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ORIGINAL ARTICLE

Molecular Profile of Colorectal Cancer Patients in Bali Based on Methylation of O^6-Methylguanine DNA Methyltransferase Promoter Region and Mutation of BRAF and Kirsten RAt Sarcoma Viral Oncogene Homolog Gene

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Background: Analysis of O^6-methylguanine DNA methyltransferase (MGMT) methylation considers as a predictive marker for chemotherapy sensitivity. Mutation in BRAF and Kirsten RAt sarcoma viral oncogene homolog (KRAS) gene is also crucial in colorectal tumorigenesis which is associated with primary resistance to epidermal growth factor receptor therapy. This study was aimed to identify the methylation of MGMT gene and to detect BRAF and KRAS mutations in colorectal cancer (CRC). **Methods:** The methylation level of MGMT gene was measured by pyrosequencing from bisulfite-treated DNA. Direct sequencing was performed to identify BRAF and KRAS mutations. The expression of MGMT was evaluated by immunohistochemistry. **Results:** Analysis of MGMT methylation showed that 15 (30%) samples were classified as Group 1 (mean range ≥27%, methylated), 27 (54%) samples were Group 2 (mean range: 10%–26%, intermediate), and 8 (16%) samples were Group 3 (mean range <10%, unmethylated). Direct sequencing showed no mutation (V600E) of BRAF gene and 11 (22.5%) samples with mutated KRAS which was 9 (18.4%) and 2 (4.1%) samples mutated at codons 12 and 13, respectively. Immunohistochemistry results showed that from 29 methylated MGMT, 19 (65.5%) samples had low expression and 10 (34.5%) samples had high expression of MGMT. **Conclusions:** Altogether, our result showed that most of the samples showed MGMT methylation and it tended to decrease the expression of MGMT. In addition, BRAF and KRAS mutations were exclusively occurred. These data give a contribution to the situation of MGMT methylation and BRAF and KRAS mutations in CRC patients in Denpasar whose data are limited. Further studies are needed to identify the key molecular pathway of CRC that will be potential for CRC management.

Key words: BRAF, colorectal cancer, epigenetic, Kirsten RAt sarcoma viral oncogene homolog, O^6-methylguanine DNA methyltransferase

INTRODUCTION

Molecular characterizations of colorectal cancer (CRC) provide an understanding of this disease and open a possibility for invention of molecular marker for early detection, or prevention, or for therapy and survival prediction.[1] Epigenetic alteration through DNA methylation prevents gene expression and leads to loss of its function.[2] Hypermethylation of various genes has been described in colorectal carcinoma, including gene encodes for O^6-methylguanine DNA methyltransferase (MGMT). MGMT enzyme is encoded by a single gene located in chromosome 10 (10q26); this gene consists of five exons. MGMT-encoded gene has a promoter containing CpG islands.[3] This protein plays a critical role in DNA repair by eliminating alkyl groups from O^6-guanine to cytosine at the same strand of DNA.[4,5] Therefore, MGMT protects normal cells from carcinogenic agents. On the other hand, MGMT leads to the resistance of malignant cells to alkylating agents.[6]

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Kirsten RAt sarcoma viral oncogene homolog (KRAS) and BRAF are key mediators in epidermal growth factor receptor (EGFR) pathway. They play an important role in CRC pathogenesis and are associated with primary resistance to EGFR therapy and prognostic value.[7]Gene encodes KRAS is located on the short arm of chromosome 12 (12p12). KRAS is a small GTPase and a major downstream component that relays a signal originating at receptor tyrosine kinases such as the EGFR family. It can also activate other signaling pathways, PIK3CA-AKT-mTOR axis.[8] Approximately 40% of CRCs exhibit missense mutations; mutations at exon 2 at codons 12 and 13 are the most common KRAS mutations.[8,9] BRAF gene is located at chromosome 7 (7q34). It is a serine-threonine kinase and can be activated by KRAS. BRAF is the top-level component of the RAF-MEK-ERK (MAPK) kinase cascade which regulates proliferation, differentiation, and cell motility.[8] Approximately 4.4% of CRCs show BRAF mutations, mostly V600E mutation at exon 15.[7]

Data on MGMT methylation as well as BRAF and KRAS mutations in CRC patients in Denpasar are restricted. Therefore, through this study, we provide the data that will give contribution to ongoing discussion on molecular CRC pathogenesis, and importantly, it will also benefit for cancer management, especially for the management of CRC.

