI-H CRCs has been reported to be approximately 12-38% [64-68]. This conflicting finding is likely due to the variability in the frequency of specific mutations in KRAS codons 12 and 13. A study by Asaka et al (2009) investigated KRAS mutations in different MSI status (MSI-H, MSI-L, and MSS) and reported that 93% G to A KRAS mutation occurred in MSI-H tumors compared to MSI-L and MSS [69]. Although we did not specifically examine this hotspot mutation, this phenomenon likely explains our findings.

We also observed the concomitant KRAS and BRAF mutation on 5.73% of total CRC cases. Despite previously thought as a rare event, there are a growing number of studies reporting the co-mutation, including a study by Gong et al (2017) showing the incidence rate of 1.4% of 138 metastatic

Table 3. Clinicopathology Association With PIK3CA and KRAS Oncogene Status

Characteristic	Overall, N = 244[a]	PIK3CA			KRAS		
		Mutant, N = 107[a]	Wild-type, N = 137[a]	P-value[b]	Mutant, N = 151[a]	Wild-type, N = 93[a]	P-value[b]
Age				0.8			0.3
< 50	53 (21.72%)	22 (20.56%)	31 (22.63%))		36 (23.84%)	17 (18.28%)	
≥ 50	191 (78.28%)	85 (79.44%)	106 (77.37%)		115 (76.16%)	76 (81.72%)	
Sex				0.040*			0.6
Female	123 (50.41%)	62 (57.94%)	61 (44.53%)		74 (49.01%)	49 (52.69%)	
Male	121 (49.59%)	45 (42.06%)	76 (55.47%)		77 (50.99%)	44 (47.31%)	
Tumor site				0.083			0.022*
Left	192 (79.01%)	79 (73.83%)	113 (83.09%)		112 (74.17%)	80 (86.96%)	
Right	51 (20.99%)	28 (26.17%)	23 (16.91%)		39 (25.83%)	12 (13.04%)	
Unknown	1	0	1		0	1	
Stage				0.6			> 0.9
I	12 (5.08%)	5 (4.76%)	7 (5.34%)		7 (4.86%)	5 (5.43%)	
II	70 (29.66%)	33 (31.43%)	37 (28.24%)		43 (29.86%)	27 (29.35%)	
III	56 (23.73%)	28 (26.67%)	28 (21.37%)		35 (24.31%)	21 (22.83%)	
IV	98 (41.53%)	39 (37.14%)	59 (45.04%)		59 (40.97%)	39 (42.39%)	
Unknown	8	2	6		7	1	
Tumor (T) status				> 0.9			> 0.9
1	3 (1.23%)	1 (0.94%)	2 (1.46%)		2 (1.33%)	1 (1.08%)	
2	25 (10.29%)	10 (9.43%)	15 (10.95%)		17 (11.33%)	8 (8.60%)	
3	156 (64.20%)	71 (66.98%)	85 (62.04%)		94 (62.67%)	62 (66.67%)	
4	59 (24.28%)	24 (22.64%)	35 (25.55%)		37 (24.67%)	22 (23.66%)	
Unknown	1	1	0		1	0	
Lymph node (N) status				0.3			0.7
0	121 (51.05%)	49 (46.67%)	72 (54.55%)		77 (53.10%)	44 (47.83%)	
1	84 (35.44%)	38 (36.19%)	46 (34.85%)		49 (33.79%)	35 (38.04%)	
2	32 (13.50%)	18 (17.14%)	14 (10.61%)		19 (13.10%)	13 (14.13%)	
Unknown	7	2	5		6	1	
Metastasis (M) status				0.3			0.8
0	139 (58.90%)	66 (62.86%)	73 (55.73%)		86 (59.72%)	53 (57.61%)	
1	97 (41.10%)	39 (37.14%)	58 (44.27%)		58 (40.28%)	39 (42.39%)	
Unknown	8	2	6		7	1	
Histological grading				0.4			0.038*
1	107 (44.77%)	47 (45.19%)	60 (44.44%)		67 (45.89%)	40 (43.01%)	
2	99 (41.42%)	40 (38.46%)	59 (43.70%)		55 (37.67%)	44 (47.31%)	
3	31 (12.97%)	17 (16.35%)	14 (10.37%)		24 (16.44%)	7 (7.53%)	
4	2 (0.84%)	0 (0.00%)	2 (1.48%)		0 (0.00%)	2 (2.15%)	
Unknown	5	3	2		5	0	
Lymphovascular invasion status				0.3			> 0.9
0	51 (46.36%)	27 (51.92%)	24 (41.38%)		29 (46.77%)	22 (45.83%)	
1	59 (53.64%)	25 (48.08%)	34 (58.62%)		33 (53.23%)	26 (54.17%)	

