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The prevalence of *KRAS* and *BRAF* mutation in colorectal cancer patients in Bali

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ABSTRACT Mutations in the *KRAS* (Kirsten rat sarcoma viral oncogene homolog gene) and *BRAF* (v-Raf murine sarcoma viral oncogene homolog B1) gene play a significant role in primary resistance to colorectal cancer therapy. Around 85-90% of *KRAS* mutations in colorectal cancer occur in exon 2 (codon 12 and 13), whereas approximately 96% of *BRAF* mutations occur in exon 15 codon 600 (V600E). This study aimed to determine the prevalence and mutation characteristics of the *KRAS* and *BRAF* genes in colorectal cancer patients in Bali. The DNA was isolated from 44 formalin-fixed paraffin-embedded colorectal cancer samples which were stored in the Department of Pathology, Sanglah General Hospital in 2017. Detection of mutation was carried out by polymerase chain reaction (PCR) and direct sequencing. Out of 44 samples, only 27 were successfully amplified and sequenced. Our findings showed six samples (22.2%) with mutated *KRAS* at codons 12 and 13 (including two samples with G12D, one sample with G12V, and three samples with G13D). Interestingly, we found three samples (11.1%) of *BRAF* mutation, including two samples with V600E mutation and one with V600L mutation. Taken together, our results showed that *KRAS* and *BRAF* mutations were identified and occurred exclusively. Further studies are essential to identify the correlation of these mutations with colorectal cancer prognosis and response to chemotherapy.

KEYWORDS *BRAF*; colorectal cancer; gene mutation; *KRAS*

1. Introduction

Colorectal cancer (CRC) is a malignancy that occurs in the colon or rectum. Colorectal cancer ranks third in most types of cancer suffered in the world. The number of new cases of colorectal cancer in the world reached 1.8 million in 2018 (Globocan- The Global Cancer Observatory 2019), whereas in Indonesia, there have been 30,017 cases (World Health Organization 2019). The absence of population-based data in Indonesia leads to an unclear overview of the incidence of CRC. Various reports show increases in the number of cases of CRC as one of the ten most common cancers (Warsinggih et al. 2020). These indicate that colorectal cancer is one of the concerned health problem.

Current evidence showed both genetic and epigenetic contribute to the development of colorectal cancer pathogenesis. It consists of three main pathways, namely chromosomal instability (CIN), group microsatellite instability (MSI), and methylation on CpG island/CpG island methylator phenotype (CIMP). From all these three mechanisms, most colorectal cancer development is related to the CIN pathway. The CIN pathway involves alterations in the RAS/RAF/MEK/ERK signaling cascade that regulates cell proliferation, differentiation, motility, and apoptosis (Kuipers et al. 2015).

The mutated *RAS* gene is found in more than 30% of cancers that occur in humans. Mutations in the *KRAS* gene occur with the highest frequency (21.6%), followed by *NRAS* (8.0%) and *HRAS* (3.3%) (Arrington et al. 2012). In colorectal cancer, *KRAS* genes that undergo mutations contribute to 40% of cases. This protein can convey external signals to the nucleus by encoding a protein bound to guanosine triphosphate (GTP) that regulates cell division (Arrington et al. 2012). If a mutation occurs, the *KRAS* gene will be impaired to switch between active and inactive states. Mutations due to changes nucleotide bases in codons 12 and 13 will change the encoded amino acids, causing the *KRAS* gene to be constitutively active and undergo proliferation. Approximately 85-90% of *KRAS* mutations in exon 2 (codon 12 and 13) contribute to colorectal cancer. *KRAS* mutation G12D, G12V, and G13D were the most prevalent. Notably, not all mutations of *KRAS* predict poor prognosis in patients with CRC. Only G12D

and G12V mutations in codon 12 of *KRAS* were independent prognostic factors of worse overall survival and progression-free survival for CRC patients (Li et al. 2019).