METHODS

Samples

Samples used in this study were 50 stored formalin-fixed paraffin-embedded samples which were histologically confirmed as CRC specimens. The study was reviewed and approved by the local ethics committee of the institute (IRB number 1952/UN.14.2/KEP/2017, approved on August 21, 2017). The informed patient consent was waived by obtaining approval from the institutional review board committee due to the retrospective nature.

DNA extraction from formalin-fixed paraffin-embedded samples

DNA was extracted following blackPREP FFPE DNA Kit (Analytik Jena) guidelines. Two to five sections of 10 µm thickness of FFPE sample were lysed with 400 µl solution MA and 40 µl proteinase K. The reaction was then incubated at 65°C for 1 h and then at 90°C for 1 h in a thermal mixer under continuous shaking at 1,000 rpm. After incubating the sample for 5 min at room temperature, samples were centrifuged at maximum speed for 2 min and transferred into a new 1.5 ml reaction tube. Ethanol absolute (400 µl) was mixed vigorously and then applied onto a spin filter with a 2.0 ml receiver tube and centrifuged at 12,000 rpm for 1 min. A sequential washing step was done using washing solution C and washing solution BS. After washing with 650 µl ethanol absolute (96%–99%) and centrifuged at 12,000 rpm for 1 min, DNA was eluted in 100 µl elution buffer. A 1 µl of DNA was applied to measure its concentration.

Bisulfite modification

Isolated DNA (200–500 ng) was subjected to bisulfite conversion according to the manufacturer's protocol (EZ DNA Methylation Kit, Zymo). A total volume of 50 µl, consisting of DNA, M-dilution buffer, and ddH2O, was incubated at 37°C for 15 min. CT conversion buffer was added (100 µl) and incubated at 50°C for 16 h. After bisulfite conversion, DNA was cleaned up. Briefly, after 16 h incubation, tubes were placed on ice for 10 min and the solution then transferred to column that has been prepared with 400 µl M-binding buffer. Tubes were centrifuged at 10.000 rpm for 30 s. After washing with 100 µl M-wash buffer and centrifuged at 10.000 rpm for 30 s, samples were incubated with 200 µl M-desulfonation buffer at room temperature for 15 min. Samples were washed twice with 200 µl M-wash buffer and then DNA was eluted in 30 µl elution buffer.

Pyrosequencing

Pyrosequencing was performed on a PyroMark Q24 (Qiagen) with all primers (forward and reverse MGMT primers as well as MGMT sequencing primer) provided with the PyroMark™ Q24 MGMT Kit (Qiagen). The promoter region of MGMT gene was amplified using PyroMark PCR Kit (Qiagen). PyroMark PCR master mix containing HotStart Taq DNA polymerase, buffer, MgSO4, and dNTP (1x), coral load concentrate (1x), forward and reverse MGMT primers (each 0.2 µM; reverse primer was biotin-conjugated), 5 µl bisulfite-converted DNA template, and ddH2O were mixed to a total volume of 25 µl. Amplification of MGMT gene was performed with initial denaturation (to activate the Taq DNA polymerase) at 95°C for 15 min, followed by 50 cycles of denaturation at 95°C for 20 s, annealing at 53°C for 20 s and extension at 72°C for 20 s, and a final extension step at 72°C for 5 min. It produced a 104 bp amplicon length.

