# Association of *BMP4* RS444235 Gene Polymorphism and the Stage and Grade of Colorectal Cancer at Prof. dr. I G.N.G Ngoerah General Hospital

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**Abstract**

Aging is one of the main risk factors for cancer, with one of the most common in the elderly population is colorectal cancer, which is the third most common cancer and the fourth most common cause of death in the world. One of the biomarkers studied which is related to the balance of colorectal cancer tumorigenesis is bone morphogenetic proteins (BMPs). The *BMP4* gene polymorphism, namely *BMP4* rs4444235, has been associated with an increased risk of colorectal cancer. This study used a cross-sectional analytical method to analyze the relationship between the *BMP4* rs44444235 gene polymorphism and colorectal cancer at Prof. dr. I G.N.G. Ngoerah General Hospital using 41 FFPE samples from colorectal cancer. The identification of *BMP4* rs4444235 gene polymorphism was carried out using the Polymerase Chain Reaction (PCR) and sequencing. The data obtained was then processed using SPSS 25.0. Out of 30 colorectal cancer patients, 17 samples (56.7%) were male and 13 samples (43.3%) were female, with 24 samples (60%) had advanced stage colorectal cancer and 6 samples (20%) had early stage. A number of 28 samples (93.3%) were of low-grade colorectal cancer and 2 samples (6.7%) were of high grade. *BMP4* gene polymorphism rs4444235 of CC genotype were found in 8 samples (26.7%), a CT genotype in 14 samples (46.7%), while a TT genotype in 8 samples (26.7%). The test showed the *BMP4* rs4444235 gene polymorphism was not associated with the stage and grade of colorectal cancer ($p>0.05$), with p value of 0.547 and 0.294 for stage and grade of colorectal cancer respectively.

Taken together, *BMP4* rs4444235 gene polymorphism was identified, however there was no association found with the stage and grade of colorectal cancer at Prof. dr. I G.N.G. Ngoerah General Hospital.

*Keywords:* stage; grade; colorectal cancer; polymorphism; *BMP4* rs4444235 gene

## 1. INTRODUCTION

Aging is synonymous with a decrease in the physiological function of organs which can then be followed by a decrease in a person's quality of life. Along with advances in science and technology, especially in the health sector, it is known that aging is also a major risk factor for cancer. One of the most common cancers in the elderly population is colorectal cancer, which is the third most common cancer and the fourth most common cause of death in the world. An increased risk of developing colorectal cancer is known to occur in subjects who are older, have a history of chronic diseases, and have an unhealthy lifestyle (Mármol *et al*., 2017; Sayuti & Nouva, 2019). It is known that 396,914 new cases of colorectal cancer were recorded with 234,511 deaths in Indonesia (Zannah *et al*., 2021).

One of the pathogenesis of colorectal cancer is the molecular pathway in colorectal carcinogenesis which involves several complex genetic and epigenetic modulations that cause changes from normal colonic mucosa into benign polyps and then into malignant tumors (Kasi *et al*., 2020). The heritability of genetic predisposition to colorectal cancer is estimated at 12-35%. For individuals with certain hereditary cancer syndromes, the lifetime risk for colorectal cancer can approach 50-80% in the absence of endoscopic and/or surgical intervention (Valle *et al*., 2019; Yusuf *et al*., 2021). In the pathogenesis of colorectal cancer, one of the biomarkers studied which is related to the balance of colorectal cancer tumorigenesis is bone morphogenetic proteins (BMPs). Bone morphogenetic proteins are a class of molecules with more than 20 diverse growth factor proteins, belonging to the transforming growth factor-β (TGF-β) family and

are strongly associated with bone formation and disease development. Aberrant expression of BMPs has been reported in several cancer tissues (Bach *et al*., 2018).

The *BMP4* gene polymorphism, namely *BMP4* rs4444235, has been associated with an increased risk of colorectal cancer. The results of meta-analysis studies have identified an increased risk of colorectal cancer in T-C alterations at rs4444235, a 9.4 kb locus upstream of the transcription start site of the *BMP4* gene. The homozygous C allele CC and heterozygous CT were found to have a positive correlation with an increased risk of colorectal cancer, compared to the homozygous TT allele (Li *et al*., 2012). Apart from having an important role in identifying the molecular phenotype of colorectal cancer, the study of polymorphisms also has an important role in determining cancer treatment (Valle *et al*., 2019).