BRAF mutation mostly (96%) occurs in exon 15 of codon 600 or known as V600E mutation. Epidemiological studies showed that mutations in the *BRAF* gene occur in 5-15% of colorectal cancer. In addition, a case study conducted on 2,530 patients found that the prevalence of *BRAF* mutations was 9.1% (Seligmann et al. 2017). *BRAF* mutations can be a biomarker to determine the prognosis and therapy of colorectal cancer. In terms of prognosis, *BRAF* mutations are associated with poor prognosis and reduced overall survival in colorectal cancer patients (Wang et al. 2019). As for treatment, *BRAF* mutation plays a role in choosing the type of regiment therapy that affects the outcomes (Kopetz et al. 2015).

As concluded from various findings , the *KRAS* exon two mutation and BRAF V600E mutation is an important predictive and prognostic biomarker in colorectal cancer. However, data on the prevalence and mutation characteristics of exon two codon 12 and 13 *KRAS* gene also *BRA*F V600E in colorectal cancer patients in Bali are limited. Thus, we sought to identify the prevalence and characteristics of amino acid alterations of KRAS and BRAF mutations through this study.

2. Materials and Methods

2.1. Ethics

The study was reviewed and approved by the ethics committee of the Faculty of Medicine, Udayana University (number: 400/UN14.2.2.VII.14/LP/2020).

2.2. Samples

Samples used in this study were 44 formalin-fixed paraffin-embedded (FFPE) stored at the Department of Pathology, Sanglah General Hospital, which were histologically confirmed as colorectal cancer specimens in 2017.

2.3. DNA extraction from FFPE samples

DNA was extracted according to the Black Prep FFPE DNA Kit (Analytic Jena GmbH, Germany). Briefly, 2×10 µm FFPE slices were lysed with 400 µL Lysis Solution MA and 40 µL Proteinase K. After incubation at 65 °C for one hour, samples were then incubated at 90°C for one hour in a thermal mixer at 1,000 rpm. Following incubation for 5 min at room temperature, the sample was centrifuged at 13,000 rpm for 2 min. The supernatant was transferred into a 1.5 mL microcentrifuge tube and 400 µL absolute ethanol 99% was added. The sample was transferred into the spin column and centrifuged at 12,000 rpm for 1 min. The sequential washing steps were carried out using 500 µL Washing Solution C and 650 µL Washing Solution BS, each centrifuged at a speed of 12,000 rpm for 1 min. After washing with 650 µL of 99% absolute ethanol and centrifuged at a speed of 12,000 rpm for 1 min, DNA was eluted in 100 µL elution buffer and centrifuged at 12,000 rpm for 1 min. The concentration of isolated DNA was then measured using SimpliNano (Biochrom).

2.4. KRAS and BRAF PCR amplification

Exon 2 of the *KRAS* gene was amplified using the following primers (Macrogen, Korea): forward 5'GGTACTGGTGGAGTATTTGATAGTG3' and reverse primer 5'CATGAAAATGGTCAGAGAACC3', whereas the *BRAF* gene was amplified using forward primer 5'TGCTTGCTCTGATAGGAAAATGA3' and reverse primer 5'TGCTTTCTCTGATAGAAAAATGA3'. Amplification was carried out in a total volume of 10 µL containing 5 µL master mix, 0.2-0.3 µL for each forward and reverse primer either *KRAS* gene or *BRAF* gene (10 µM), 0-1.6 µL ddH2O and 3-4,6 µL of 10 ng/µL DNA. PCR program or *KRAS* was carried out at 95 °C for 5 min and followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at a temperature range of 50-56 °C for 60 s and extension at 72 °C for 30 s and a final elongation step at 72 °C for 5 min. For the *BRAF* PCR amplification, the program was carried out at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 40 s and a final extension step at 72 °C for 5 min. The length of the amplicon for *KRAS* was 288 bp, whereas *BRAF* was 165 bp. PCR product was applied into 2% gel agarose dissolved in 1X TBE buffer.

2.5. KRAS and BRAF direct sequencing

KRAS mutations in exon two and *BRAF* V600E mutation were identified by direct sequencing. PCR products were sent to the Genetika Science Laboratory, Jakarta. Direct sequencing was done using BigDye (Applied Biosystems).