After confirming the PCR products using gel electrophoresis, 10–20 µl of it was subjected to the pyrosequencing sample preparation step. DNA was mixed with streptavidin-coated Sepharose beads and binding buffer by vortexing. The step was followed by strand separation and washing using vacuum tool (Qiagen). The single-strand DNA was then mixed with 0.3 µM sequencing primer (diluted using annealing buffer), incubated at 80°C for 2 min, and cooled down at room temperature for 5 min. Cartridge was prepared according to

protocol and the program run as prepared. The sequencing result was analyzed using the PyroMark Q24 software. For statistical analysis, two subgroups were defined according to an average of CpG residue methylation: first, a methylated group for sample with an intermediate status (mean range of 10%–26%) or with a methylated status (mean range ≥27%), and second, an unmethylated group for which the mean range was <10%.[10]

Immunohistochemistry

Detection of MGMT was performed in formalin-fixed and paraffin-embedded tumors using monoclonal mouse anti-human MGMT antibody (MT3.1, Abcam). We used 1:100 dilution and overnight incubation at room temperature. Diaminobenzidine was used as chromogen, resulting in a brown-colored precipitate at the antigen site. After counterstaining with Mayer's hematoxylin, the sections were embedded with xylene. Tonsil tissue was used as a positive control. The intensity of MGMT expression was grouped as low and high expression; it was scored high when the nucleus of tumor cells was completely stained and low when showed partially or lack of staining regardless of percentage of tumor cells stained. H and E staining was performed to ensure that only tumor tissues were analyzed.

BRAF amplification and direct sequencing

Exon 15 of BRAF gene containing the V600E mutation was amplified using the following primers: forward primer 5' TGCTTGCTCTGATAGGAAAATGA 3' and reverse primer 5' TGGATCCAGACAACTGTTCAAA 3'. Amplification was carried out in a total volume of 25 μl which contains 1x colorless buffer, 0.2 μM of each primer, 0.2 mM of dNTPs, 1.5 mM $MgCl_2$, and 1.25U GoTaq DNA polymerase. PCR was run at 95°C for 5 min and followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 40 s, and a final extension step at 72°C for 5 min. Amplicon size was 165 bp. Direct sequencing was performed using BigDye (Applied Biosystems).

Kirsten RAt sarcoma viral oncogene homolog amplification and direct sequencing

The hotspot KRAS mutation at exon 2 was identified by direct sequencing. Exon 2 of KRAS gene was amplified using the following primers: forward primer 5' GGTACTGGTGGAGTATTTGATAGTG 3' and reverse primer 5' CATGAAAATGGTCAGAGAAACC 3'. Amplification was carried out in a total volume of 15 μl which contains 1x colorless buffer, 0.2 μM of each primer, 0.2 mM of dNTPs, 1.5 mM $MgCl_2$, and 1.25U GoTaq DNA polymerase. PCR was run at 95°C for 5 min and followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 50°C for 60 s and extension at 72°C for 30 s, and a final extension step at 72°C for 5 min. The length of the amplicon was 288 bp. Direct sequencing was performed using BigDye (Applied Biosystems).

Analyzing DNA fragments by agarose gel electrophoresis

PCR product was mixed with 6 × DNA loading dye and separated in 1.8%–2% agarose. After 35 min, gel was soaked into solution contained 1 μg/ml ethidium bromide for 15 min. DNA marker was separated in parallel with the samples. Bands were visualized under ultraviolet light illumination and documented using a digital imaging system.

Statistical analysis

The Chi-square test was used to evaluate the associations between the MGMT promoter methylation status (methylated vs. unmethylated) and sex, grade, KRAS mutation, and MGMT expression. *P* values lower than 0.05 were considered statistically significant.