(continued)

Table 3. *(continued)*

Characteristic	Overall, N = 244[a]	PIK3CA			KRAS		
		Mutant, N = 107[a]	Wild-type, N = 137[a]	P-value[b]	Mutant, N = 151[a]	Wild-type, N = 93[a]	P-value[b]
Unknown	134	55	79		89	45	
Pathological morphology				0.2			0.7
Adenocarcinoma	239 (97.95%)	103 (96.26%)	136 (99.27%)		147 (97.35%)	92 (98.92%)	
Mucinous carcinoma	5 (2.05%)	4 (3.74%)	1 (0.73%)		4 (2.65%)	1 (1.08%)	
TILs				0.023*			0.6
High	80 (39.41%)	26 (30.23%)	54 (46.15%)		46 (36.80%)	34 (43.59%)	
Medium	78 (38.42%)	34 (39.53%)	44 (37.61%)		51 (40.80%)	27 (34.62%)	
Low	45 (22.17%)	26 (30.23%)	19 (16.24%)		28 (22.40%)	17 (21.79%)	
Unknown	41	21	20		26	15	
BMI (kg/m^2)				0.3			0.081
< 18.5	72 (30.77%)	33 (32.67%)	39 (29.32%)		42 (28.97%)	30 (33.71%)	
≥ 25	33 (14.10%)	18 (17.82%)	15 (11.28%)		18 (12.41%)	15 (16.85%)	
18.5 - 22.9	97 (41.45%)	36 (35.64%)	61 (45.86%)		69 (47.59%)	28 (31.46%)	
23 - 24.9	32 (13.68%)	14 (13.86%)	18 (13.53%)		16 (11.03%)	16 (17.98%)	
Unknown	10	6	4		6	4	
Hemoglobin level (g/dL)				> 0.9			0.9
< 10	48 (20.51%)	21 (20.59%)	27 (20.45%)		30 (21.13%)	18 (19.57%)	
≥ 10	186 (79.49%)	81 (79.41%)	105 (79.55%)		112 (78.87%)	74 (80.43%)	
Unknown	10	5	5		9	1	
ECOG		0.5			0.7		
0 - 1	154 (73.33%)	72 (75.79%)	82 (71.30%)		98 (75.38%)	56 (70.00%)	
2	38 (18.10%)	14 (14.74%)	24 (20.87%)		22 (16.92%)	16 (20.00%)	
3 - 4	18 (8.57%)	9 (9.47%)	9 (7.83%)		10 (7.69%)	8 (10.00%)	
Unknown	34	12	22		21	13	

[a]n (%). [b]Fisher's exact test. *P-value < 0.05. BMI: body mass index; ECOG: Eastern Cooperative Oncology Group; TILs: tumor-infiltrating lymphocytes.

CRC [70]. Our cohort was highly enriched for metastatic disease (41.1%) which may contribute to the higher frequency of co-mutation. However, it is important to note that most patients of our cohort presented with advanced disease status. To date, there is no optimal treatment for metastatic CRC harboring both KRAS and BRAF [71]. Consequently, our high co-mutation rate in the Indonesian population can shed new light that warrants further investigation. This study and others have shown that KRAS mutation was more frequent in right-sided CRC [48, 72, 73]. The predilection of KRAS mutations for the right side of the colon may be influenced by the fact that the right and left sides of the colon have different biology and histopathology in their respective embryological origins [48, 74, 75]. Right-sided CRC often has flat histopathology and a DNA MMR pathway deficiency [76]. This may explain the significant association of mutant KRAS and MSI in our cohort.

Our study reports the PIK3CA (exon 9 and 20) mutation frequency of 43.85%, which is lower compared to a previous study in other regions of Indonesia with smaller sample size, reporting 70.9% mutation [77]. An NGS-based study on Indonesian patients with advanced CRC revealed that all patients (100%, n = 22) harbor PIK3CA mutation, distinctively in exon 2, 5, 7, 8, 10, 19, and 21, in addition to commonly reported exon 9 and 20 [53]. Similar to our result, a meta-analysis of 44 studies enrolling 17,621 patients has reported that mutations of PIK3CA exon 9 and 20 were associated with MSI, KRAS mutation and right-sided colon [78]. Tumor with MSI is usually associated with a high number of TILs due to the generation of neoantigens, hence it serves as a good candidate for immunotherapy [79-81]. However, in this study, we found a subset of cases with lower numbers of TILs in PIK3CA mutant tumors, suggesting an immune evasion mechanism at play.