3. Results and Discussion

3.1. Characteristics of patients

Twenty-five (57%) patients were male and 19 (43%) patients were female and most of them (34 out of 44, or 77%) were more than 50 years old. Based on colorectal cancer histological type, we identified 37 (84%) samples classified as adenocarcinoma, 6 (14%) samples were mucinous adenocarcinoma and 1 (2%) sample were intramucosal adenocarcinoma (Table 1).

3.2. KRAS and BRAF mutation

Out of 44 samples, there were only 27 samples in which DNA could be successfully amplified. The overall *KRAS* mutation rate was 22.2% (6/27). From all 6 *KRAS* mutated samples, three samples showed mutations in codon 12, including two samples with G12D (Figure 1a) and 1 sample with G12V (Figure 1b). Another three samples exhibited mutation in codon 13, G13D (Figure 1c).

Interestingly, we found the overall *BRA*F V600E mutation rate was 7.4% (2/27) and the *BRAF* V600L mutation rate was 3.7% (1/27). *BRAF* V600E mutation marked with

TABLE 1 Characteristic of samples based on age, gender and histological type

Characteristics	N (%)
Age (years)	
<50	10 (23)
>50	34 (77)
Gender	
Male	25 (57)
Female	19 (43)
Histology	
Adenocarcinoma, NOS	37 (84)
Mucinous adenocarcinoma	6 (14)
Intramucosal adenocarcinoma	1 (2)

NOS, not otherwise specified

alteration of valine (GTG) to glutamic acid (GAG) (Figure 2a and 2b). The *BRAF* V600L mutation was marked with alteration of valine (GTG) to leucine (TTG) (Figure 2c). The frequency and amino acid change of *KRAS* and *BRAF* mutation are shown in Table 2.

3.3. Discussion

Patient data in the form of age, gender, and histology characteristics were taken from the Department of Pathology, Sanglah Hospital's medical record in 2017. Samples were found to be in the age range of 23 to 80 years old. The results showed that the incidence of colorectal cancer was more in the age group of more than 50 years old. This shows conformity with previous publications that most sufferers are over 50 years old (Kuipers et al. 2015).

Our results showed that the incidence of colorectal cancer was higher in men than in women. Indrayani and Sriwidyani (2017) showed that cases of colorectal cancer in males were found in 16 (70%) of 23 samples. These findings are consistent with data published by Globocan-The Global Cancer Observatory (2019) that stated the standardized incidence rate of colorectal cancer age in the world for male are higher than female (23.6 compared to

TABLE 2 Frequency of *KRAS* and *BRAF* mutations

Nucleotide change	Amino acid change	No. of mutated cases
***KRAS* (n=27)**		
KRAS codon 12		
c.35G>A	p.G12D	2
c.35G>T	p.G12V	1
KRAS codon 13		
c.38G>A	p.G13D	3
***BRAF* (n=27)**		
BRAF codon 600		
c.1799T>A	p.V600E	2
c.1798G>T	p.V600L	1