*BMP4* rs4444235 gene polymorphism is known to play a role in the pathogenesis, prognosis and therapy of colorectal cancer, however studies related to this polymorphism have not been published in Indonesia, especially in Bali. This research data can benefit for future research of the *BMP4* rs4444235 gene polymorphism in colorectal cancer patients. Apart from that, this research data can also be linked to the clinicopathological condition of colorectal cancer patients which can later become a molecular key for colorectal cancer management in Indonesia, especially in Bali.

## 2. METHODS

### *2.1. Study Design*

This research was conducted using a cross-sectional analytical method. The research subjects were patients confirmed histologically as colorectal cancer at Prof dr. I G.N.G Ngoerah General Hospital in 2018-2020. The samples needed in this study were 30 Formalin-Fixed Paraffin-Embedded (FFPE) samples stored at the Department of Pathology, Faculty of Medicine, Udayana University. The research was conducted at the Integrated Biomedical Laboratory, Faculty of Medicine, Udayana University, and approved by ethical committee no. 821/UN14.2.2.VII.14/LT/2023.

### *2.2. Experimental Procedure*

#### *2.2.1. DNA Extraction from FFPE*

DNA was extracted using Black Prep FFPE DNA Kit, with following steps: (1) FFPE sample was sliced with a thickness of around 10 µm in 2 to 5 slices with a microtome; (2) FFPE slices were placed in a 1.5 mL microcentrifuge tube and centrifuged at a speed of 13,000 rpm; (3) FFPE samples were lysed with 400 µL of MA solution and 40 µL of Proteinase K and vortexed for 10 seconds; (4) The sample was then incubated at 65° C and 90° C for 1 hour each in a thermal mixer and centrifuged at a speed of 1,000 rpm; (5) Sample was incubated for 5 minutes at room temperature, then centrifuged at maximum speed for 2 minutes and transferred to a new 1.5 mL test tube; (6) Absolute ethanol (400 µL) was then mixed and put into a vortex for 10 seconds, then applied to a spin filter with a 2 mL receiving tube and centrifuged at 12,000 rpm for 1 minute; (7) The original filtrate receiving tube was discarded and a spin filter was applied to the new receiving tube. Sequential washing steps were carried out using washing solution C and washing solution BS; (8) The receiving tube was removed and a spin filter was applied to the elution tube; (9) The DNA was then eluted in 100 µL of elution buffer. A total of 1 µL of DNA was used to measure the concentration, using a spectrophotometer (Biochrom).

#### *2.2.2. Identification of BMP4 rs4444235*

*BMP4* rs4444235 was identified using PCR amplification and sequencing. Amplification was carried out in a total volume of 10 µL containing 5 µL of green master mix, 0.3 µM of each primer, 0-1.6 µL of ddH2O, and 3-4.6 µL of isolated DNA, with a concentration of 10 ng/µL. PCR was carried out at 95˚C for 5 minutes and followed by 40 cycles of denaturation at 95˚C for 15 seconds, annealing at 50˚C for 60 seconds and at 72˚C for 30 seconds, and finally at 72˚C. C for 5 minutes. PCR product was visualized using 1.5% gel electrophoresis. PCR products were then sequenced in Genetic Science Laboratory, Jakarta. Genotype of *BMP4 rs4444235* was evaluated using Chromas software. The sequencing results were then checked using the Basic Local Alignment Search Tool (BLAST). BLAST was accessed via the website https://blast.ncbi.nlm.nih.gov/Blast.cgi.

*2.3. Data Analysis*

Chi-square test was used to determine the relationship between *BMP4* rs4444235 polymorphism to the stage and grade of colorectal cancer. The significance limit used was $p < 0.05$ (95% confidence interval), while Fisher-exact test was used on data that did not meet the requirements of Chi-square.