RESULTS

Characteristics of patients

Of the 50 patients, females were slightly more than males (n = 26, 52% vs. n = 24, 48%); age more than 50 years was higher than age <50 years (n = 39, 78% vs. n = 11, 22%). Based on cell differentiation, CRC can be classified into well-differentiated, moderately differentiated, and poorly differentiated CRC. In our study, we identified 20 (40%) samples classified as well differentiated, 15 (30%) samples were moderately differentiated, and 15 (30%) samples were poorly differentiated [Table 1].

O^6-methylguanine DNA methyltransferase promoter methylation

Methylation of MGMT promoter region was identified for 5 CG sites using pyrosequencing. We grouped the results as follows. Samples with considerable methylation at almost all of the CpG sites tested (more than and equal to 27% methylation) belonged to Group 1 (methylated), samples with elevated methylation at only some CpG sites that had average methylation range 10%–26% were Group 2 (intermediate), and samples with almost completely unmethylated (<10% methylated) were Group 3 (unmethylated). We found that 15 (30%) samples were classified as Group 1 (methylated), 27 (54%) samples were Group 2 (intermediate methylated), and the rest 8 (16%) samples were Group 3 (unmethylated). The pyrosequencing histograms for Groups 1 and 3 are shown in Figure 1.

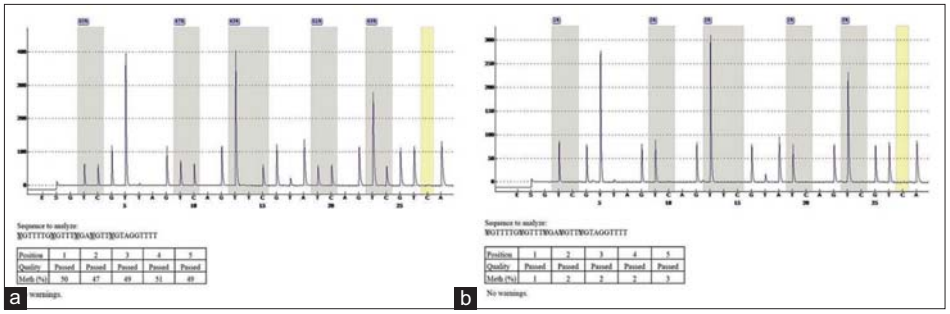

Figure 1: Representative histograms for methylated group (a) and unmethylated group (b). Bisulfite-converted DNA was amplified using O^6-methylguanine DNA methyltransferase primer pairs and then subjected to pyrosequencing. Blue boxes described the number of percentage of methylated DNA for each of 5 CG sites tested (gray boxes). Yellow box was the control of bisulfite conversion

Expression of O^6-methylguanine DNA methyltransferase

MGMT expression was examined using immunohistochemistry. We observed MGMT staining in nucleus of cells, shown by brown color [Figure 2]. Data on MGMT expression detected by immunohistochemistry were available for 66% (33/50) of the patients. Out of these 33 samples, 21 (63.6%) and 12 (36.4%) samples showed low and high intensity, respectively. From those in methylated group samples, 19 (65.5%) samples showed low intensity and 10 (34.5%) samples showed high intensity of MGMT expression.

BRAF and Kirsten RAt sarcoma viral oncogene homolog mutation

BRAF mutation at exon 15 (V600E) was not identified in all 50 samples. All samples showed wild-type GTG sequence (valine) at this position. One sample DNA showed unreadable electropherogram for KRAS; thus, we excluded it from calculation. The overall KRAS mutation rate was 22.5% (11/49). Of 11 KRAS-mutated samples, 9 samples showed mutations in codon 12, including 5 samples with G12D and 4 samples with G12V. Two of 11 samples exhibited mutation in codon 13, G13D [Table 2 and Figure 3].

Association of O^6-methylguanine DNA methyltransferase methylation, patient characteristics, mutations, and O^6-methylguanine DNA methyltransferase expression

To analyze the association between MGMT methylation, patient characteristics, mutations, and MGMT expression, three groups of methylation were reclassified into two groups: methylated (Group 1 and Group 2) and unmethylated (Group 3). Statistical analysis was not performed for parameters with zero value.