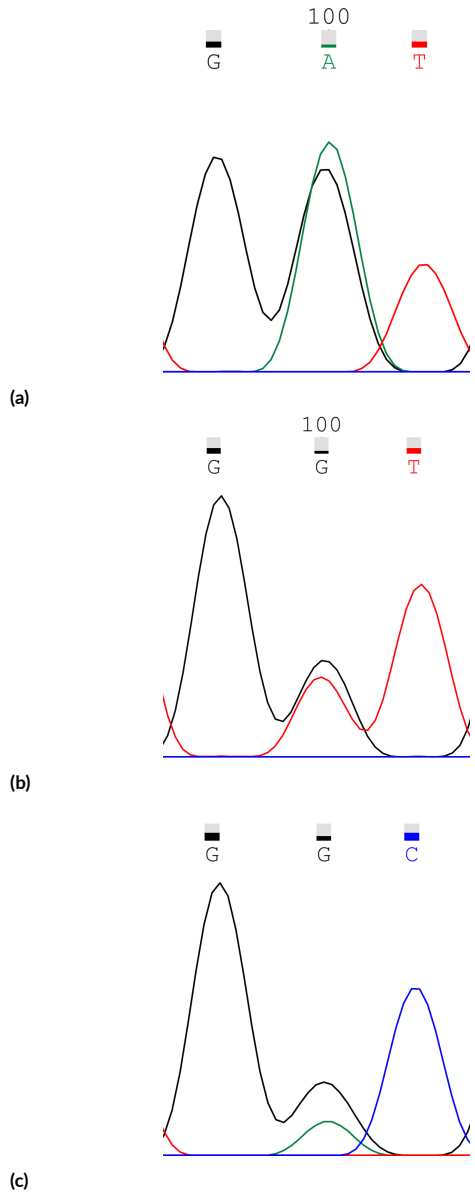

FIGURE 1 *KRAS* mutations at exon 2 codon 12 and 13 from 3 different samples. The electropherograms display mutations of *KRAS* G12D (G→A) (a), *KRAS* G12V (G→T) (b), and *KRAS* G13D (G→A) (c).

16.3 per 100,000 people). The results of the study are also consistent with data from the World Health Organization (2019) that the incidence rate of standardized colorectal cancer in Indonesia for males is higher than females of 7.7 and 4.4 per 100,000 people. The data show that the incidence of colorectal cancer is consistently higher in males than in the female.

In our study, the most prominent type of colorectal cancer was adenocarcinoma, followed by mucinous adenocarcinoma and intramucosal adenocarcinoma. This result shows concordance with studies that about 95% of the histology of colorectal cancer is adenocarcinoma, and the rest are other types, namely mucinous carcinoma and adenosquamous carcinoma (American Institute of Cancer Research 2018).

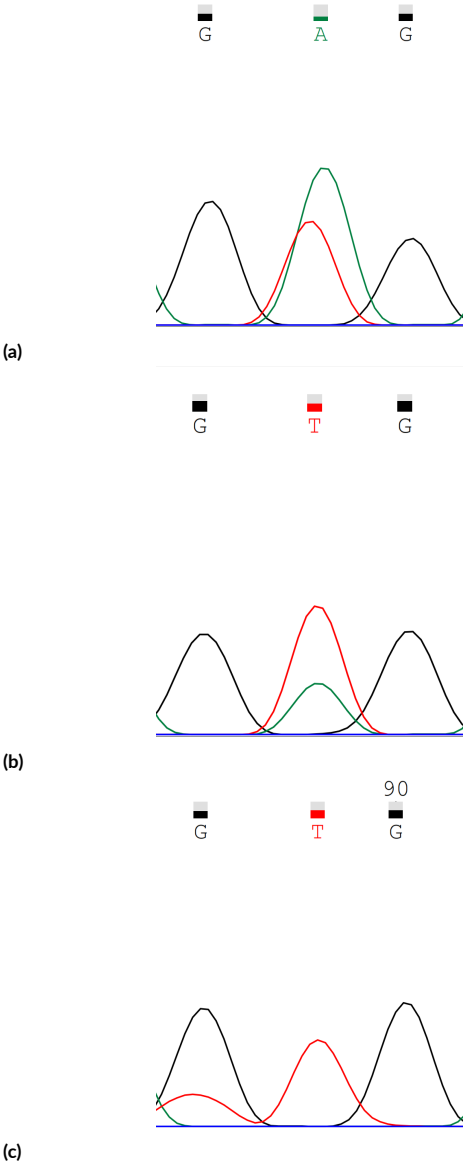

FIGURE 2 *BRAF* mutation at exon 15 of three samples. The electropherogram shows V600E mutation (T→A) (a and b) and V600L mutation (G→T) (c).

