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The Association Between CYP24A1 rs4809957 Gene Polymorphism and Grade of Colorectal Cancer at Prof. Dr. I G.N.G Ngoerah Hospital in Bali

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ABSTRACT

Introduction: Colorectal cancer (CRC) is a malignancy in the large intestine, including the colon and rectum. One of the pathogenesis of CRC is the molecular pathway. This study aimed to evaluate the association between the CYP24A1 rs4809957 gene polymorphism and the histopathological grade of CRC patients at Prof. Dr. I G.N.G. Ngoerah Hospital in 2018-2019. ***Methods:*** This research was an observational analytic study with a cross-sectional design. This study used 34 samples of stored biological material from CRC tissue. CYP24A1 polymorphism (rs4809957) was identified using qPCR. ***Results:*** The results showed that the mean ± SD of the patient's age was 61.09 ± 11.79 years. A total of 18 patients (52.9%) of the patient sample were male, and 16 patients (47.1%) were female. The histopathological grade of CRC in this study consisted of 30 samples (88.2%) of low grade and 4 (11.8%) of high grade. There were 21 patients (61.8%) who had a history of smoking. In addition, 20 patients (58.8%) had a history of consuming alcoholic beverages. This study's CYP24A1 rs4809957 gene polymorphism comprised the AA+AG genotype in 23 samples (67.6%) and GG in 11 (32.4%). There was no significant association between the CYP24A1 rs4809957 gene polymorphism and the histopathological grade of CRC with a p-value = 0.580 and PR (95%CI) = 2.33 (0.83 – 19.42). ***Conclusion:*** This study concluded that there was no significant association between the CYP24A1 rs4809957 gene polymorphism and the histopathological grade of CRC patients.

Keywords: colorectal cancer; CYP24A1 rs4809957; gene polymorphism; histopathological grade

INTRODUCTION

Aging is a phenomenon that increases the risk of organ failure with age. Aging is a universal biological process that manifests as a decline in functional capacity and an increased risk of morbidity and mortality.[1] Aging occurs when tissue loses the ability to repair or replace itself and loses the ability to maintain normal structure and function. Thus, as a result, the tissue cannot survive and cannot repair the damage it suffers.[2] Aging is accompanied by a decline in physiological organ function over time and is a major risk factor for cancer development.

Aging and cancer are closely interconnected, and many of the same strategies and drugs can be used to target both.[3] One of the aging-associated cancers is colorectal cancer (CRC).

CRC is a malignancy originating from large intestinal tissue, including the colon and rectum, which is often associated with the aging process, especially T cell dysfunction involving telomere defects and telomerase activity.[4] According to the American Cancer Society, CRC is ranked third for the highest number of cases and fourth for cancer-related deaths.

Based on the GLOBOCAN survey in 2020, CRC ranks third with 10% of the highest cancer incidence and is the second leading cause of death with 9.4% after lung cancer. In Indonesia in 2018, CRC was in fourth position with 30,017 cases (8.6% of all cancer cases in Indonesia).[5] Meanwhile, research in Bali found 44 cases of colorectal cancer in 2017 at RSUP Prof. Dr. Dr. I G.N.G. Goerah Denpasar.[6]

Several known risk factors are associated with CRC development, such as elderly, history of chronic diseases, and lifestyle.[7] As a risk factor for CRC, elderly are also known to be the most significant main risk factor for morbidity and mortality.[3] Elderly is also associated with gradual loss of function or degeneration at the body's molecular, cellular and tissue levels.[8] If viewed based on histopathological aspects, low grade is the highest CRC grading compared to high grade.[9]

Degeneration at the molecular and cellular levels related to aging is thought to play a role in the pathogenesis of CRC, which is still being investigated. One of the pathogenesis pathways of CRC is the molecular pathway. The molecular pathway involves several complex genetic and epigenetic modulations that cause normal colonic mucosa to metamorphose into benign polyps and malignant tumors.[10] In the development of CRC, 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) plays an important role in inhibiting proliferation, invasion and metastasis. 24-hydroxylase, encoded by the CYP24A1 gene, is the key enzyme that catabolizes 1,25(OH)2D3 to the less active calcitroic acid. CYP24A1 is overexpressed in several types of human tumors, and changes in CYP24A1 expression are associated with cancer progression. Additionally, increased tumor expression of CYP24A1 correlates with a worse prognosis. CYP24A1 may be a candidate oncogene and an important biomarker for cancer diagnosis and prognosis.[11]