Interestingly, programmed death-ligand 1 (PD-L1) expression was correlated with PIK3CA mutations, suggesting that cancers with PIK3CA mutations and PD-L1 expression are immunotherapy candidates [28]. Inhibition of the PI3K-AKT pathway may improve effector T-cell infiltration in PI3K-altered CRC. Combining PI3K inhibitors with anti-PD-1

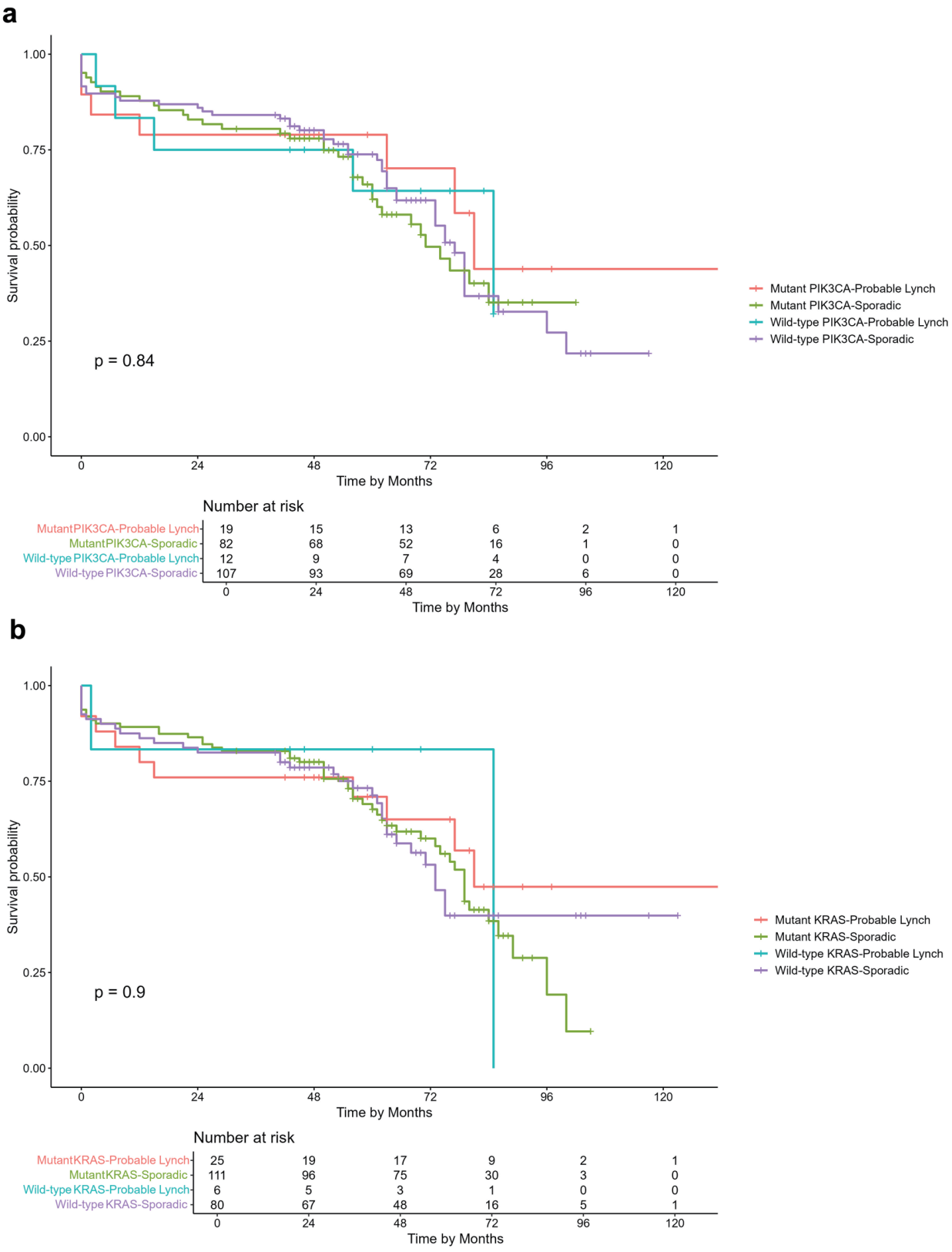

Figure 1. Kaplan-Meier overall survival curves of CRC patients. (a) Overall survival comparison based on PIK3CA mutation and probable Lynch syndrome status. (b) Overall survival comparison based on KRAS mutation and probable Lynch syndrome status.

could enhance treatment efficacy and $CD8^+$ T cell proliferation [82]. It is also worth noting that PIK3CA mutations are prevalent in the early stages of MSI, while it tends to occur later in MSS [83]. These suggest that the combination of PIK3CA mutation, MSI status, and PD-L1 expression could potentially be used to guide treatment selection or prognosis improvement for certain subsets of CRC patients.