No association was observed neither between MGMT promoter methylation and patients' characteristics, including sex and grade, and nor with MGMT expression ($P > 0.05$). There was an association between MGMT promoter methylation and KRAS mutation ($P < 0.05$) [Table 3].

Table 1: Characteristics of colorectal cancer patients based on sex, age, and cell differentiation

Characteristics	Frequency, ***n*** (%)
Sex	
Male	24 (48)
Female	26 (52)
Age (years)	
20-30	1 (2)
31-40	1 (2)
41-50	9 (18)
51-60	18 (37)
>60	21 (43)
Histopathology	
Well differentiated	20 (40)
Moderately differentiated	15 (30)
Poorly differentiated	15 (30)

Table 2: Frequency of BRAF and KRAS mutations

Nucleotide change	Amino acid change	Number of mutated cases (%)
KRAS (*n*=49)		
KRAS codon 12		9 (18.4)
c.35G>A	p.G12D	5
c.35G>T	p.G12V	4
KRAS codon 13		2 (4.1)
c.38G>A	p.G13D	2
BRAF (*n*=50)		
BRAF codon 600		
c.1799T>A	p.V600E	0 (0)

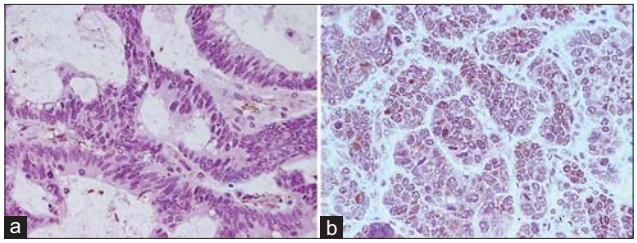

Figure 2: Low (a) and high (b) intensity of O^6-methylguanine DNA methyltransferase expression (brown-colored staining) as shown by immunohistochemistry using anti-O^6-methylguanine DNA methyltransferase antibody (×400)

DISCUSSION

A study of CRC profile in Indonesia was conducted by Khairina *et al*. using data collected from population based on cancer registry in 14 provinces (26 cities/districts) in Indonesia, which showed that colon cancer was higher in males (54%) than females (46%), which peak cancer cases occurred in the age of 50–54 years. The type of cancer morphology was adenocarcinoma.[11] Another study in Indonesia demonstrated that 49.6% of CRC cases occurred in younger age (<40 years old) and incidence in males was slightly higher than females (50.4% vs. 49.6%).[12]

CRC is a multistep disorder that arises from the accumulation of genetic and epigenetic alterations.[13] There are three different mechanisms lead to CRC, namely chromosomal instability (CIN), microsatellite instability (MSI) and CpG island methylator phenotype (CIMP). [14] Promoter hypermethylation is associated with CIMP, either microsatellite-stable (MSS) or MSI subtype.[15] These three pathways often overlap in CRC pathogenesis and play a role in prognosis and response to therapy. Patients with serrated adenoma precursor lesion, BRAF mutation, MSI (+), and CIMP (+) have a better prognosis than those with tubular adenoma, APC, and p53 mutation, negative for CIN and CIMP. Poor prognosis and poor therapy response for 5-fluorouracil and cetuximab occurred in patients with CIMP and predominant KRAS mutation (occasionally with BRAF mutation).[16]

Promoter hypermethylation of various genes has been described in CRC, including gene encodes for MGMT. MGMT is a ubiquitously expressed DNA repair enzyme which eliminates O6-methylguanine, promutagenic residues, from DNA.[6,17] Therefore, MGMT protects normal cells from carcinogenic agents. However, expression of MGMT leads to the resistance of malignant cells to chemotherapy using alkylating agents.[6] Mutations of MGMT gene have been rarely identified. This suggests that the inactivation of MGMT expression or loss of its function is primarily induced by hypermethylation of its promoter in human cancers, including CRC.[17]