Out of 44 samples, there were 17 samples in which DNA could not be successfully amplified. The reason might be because the DNA was degraded due to the use of FFPE. Evaluation of the degree of DNA degradation is of major importance when handling FFPE samples. Solassol et al. (2011) compared the degradation level of DNA isolated from frozen samples and FFPE using a 2% agarose gel electrophoresis. They found that the frozen samples were not degraded, whereas the FFPE samples were partially fragmented. However, they observed a correct PCR amplification in both the FFPE and frozen tissues, demonstrating that the PCR conditions were adapted to the FFPE samples. In our study, we re-isolated and re-PCR amplified for the samples with suspected DNA degradation, but we still obtained PCR amplification unsuccessfully and/or the sequencing data. Thus, the samples could not be analyzed. Therefore, we excluded those samples for analysis.

The results of other studies showed a different prevalence of *KRAS* mutation which is 31% (Phipps et al. 2013), 40% (Imamura et al. 2014), and 33.3% (Phua et al. 2015). In contrast, studies in Indonesia with a small size of samples showed a prevalence of *KRAS* mutation was 30% (Mastutik et al. 2016) and 60.9% (Indrayani and Sriwidyani 2017). The RASCAL study (Arrington et al. 2012) reported mutations in codon 12 in 27.7% of cases. Jones et al. (2017) stated that mutations in codon 12 were found at the most, as many as 34.6% of cases. However, it is different from research published by Indrayani and Sriwidyani (2017) that the mutation of exon two codon 12 *KRAS* gene was found in 9 (39%) of 23 samples. Our findings showed that 2 (7.4%) out of 27 samples had G12D mutations, and 1 (3.7%) of 27 samples had G12V mutations. The results agreed with the RASCAL study that codon 12 with the highest prevalence were G12D mutations, followed by G12V, G12C, G12S, G12A, and G12R (Arrington et al. 2012). Jones et al. (2017) also described that the G12D mutation was more than G12V mutations (36% and 30.1%, respectively).

G13D variation is the most common variation in exon two codon 13 *KRAS* gene mutation. A report from Singapore General Hospital from June 2010 to October 2012 showed the prevalence of mutations in codons 13 as many as 8.9% (4 out of 45 samples) and 100% (4 out of 4 samples) had variations in the G13D mutation (Phua et al. 2015). Indrayani and Sriwidyani (2017) analyzed *KRAS* mutation from 23 CRC FFPE samples in Sanglah Hospital using HRM PCR. They found 14 (60.9%) cases had *KRAS* mutation with 9 cases with codon 12 mutation, 4 cases with codon 13 mutation, 1 case with codon 59 mutation and 1 case with codon 117 mutation. However, they did not provide specific data on the characteristics and type of those mutations.

The frequency of the *BRAF* V600E mutation found in this study is different from the study conducted in the Middle East with the number of *BRAF* gene mutation were 19 (2.5%) out of 757 samples and 17 (90%) samples were *BRAF* V600E mutations (Siraj et al. 2014). Shimada et al. (2018) showed that the *BRAF* V600E mutation was found in 7 out of 98 samples. In addition, a study conducted by Taniguchi et al. (2020) showed the percentage of *BRAF* V600E mutations was 34 (10.5%) out of 324 samples. Difference results were also found in the study conducted in Mexico, Latin America and the Caribbean population, which was 4% and 7.8%, respectively (Hernández-Sandoval et al. 2020). Meanwhile, different results were also found in Indonesia, showing *BRAF* V600E mutation in 6 (14%) of 43 samples (Warsinggih et al. 2020) and another study identified no *BRAF* mutation (Ni Nyoman et al. 2020). Another mutation of *BRAF* non-V600E was found in this study, with a change in a valine (GTG) to leucine (TTG) or known as V600L. A study conducted by Mao et al. (2012) in the Chinese patients showed different results with the frequency of V600L mutations in 6 (10.2%) out of 59 cases of colorectal cancer. This study also found

other types of non-V600E mutations, namely V600Q in 2 (3.4%) cases and V600V in 1 (1.7%) case. *BRAF* mutation is much higher in this study than in other studies and frequently overlaps with *KRAS* mutations.