Based on the course of the disease, CRC is the most preventable and curable cancer of all human cancers. This is because a pre-clinical phase characterizes CRC until it becomes cancer over a long period of around 10 to 20 years.[12,13] Thus, the application of AAM in the pathogenesis of CRC, which is related to the CYP24A1 rs4809957 pathway, is an interesting thing to study, one of which is the association between the CYP24A1 rs4809957 polymorphism and CRC grading, which contributes to the prognostic value of the disease.

However, research on the association between the CYP24A1 rs4809957 polymorphism and CRC grade is still limited. Sun et al. (2016), in China with 99 samples, stated that CYP24A1 polymorphisms were more common in CRC with well and moderate differentiation, but there was no significant association between them.[11] Other research in China with 528 samples shows that this gene polymorphism is significantly related to medium-grade CRC and is protective against CRC.[14] These differences in findings have caused research regarding the association between the CYP24A1 rs4809957 polymorphism and the histopathological grade of CRC patients to become the focus of recent research that has not been carried out, especially in Indonesia.

The limited information about the CYP24A1 rs4809957 gene polymorphism cannot be separated from the lack of recent epidemiological data regarding the CYP24A1 rs4809957 gene polymorphism profile in CRC patients. Therefore, data describing the CYP24A1 rs4809957 gene polymorphism in CRC patients in Bali Province is needed. This research data can then become basic information about the CYP24A1 rs4809957 gene polymorphism in CRC patients, which can later be linked to other research in the future. This research data can also be linked to CRC patients' clinicopathological condition, which can later become a molecular key for CRC management in Indonesia, especially in Bali Province. This study aimed to prove the association between the CYP24A1 rs4809957 gene polymorphism and the histopathological grade of CRC patients in 2018-2019 at RSUP Prof. Dr. I G.N.G. Ngoerah.

METHODS

Study Design and Data Collection

This study used a cross-sectional design conducted at the Integrated Biomedical Laboratory Unit, Faculty of Medicine, Udayana University (January – August 2023). We used stored biological material in the form of CRC tissue specimens in the form of Formalin-Fixed Paraffin-Embedded (FFPE) treated at RSUP Prof. Dr. I G.N.G Ngoerah from 2018-2019 and kept at the Integrated Biomedical Laboratory, Faculty of Medicine, Udayana University. The subject inclusion criteria included (1) Having complete medical records (clinical data, histopathology) according to the variables evaluated in this study, (2) FFPE samples from CRC patients confirmed to be in good condition by an anatomical pathology specialist. This study's histopathological grade of CRC is classified as low grade and high grade.

Ethical Clearance

Research Ethics Committee Faculty of Medicine, Udayana University declared this study ethically feasible with No: 400/UN14.2.2.VII14/LP/2020.

Research Procedure

- **DNA Extraction**

DNA was extracted from tumor tissue following the manual instructions (Thermo Scientific GeneJET FFPE DNA Purification Kit #K0882). Briefly, 2-3 sections of FFPE tissue were added with 200 µL of digestion buffer into a microcentrifuge tube. The sample is incubated at 90°C for 3 minutes until it is completely dissolved. The sample was then cooled to room temperature and incubated at 65°C for 50 minutes in a thermoshaker with a vortex (300-400 rpm). After incubating the sample at 90°C for 40 minutes, centrifugation was performed at 6000×g for 1 minute and 200 µL of the digested lysate was transferred into a new 1.5 mL microcentrifuge tube. A total of 10 µL of RNase A solution was added and mixed thoroughly by vortexing.

After 10 minutes of incubation at room temperature, 200 µL of binding buffer and 400 µL of ethanol (96-100%) were added. The lysate was then transferred to a GeneJET DNA Purification Column and centrifuged for 1 minute at 6000 × g. The washing stage was carried out using 500 µL Wash Buffer 1 (with the addition of ethanol) and 500 µL Wash Buffer 2 (with the addition of ethanol). DNA is purified using 60 µL of elution buffer. The concentration of pure DNA was measured using SimpliNano (Biochrom). DNA is stored at -20 °C until use.