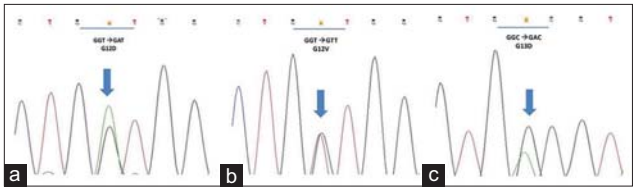

Figure 3: Kirsten RAt sarcoma viral oncogene homolog mutations at exon 2 codons 12 and 13 from 3 different samples. The electropherograms display mutations of Kirsten RAt sarcoma viral oncogene homolog G12D (a), Kirsten RAt sarcoma viral oncogene homolog G12V (b) and Kirsten RAt sarcoma viral oncogene homolog G13D (c). Arrows show heterozygosity. R = A or G; K = G or T

Table 3: Association between O^6-methylguanine DNA methyltransferase methylation, patient characteristics, KRAS mutation, and O^6-methylguanine DNA methyltransferase expression

	MGMT promoter	
	Methylated, *n* (%)	Unmethylated, *n* (%)
Number of patients	42 (84)	8 (16)
Age (years)		
<50	7 (100)	0
≥50	35 (81.4)	8 (18.6)
Sex		
Male	21 (87.5)	3 (12.5)
Female	21 (80.8)	5 (19.2)
*P**	0.517	
Grade†		
1	17 (85)	3 (15)
2	11 (73.3)	4 (26.7)
3	14 (93.3)	1 (6.7)
*P**	0.324	
BRAF		
Mutation	0	0
No mutation	42 (84)	8 (16)
KRAS		
Mutation	7 (63.6)	4 (36.4)
No mutation	34 (89.5)	4 (10.5)
*P**	0.041	
Immunohistochemistry (*n*=33)		
Low	19 (90.5)	2 (9.5)
High	10 (83.3)	2 (16.7)
P‡	0.545	

**P* value for the differences between methylated and unmethylated groups. †Grade 1, well differentiated; Grade 2, moderately differentiated; Grade 3, poorly differentiated, ‡*P* value for differences between low and high MGMT expression in the methylated group. MGMT: O6-methylguanine DNA methyltransferase

Methylation of promoter is one of the major posttranscriptional mechanisms that could affect the

levels of expression of MGMT protein. There are 97 CpG regions located at the promoter region of MGMT gene with various methylations. Everhard *et al*. described six sites of CpG (CpG −228, −186, +95, +113, +135, and + 137) as well as two other regions (position −186 to −172 and from +93 to +153) and exhibited the correlation between methylation and MGMT gene expression.[18] In this study, we identified MGMT promoter methylation in five CG sites using a commercially pyrosequencing kit. The assay detects the level of methylation in region +17 to +39 in exon 1 of the MGMT gene (Ensembl ID: OTTHUMT00000051009). The role of MGMT in CRC carcinogenesis is widely accepted, and reduced MGMT expression has been identified in tumor versus normal colon tissues.[19] However, mechanism by which MGMT expression is controlled remains unclear. It has been described that epigenetic silencing of MGMT by hypermethylation of the promoter region can lead to G: C to A:T mutations in p53, KRAS, and PIK3CA, leading to progression of the tumor to more advanced stages.[20-22] Methylation is not the only mechanism of regulation of MGMT expression. Polymorphisms of MGMT promoter may reduce MGMT expression, activity, and/or sensitivity and have been correlated with progression-free survival in CRC.[23-25] There is an association between methylation of MGMT gene and C/T polymorphism (rs16906252) at exon 1 in CRC,[23,24] which in this study has not been identified. Whether polymorphism of promoter region regulates the expression of MGMT, exclusively or inclusively, along with promoter methylation is another important aspect to be identified.