The difference percentage of mutation found in this study may be affected by the small number of samples. The larger number of samples used will result in the possibility of more mutation cases. In addition, the difference in the frequency of mutations found can also be attributed to race or ethnicity. *KRA*S mutation rates were highest in tumors from blacks (44.1%), followed by tumors from Asians (27.8%) and whites (34.9%). *BRAF* V600E mutation was higher in white people (13.9%) than in black people (6.4%) and Asians (5.6%). Wild-type *KRAS* and *BRAF* tumors were most common among Asians (66.7%), and the frequency differed compared with tumors from blacks (49.5%) or whites (51.2%) (Yoon et al. 2015).

Up to now, *KRAS* mutations have been identified as a predictive marker of resistance to anti-EGFR in patients with metastatic CRC, and the use of anti-EGFR is restricted to the patients with wild-type *KRAS* (Mao et al. 2012). Although the *KRAS* status helps identify patients who are unlikely to benefit from anti-EGFR therapy, not all patients with wild-type KRAS respond to anti-EGFR therapy (Zhao et al. 2017). It remains unclear why a large number of patients with wild-type *KRAS* tumors are still not responsive to the treatment. Major downstream pathways activated by EGFR, including the RAS-RAF-MAPK and PI3K-PTEN-AKT signaling pathways, are important for generating resistance to anti-EGFR. *BRAF*, a downstream effector of *RAS* in the EGFR pathway, has been a subject of focus. Mutations of *KRAS* and BRAF genes are frequently mutually exclusive in colorectal cancer (Mao et al. 2012).

Early detection of mutations in colorectal cancer patients is very important to improve the outcome. Changes in coded amino acids resulting from changes in the nucleotides that make up the codon cause either the *KRAS* or *BRAF* gene to be in a constitutively active state. Circumstances such as those that trigger excessive proliferation and inhibition of apoptosis, thus leading to the emergence of cancer and resistance to anti-EGFR therapy and RAF inhibitor therapy, such as panitumumab and cetuximab in the treatment of colorectal cancer (Phua et al. 2015; Zhao et al. 2017). In addition to determining the appropriate therapy, the *KRAS* mutation has also been a predictive factor for prognosis in colorectal cancer patients. A 5-year progression-free survival in patients with *KRAS* gene mutations was lower (74.5%) than patients with normal *KRAS* genes (85.9%) (Li et al. 2019). *BRAF* mutation also contributed to determining the regimen in cancer patients. *RAF* inhibitors such as vemurafenib only produce 5% of response therapy in patients with *BRAF* mutation than wild-type patients (Kopetz et al. 2015). Therefore, identification of *KRAS* and/or *BRAF* mutation in a clinical setting is beneficial for treatment cost-effectiveness and is important to plan the proper chemotherapy for the patients. In this study, we did not analyze the correlation between *KRAS* or *BRAF* mutation and response therapy due to insufficient data in the medical record. Because this research was a retrospective study with a limited sample size and was conducted only in a single hospital, it may have introduced some selection bias. All are considered the limitation of the study.

4. Conclusions

In conclusion, our result showed that colorectal cancer cases in Bali were more common in males and increased with age. *KRAS* mutations have been identified in exon 2, and the most frequent is G13D, followed by G12D and G12V. Interestingly, in addition to *BRAF* V600E mutations in exon 15, we also identified *BRAF* V600L mutation. Although the sample size in this study is small and cannot yet be generalized to the Indonesian population, our finding contributes to the data on the prevalence and characteristics of *KRAS* and *BRAF* mutation in colorectal cancer patients in Bali, whose data is very restricted. Importantly, *BRAF* V600L mutation, as far as we know, has not been published in colorectal cancer patients in Indonesia. Further studies with a sufficient number of samples are needed to obtain conclusive data on the relationship between these gene mutations and the response to therapy and prognosis of colorectal cancer.

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Authors' contributions

NNAD designed this study and conducted manuscript proofreading before submission. NMPWS, AANSP and AYR carried out laboratory work and analyzed the data. NNAD and IWJS advised the laboratory techniques. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

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