• SNP Genotyping

The SNP genotype of CYP24A1 rs4809957 was identified using quantitative real-time PCR (qPCR) using allele-specific primers by the rhAmpTM SNP Genotyping System kit (IDT Technologies). FAM and VIC dyes are used for the reference allele (A) and alternative allele (G), respectively. Briefly, 2x master mix and 40x reporter mix (20:1 ratio) were combined for a total reaction mixture of 10ul with the following formula: master mix and 5.3µl reporter mix. For amplification, 8µl of reaction mixture was combined with 2µl of diluted DNA (10ng/ul) and run for qPCR (qTower, Analytic Jena) with the following program: enzyme activation at 95^{oC} for 10 minutes, 40 cycles of denaturation at 95^{oC} for 10 seconds, annealing at 60^{oC} for 30 seconds and extension at 68oC for 20 seconds.

• Statistical Analysis

Data analysis using SPSS software in several stages. First, the descriptive analysis describes the characteristics of the subject in general. Second, bivariate analysis used Chi-Square test to find p-value and Prevalence Ratio (PR). The Fisher Exact test is carried out if the bivariate analysis requirements are unmet. The association was declared significant if the p-value <0.05 with a 95% confidence interval.

RESULTS

Study Characteristics

The number of samples used in this research was 34 samples. The mean ± SD age of the patients was 61.09 ± 11.79 years, and the majority were aged ≥60 years, as many as 18 patients (52.9%). Most patients were male, namely 18 subjects (52.9%). Over half of the sample had a histopathological grade of low-grade CRC patients, namely 30 subjects (88.2%). Most subjects had a history of smoking, namely 21 subjects (61.8%) and a history of consuming alcoholic beverages, namely 20 subjects (58.8%). Sample characteristics are presented in Table 1.

TABLE 1: Clinical characteristics of study subjects (N=34).

Variable	N = 34 n (%)
Age	
Mean ± SD	61.09 ± 11.79
<60 years	16 (47.1)
≥60 years	18 (52.9)
Gender	
Male	18 (52.9)
Female	16 (47.1)
Histopathological grade of CRC patients	
Low grade	30 (88.2)
High grade	4 (11.8)
History of smoking	
Yes	21 (61.8)
No	13 (38.2)
History of alcoholic beverages consumption	
Yes	20 (58.8)
No	14 (41.2)

Meanwhile, looking at the results of reading the CYP24A1 rs4809957 gene polymorphism, it was found that most subjects had the AA+AG genotype, namely 23 (67.6%). A total of 11 other subjects (32.4%) had the GG genotype. The frequency distribution of the CYP24A1 rs4809957 gene polymorphism is presented in Table 2.

TABLE 2: Frequency distribution of the CYP24A1 rs4809957 gene polymorphism.

CYP24A1 rs4809957 Gene Polymorphism	N = 34 n (%)
AA+AG	23 (67.6)
GG	11 (32.4)

The Association Between CYP24A1 rs4809957 Gene Polymorphism with Histopathological Grade of CRC Patients

Based on the analysis results, it was found that the majority of AA+AG genotypes were low grade, namely 21 samples (91.3%) and high grade 2 samples (8.7%). Most of the GG genotypes were low grade, namely 9 subjects (81.8%), and high grade was 2 samples (18.2%).

The analysis used to determine the association between the CYP24A1 rs4809957 gene polymorphism and the histopathological grade of CRC patients was the Fisher's Exact test because the Chi-Square test results did not meet the requirements (less than 5 cells more than 20%). The analysis showed no significant association between the CYP24A1 rs4809957 gene polymorphism and the histopathological grade of CRC patients with a p-value = 0.580 and PR (95%CI) = 2.333 (0.283 – 19.242). The results of the analysis are presented in Table 3.

TABLE 3: The association between CYP24A1 rs4809957 gene polymorphism with histopathological grade of CRC patients.

	Histopathological Grade of CRC Patients		p-value	PR (95%CI)
	Low Grade n (%)	High Grade n (%)		
CYP24A1 rs4809957 gene polymorphism			0.580	2.333 (0.283 – 19.242)
AA+AG	21 (91.3)	2 (8.7)		
GG	9 (81.8)	2 (18.2)		

p: significance with the Fisher Exact Test.