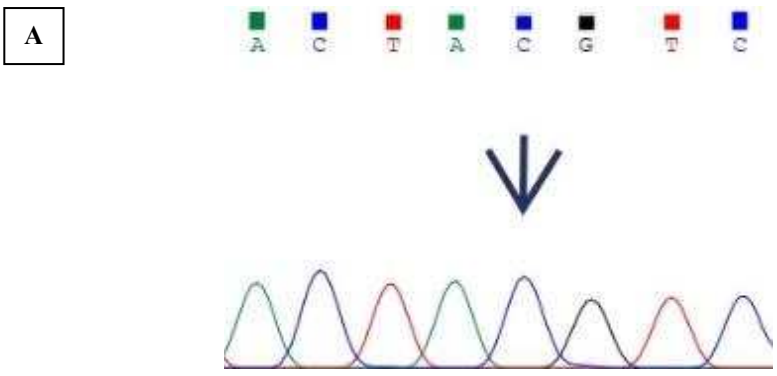
## 3. RESULTS

This research was conducted involving 30 research samples with a mean age of the research samples of 64.8 ± 11.1 years. In terms of gender, the majority of the research sample was male, 17 people (56.7%), and the remaining 13 people (43.3%) were female.

Table 1. Basic Characteristics of Samples

| Variables | n(%) |
|---|---|
| Age | 64.8±11.1 |
| Sex | |
| Men | 17 (56.7%) |
| Women | 13 (43.3%) |
| Polymorphism | |
| TT | 8 (26.7%) |
| CT | 14 (46.7%) |
| CC | 8 (26.7%) |
| Stage | |
| Early | 6 (20.0%) |
| Late | 24 (80.0%) |
| Grade | |
| High Grade | 2 (6.7%) |
| Low Grade | 28 (93.3%) |

Chromatogram illustration of the polymorphism sequencing result of homozygote CC and TT and heterozygote CT is presented in Figure 1.



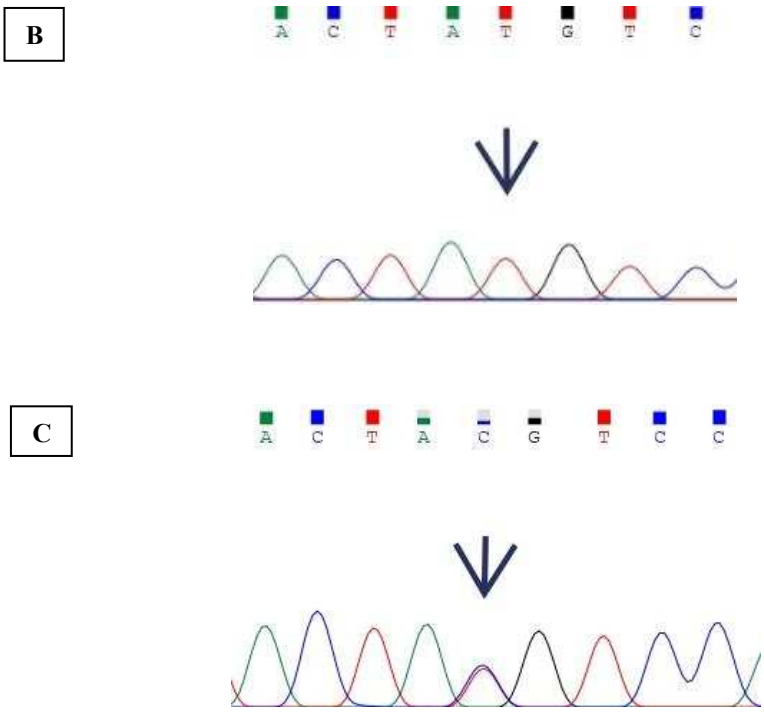



Fig. 1. Chromatogram illustration of the results of sequencing the CC *BMP4* rs 4444235 polymorphism in colorectal cancer. (A) Genotype CC (B) Genotype TT (C) Genotype CT

Based on the bivariate analysis carried out to examine the association of *BMP4* rs4444235 polymorphism to the stage of colorectal cancer using Fisher's Exact test, no significant relationship was found (p>0.05). The data is presented in Table 2.

Table 2. Bivariate Analysis of *BMP4* rs4444235 Polymorphism on Stage of Colorectal Cancer

| *BMP4* rs4444235 Polymorphism | Stage | | OR | CI95% | P |
|---|---|---|---|---|---|
| | Early | Late | | | |
| Genotype | | | | | |
| CC | 7 (87.5%) | 1 (12.5%) | - | - | 0.547 |
| CT | 10 (71.4%) | 4 (28.6%) | | | |
| TT | 7 (87.5%) | 1 (12.5%) | | | |
| Genotype | | | | | |
| CC + CT | 17 (70.8%) | 5 (83.3%) | 0.486 | 0.048 | 1,000 |
| TT | 7 (29.2.%) | 1 (16.7%) | | 4.945 | |

Based on the bivariate analysis carried out to examine the association of *BMP4* rs4444235 polymorphism to grade of colorectal cancer using Fisher's Exact test, no significant relationship was found (p>0.05). The data is presented in Table 3.