KRAS, a GTPase protein, will be activated or phosphorylated when a ligand binds to EGFR. When a ligand binds to EGFR, KRAS will activate other molecules, such as BRAF, mitogen-activated protein kinase (MEK) and ERK, facilitating cell proliferation, differentiation, or cell adaptation.[26]

There are three types of RAS protein, NRAS, HRAS, and KRAS. Mutation of RAS protein is found in more than 90% pancreatic adenocarcinoma, 30%–50% in CRC, 55% in thyroid cancer, 35% in lung cancer, and 35% in rhabdomyosarcomas. KRAS mutation occurred in the highest frequency in all cancer types, followed by NRAS and HRAS.[27,28] KRAS mutation caused cells lost its ability to breakdown GTP into GDP in cellular signaling, leading to uncontrolled cell divisions.[29] Point mutations in KRAS gene predominantly occurred in codons 12 and 13 of exon 2, or codon 61 of exon 3.[30] In CRC, 85%–90% of KRAS mutations are in exon 2 (codons 12 and 13).[9] These two codons encode glycine in wild-type protein.[31] The most common alteration in codon 12 is GGT to GAT (G12D; aspartic acid replaced glycine). Glycine residue in this position has an important role for the normal function of KRAS protein. Therefore, base substitution of the glycine will facilitate a continuous GTPase synthesis.[27]

Most of the CRC cases in Indonesia are sporadic. A study in Indonesia found that only 16.3% of 43 sporadic CRC cases harbored KRAS mutation and no BRAF mutation. This low KRAS mutation may indicate a different pattern of genetic instability in these colorectal tumors. The absence of BRAF gene mutation suggested that the samples may not belong to the MSI-H group. [29] In our study, we did not identify and classify the samples regarding to MSS or MSI.

RAF genes that consist of ARAF, BRAF and RAF1, can undergo mutation. As much as 5%–15% of RAF mutations have been described in CRC.[32] BRAF mutation will activate MEK (MEK1 and MEK2) as well as ERK (ERK1 and ERK2) pathways. RAF mutation will activate MAPK pathway, regardless of receptor and ligand binding; this mutation affects the response of anti-EGFR therapy. Furthermore, activation of MAPK pathway because of BRAF mutation leads to uncontrolled cell proliferation.[33]

Patients with normal KRAS have two times progression-free survival better than patients with mutant KRAS.[34] In metastatic CRC, it is important to do KRAS evaluation before starting anti-EGFR therapy,[9] however, mutation analysis before anti-EGFR therapy is yet done only for codons 12 and 13.[14] Up to now, KRAS mutation is a major predictor for resistance of anti-EGFR therapy but important to note that it contributes only for 30%–40% of nonresponsive patients. Therefore, detection of alterations of other oncogenes is required; BRAF mutation, PIK3CA mutation, and loss of PTEN expression may contribute to the anti-EGFR resistance with normal KRAS, even though these parameters have not been routinely performed in clinical settings.

The significance of simultaneous presence of MGMT methylation and KRAS mutation in colorectal tumorigenesis cannot be concluded yet from this study. Furthermore, the limitation of this study such as small sample size may associate with no BRAF mutation cases. Nevertheless, as far as we know, our study is the first study which provides data on molecular profile of CRC patients in Denpasar, Bali, based on promoter MGMT methylation and KRAS and BRAF mutation.

CONCLUSIONS

Taken together, CRC case in this study was almost equal between genders and increased with age. Methylation pattern of MGMT promoter varied, however, mostly showed methylated CpGs. BRAF mutation V600E was not found, however, KRAS mutation at codons 12 and 13 have been identified. Our study showed that there was an association between MGMT promoter methylation and KRAS mutation.

Further studies with larger number of samples are required to identify the correlation between MGMT promoter methylation and gene expression, and KRAS and BRAF mutations, and its correlation with response of therapy and prognosis. Key molecular biomarkers are important for understanding and predict the prognosis, treatment response, and recurrence risk, and it can potentially represent targets for personalized therapies.

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Conflicts of interest

There are no conflicts of interest.

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