Histopathological Grade Factors in CRC Patients

Confounding variables, namely age, gender, smoking history, and history of consuming alcoholic beverages, were analyzed for the histopathological grade of CRC patients and the CYP24A1 rs4809957 gene polymorphism.

Age (p = 0.604), gender (p = 1.000), smoking history (p = 0.274), and history of consuming alcoholic beverages (p = 1.000) were not significantly related to the histopathological grade of CRC patients (Table 4).

TABLE 4: Factors of histopathological grade in CRC patients.

	Histopathological Grade of CRC Patients		p-value
	Low Grade n (%)	High Grade n (%)	
Age			
<60 years	15 (93.8)	1 (6.3)	0.604
≥60 years	15 (83.3)	3 (16.7)	
Gender			
Male	16 (88.9)	2 (11.1)	1.000
Female	14 (87.5)	2 (12.5)	
History of smoking			
Yes	20 (95.2)	1 (4.8)	0.274
No	10 (76.9)	3 (23.1)	
History of alcoholic beverages consumption			1.000
Yes	18 (90.0)	2 (10.0)	
No	12 (85.7)	2 (14.3)	

p: significance with the Fisher Exact Test.

DISCUSSIONS

Genetic polymorphism is a change in DNA sequence in the general population with a frequency of >1%. Based on the classification, polymorphisms are divided according to size and structure into small-scale polymorphisms related to base sequences and large-scale polymorphisms related to chromosomal changes. Genetic polymorphisms do not directly cause a disease but act as predisposing factors.[15]

Various types of cancer, including CRC, have been identified to be associated with SNPs in individual susceptibility. SNP is a difference in the arrangement of a single nucleotide base in an individual's genome that causes genetic variation in a population. Generally, there is a similarity of up to 99.9% and only around 0.1% of the nucleotide bases that make up the human genome.[16] One SNP that is interesting to investigate is CYP24A1 rs4809957 because it is linked to CRC.

CYP24A1 is a cytochrome P450 enzyme of the inner mitochondrial membrane that uses the soluble iron-sulfur protein adrenodoxin and a flavoenzyme or so-called adrenodoxin reductase to transfer electrons derived from NADPH to the heme center.[17]

CYP24A1 is located on chromosome 20 (NG_008334.1) with a length of 27529 bp. This gene consists of 11 introns and 12 exons. The SNP position is at 24301 exon 12.[18]

Vitamin D deficiency has a role in various cancer processes, including CRC. CYP24A1, which participates in mechanisms related to specific vitamin D metabolism disorders in the body, is associated with CRC susceptibility. Additionally, CYP24A1 has been shown to promote tumorigenesis. The most common changes in sporadic CRC are demonstrated by aberrant activation of WNT/b-catenin signaling and subsequent activation of target genes. WNT/b-catenin pathway and b-catenin transcriptional activity in CRC cells WNT is inhibited by 1,25(OH)2D3 activity.[19]

CYP24A1 polymorphism causes inhibition of the catabolism of the active form of vitamin D, namely calcitriol. The higher the CYP24A1 polymorphism, the lower the activated vitamin D.[20] Calcitriol binds to VDR and produces transcriptional activation and repression of target genes, causing increased apoptosis, decreased angiogenesis and cell differentiation so that disruption of its synthesis can increase the risk of CRC.[21] Calcitriol inhibits proliferation, migration, invasiveness and angiogenesis of CRC cells, increases differentiation, and makes these cells sensitive to apoptosis. Calcitriol reduces the protumoral effect of CRC-associated fibroblasts. Calcitriol also regulates intestinal immune cells and influences the intestinal microbiota.[22] Thus, the presence of CYP24A1 polymorphisms that inhibit calcitriol catabolism can impact the histopathological grade of CRC. Allele A can influence the CYP24A1 rs4809957 polymorphism because it is the reference allele in this gene. Reference allele refers to matching an allele to a specific region of SNP in the reference genome. The reference allele plays an important role in the polymorphism of a gene, but the allele is not always the major allele.[23]