As previously reported, our cohort was significantly enriched with EOCRC, defined as < 50 years old [6]. We harnessed a robust, simple and affordable test called N_LyST to screen for probable Lynch, which fit into resource-limited settings in Indonesia [29]. The test is a polymerase chain reaction-high resolution melting analysis (PCR-HRMA)-based method, consisting of five mononucleotide markers for MSI, MLH1 promoter methylation and BRAF V600E mutation in a single PCR run. We found that there was a high proportion of probable Lynch in our Indonesian CRC cohort (13.85%) and it was strongly associated with EOCRC, despite still substantial numbers of EOCRC that were considered as non-LS/sporadic cases [6].

Similar to other studies, this study found no significant association between oncogenic KRAS and PIK3CA mutations and EOCRC [84]. Nonetheless, in this study, we found high KRAS and PIK3CA mutations, 81.25% and 59.38% respectively, in patients with probable Lynch status. These are much higher than previously reported in the literature, showing that KRAS mutations in the LS population range between 27% and 40% [27, 85-88]. We have reported the positive association of PIK3CA mutation with KRAS mutation [89]. There was a statistically significant association between probable Lynch status and KRAS mutation, but not PIK3CA mutation. It has been reported in the literature that KRAS mutation was more frequently found in LS-related MSI CRC as compared to sporadic MSI CRC [27, 86, 90, 91]. On the other hand, PIK3CA mutation was reported to be more common in somatically mutated MMR-deficient CRC [92].

We observed no differences in overall patients' survival over a follow-up period of 120 months, between mutant and wild-type subgroups based on probable Lynch and non-LS/sporadic status for both KRAS and PIK3CA. Prior studies have shown that LS patients with CRC have a better prognosis than those with sporadic CRC, arguably due to its association with MSI and neoantigen generation in enhancing the immune response [93]. Although, as reported previously, we did not see this benefit of survival in our cohort [6]. It is appealing to speculate that high occurrences of KRAS mutation in our probable Lynch patients outweigh the survival benefit of MSI. To our knowledge, there have been no studies yet that examine the association of survival rates in KRAS and PIK3CA mutations with LS in CRC.

LS is highly heterogeneous, showing high variability in age at onset (despite enriched in EOCRC), penetrance of cancer, and clinical presentations, which may be partly attributable to the molecular profiles of carcinomas. As reviewed by Helderman et al (2021), LS heterogeneity is attributable to a variety of different molecular pathways of tumor development, and only partly depends on which MMR gene is mutated [94]. It is now recognized that LS CRCs develop via one of three pathways. The first pathway is adenoma-carcinoma pathway, in which adenomas develop independently of MMR deficiency. The second and third pathway is MMR-deficient crypt foci (MMR-DCF)-adenoma-carcinoma pathway and MMR-DCF-carcinoma pathway, which both start with MMR deficiency and is either followed by adenoma formation or results directly in a carcinoma [94, 95]. As widely known, APC mutations are more closely related to the development of adenomas, while CTNNB1 mutations appear to be associated with the MMR-DCF-carcinoma pathway [90, 94].

Recent studies have provided substantial evidence linking the methylation of MMR genes to the onset of LS [96, 97]. A significant portion of this evidence is derived from studies targeting the MLH1 gene, where methylation was observed in germline tissues of HNPCC patients who did not carry a germline mutation in the MLH1 gene [98]. Additionally, heritable germline epimutations in the MSH2 gene have been documented in LS families lacking MMR germline mutations [99]. A novel mechanism for inactivating the MSH2 gene has also been proposed. In several patients suspected of having LS but lacking detectable germline mutations in the MMR genes, researchers identified a heterozygous germline deletion encompassing the polyadenylation site within the final two exons of the epithelial cell adhesion molecule (EPCAM) gene [100]. Such deletion results disrupt the 3' end of the EPCAM gene, which induces transcriptional read-through. This aberrant transcription subsequently leads to epigenetic inactivation and silencing mechanisms that ultimately inhibit the proper expression of the MSH2 gene [101]. However, we acknowledge that we did not explore this mechanism in our study due to limited resources.