Research on the association between the CYP24A1 rs4809957 polymorphism and histopathological grade of CRC is still limited. The analysis results in this study showed no significant association between the CYP24A1 rs4809957 gene polymorphism and the histopathological grade of CRC patients with a p-value = 0.580. Sun et al. (2016) research also showed the same thing, which found no significant association between CYP24A1 expression and the histopathological grade of CRC patients. It could be due to the unequal difference in sample size between the low-grade and high-grade groups in the study by Sun et al. (2016). These conditions are similar to research conducted by the author. The study by Sun et al. (2016) divided the histopathological grade of CRC patients into two groups, namely well and moderate (low grade) and poor differentiation (high grade). This study also shows that CYP24A1 expression occurs more frequently in CRC with well and moderate differentiation (low grade) than poor differentiation (high grade). It is in line with the results of this study, namely that the majority of AG genotypes were low grade, namely 21 samples (91.3%), and high grade was 2 samples (8.7%). Most of the GG genotypes were low grade, namely 9 subjects (81.8%), and high grade was 2 samples (18.2%).[11]

The CYP24A1 rs4809957 polymorphism AA genotype significantly increased the risk of CRC 2.38 (1.30-4.37) times (p = 0.005). Research by Chai et al. (2021) shows that CYP24A1 polymorphisms are more common in low-grade CRC patients (p = 0.15).[24] Sun et al. (2016) also found a similar study, which showed that CYP24A1 polymorphisms were more common in low-grade CRC patients with insignificant results (p = 0.137).[11]

Meanwhile, another study by Gong et al. (2017) in China with 528 samples showed that the CYP24A1 rs4809957 A/G polymorphism had a statistically significant association with the risk of colon polyps, colon cancer and ulcerative colitis.[14] These results also show that the CYP24A1 polymorphism is significantly related to medium-grade CRC and is protective against CRC [p = 0.002 and OR 95%CI = 0.51 (0.34–0.78)]. Research by Gong et al. (2017) divided the histopathological grade of CRC patients into three groups: low grade, medium grade and high grade.[14] Therefore, the difference in grading classification is not comparable with the research conducted by the author.

CYP24A1 rs4809957 polymorphism occurs in elderly CRC patients.[11] The allelic variations of the CYP24A1 rs4809957 polymorphism consist of allele A and allele G. Allele A is a risk factor for the development of CRC. Meanwhile, the G allele is protective against CRC.[24] Alleles that do not match the base sequence will be destructive, thereby reducing the levels of protein produced.[25] People who have CYP24A1 gene polymorphism may have a higher barrier to vitamin D absorption, which raises the possibility of developing CRC.[24]

Several factors could cause this difference in results. Most of the subjects in this study (88.2%) experienced low-grade CRC. Therefore, statistical analysis will show insignificant results because comparisons between groups of variables have a large range of values. It is associated with sample homogeneity, or most samples have the same variance.[26]

In addition, the number of subjects in this study was relatively small. The larger the sample size, the greater the potential to represent the population. However, no definite rules exist regarding the minimum number of samples representing a population. The mean and SD of analysis results with a large sample size will likely resemble the population mean and SD. However, if the sample size is relatively small, it can represent the population well if selected through randomization.[27]

Limitations in this research consist of several elements. First, the sample used was stored biological material; thus, it was impossible to select the latest sample using pure Balinese ethnic criteri

or further study other variables to obtain more comprehensive results. Second, the number of samples used in this research is relatively small. Further research can be carried out using a larger sample size. This is the first research in Bali to analyze the association between the CYP24A1 rs4809957 gene polymorphism and the histopathological grade of CRC patients.

CONCLUSIONS

There was no significant association between the CYP24A1 rs4809957 gene polymorphism and the histopathological grade of CRC patients in 2018-2019 at RSUP Prof. Dr. I G.N.G Ngoerah. Further research could be carried out to evaluate patient characteristic data more comprehensively, such as family history, diet, activity level, and previous disease history. It involves other confounding variables to analyze independent factors on the histopathological grade of CRC. Research involving a larger number of samples or different locations (multi-center) can be carried out to evaluate comparisons of findings between studies so that we can determine the diversity of results found and become the basis for further studies such as meta-analysis.

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