Understanding the molecular landscape of LS, such as RAF/MEK/ERK and PI3K/PTEN/AKT signaling, will allow more detailed stratification of LS patients and will facilitate the provision of optimal care to each patient, including the diagnosis, surveillance, and treatment. For instance, activating PIK3CA variants are potentially susceptible to preventive aspirin therapy by inducing the transcription of COX2 gene increasing the production of PGE2 [90, 102]. This is in addition to known resistance of anti-EGFR of CRC harboring KRAS, BRAF and PIK3CA mutations [103, 104]. However, it is not yet known whether cancers that develop during aspirin therapy have a specific molecular signature [105].

In summary, despite the limitations of this study, including being conducted in a single-center, tertiary hospital, while utilizing a low throughput (PCR-based) workflow for mutation detections, this study has contributed to the better understanding of CRC molecular features in an underrepresented population in current global literatures. The use of consecutive sampling at a tertiary hospital introduces potential ascertainment bias, likely over-representing those with advanced or treatment-resistant disease, as the center primarily receives referrals for patients who already underwent resection elsewhere. Acknowledging these limitations, future studies should include more representative samples from diverse settings and use alternative sampling methods to minimize bias. Our initial findings described in this paper and previous reports from our and other studies have indicated distinct genetic make-up such as high probability of LS, high frequency KRAS, and PIK3CA mutations, and lower BRAF mutation among CRC in Indonesia. This may underpin its unique clinical characteristics

such as higher number of young patients and advanced disease stage. Further comprehensive multicenter analysis using high throughput techniques such as NGS is important to provide a more complete picture of the CRC carcinogenesis in Indonesia, with particular emphasis on EOCRC and LS. Understanding the global molecular landscape of CRC may reveal new knowledge that could challenge the current dogma, hence improving the effort to provide better care for the disease.

Conclusion

The high probability of LS in Indonesian CRC patients is associated with KRAS and PIK3CA mutations. It improved our understanding of CRC molecular features in an underrepresented population in global literature.

Supplementary Material

Suppl 1. Kaplan-Meier overall survival curves of CRC patients stratified by microsatellite instability (MSI) status. (A) Overall survival curves stratified by PIK3CA mutation and MSI status; (B) overall survival curves stratified by KRAS mutation and MSI status.

Suppl 2. Kaplan-Meier overall survival curves of CRC patients based on PIK3CA and KRAS mutation status. (A) Overall survival curves of PIK3CA mutant versus wild-type; (B) overall survival curves of KRAS mutant versus wild-type.

Suppl 3. Kaplan-Meier overall survival curve of CRC patients with Probable Lynch Syndrome versus sporadic cases.

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Conflict of Interest

MI was appointed as specialist committee member to the Diagnostics Assessment Committee of the National Institute for Health and Care Excellence (NICE), which produced the guidance DG27 on Lynch Syndrome testing. DSH, AMR, and MI are unpaid scientific advisors of PathGen Diagnostik Teknologi. Other authors have no competing interest to declare.

Informed Consent

All subjects provided written informed consent.

Author Contributions

Conception or design of the work: DSH, SHH, and SS; methodology: DSH, NY, GA, AMR, and SHH; software: GA, HH, MZA, AMR, WK, and SS; validation: DSH, NY, GA, AMR, SHH, and SS; formal analysis: DSH, NY, GA, HH, MZA, AMR, SHH, and SS; investigation: NY, GA, HH, AMR, WK, and SS; resources: DSH, NY, AYH, JK, SHH, and SS; data curation: DSH, NY, GA, VL, HH, ZH, ANG, AMR, and SS; writing-original draft preparation: DSH, VL, HH, ZH, ANG, and SS; writing-review and editing: DSH, AYH, MI, JK, SHH, and SS; visualization: GA, MZA, and SS; supervision: DSH, NY, MI, SHH, and SS; project administration: GA, VL, HH, ZH, and AMR; funding acquisition: DSH, MI, SHH, and SS; approval of the version to be published: all authors.

Data Availability

All data and related metadata underlying the findings reported in this manuscript have been provided as part of the submitted article. Any additional data that might support the findings of this study are available from the corresponding author, S.S., upon reasonable request.

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