Table 3. Bivariate Analysis of *BMP4* rs4444235 Polymorphism on Grade of Colorectal Cancer

| *BMP4* rs4444235 Polymorphism | Grade | | OR | CI95% | P |
|---|---|---|---|---|---|
| | Early | Late | | | |
| Genotype | | | | | |
| CC | 0 (0.0%) | 8 (100%) | - | - | 0.294 |

| | | | | | |
|---|---|---|---|---|---|
| CT | 2 (14.3%) | 12 (85.7%) | | | |
| TT | 0 (0.0%) | 8 (100%) | | | |
| Genotype | | | | | |
| CC + CT | 2 (100%) | 20 (71.4%) | - | - | 1,000 |
| TT | 0 (0.0%) | 8 (28.6%) | | | |

## 4. DISCUSSION

### *4.1. Samples Characteristics*

This study found that the majority of men tend to experience colorectal cancer. This is in line with previous studies which found that the ratio between men and women and the incidence of colorectal cancer was 67:50, indicating that more male patients suffer from colorectal cancer. However, there were no statistically significant differences in these two groups (Savu *et al*., 2023). Another study also obtained similar results to the current study, that a higher percentage of male patients experienced colorectal cancer compared to women, namely 65.2% in the non-mucinous adenocarcinoma (NMAC) colorectal cancer group and 75.3% in the mucinous carcinoma (MAC) type group (Lan *et al*., 2021). This study also found that the average age of colorectal cancer patients was 64.83 ± 11.194 years. Previous studies by Dwijayanthi *et al*. also supports the results of the current study which states that the majority of colorectal cancer patients were patients over 50 years of age (92.3%) (Dwijayanthi *et al*., 2020).

Previous studies stated that there was a relationship between old age and increased signaling in the *BMP4* gene. Aging is known to be associated with decreased neurogenesis in the hippocampus and decreased cognitive function associated with the hippocampus. Furthermore, the study found that there was a 10-fold increase in *BMP4* gene expression with increasing age in the dentate gyrus of experimental mice. On the other hand, this study also found a decrease in BMP gene expression inhibitors. BMP signaling plays a role in impaired neurogenesis and decline in cognitive function associated with aging (Meyers *et al*., 2016). Judging from the polymorphism variants, this study showed that the CT heterozygous variant was the most common variant found in 14 samples (46.7%), with CC and TT homozygous variants each in 8 samples (26.7%). A previous study conducted by Yang *et al*. in 2014 in Taiwan, stated that the *BMP4* rs4444235 variant found that the C allele was more likely to be retained in tumor tissue in colorectal cancer, and this was found to be statistically significant with a p value <0.05 (P=0, 0023) (Yang *et al*., 2014).

This is also in line with previous studies which stated that the *BMP4* rs4444235 polymorphism may be associated with colorectal cancer with the C allele as a possible risk factor with an OR of 1.11 (IK95: 1.08-1.15). The study also analyzed the variants of the C allele, with homozygous CC genotype and heterozygous CT genotype variants, and the ORs were 1.063 (95% CI = 1.034–0.092), 1.081 (95% CI = 1.028–1.136), and 1.166 (95% CI = 1.081–1.258) for C versus T, CT versus TT, and CC versus TT comparisons respectively. Furthermore, this meta-analysis study also analyzed the association between the C allele in *BMP4* rs4444235 and the risk of colorectal cancer based on ethnicity, geographic region, and control source. This study found that there was an increase in the frequency of the C allele in Caucasians and Asians compared to controls, but there was a decrease in cases in Africans compared to controls. This indicates that the C allele can be a risk factor in individuals with Caucasian and Asian races but may have the possibility of being a protective factor in African races. This difference in results could occur because the study used limited study subjects or at a hospital location in a certain area there was high multiethnicity so that the results of the study used were less representative (Li *et al*., 2012).

Previous studies showed that germline mutations in the *BMP4* gene can affect the function of the *BMP4* protein, conferring a juvenile polyp carrier phenotype, that *BMP4* can increase cell adhesion to fibronectin and collagen, enhance the metastatic and invasive potential in humans of the colorectal cancer cell line HCT 116, and that *BMP4* can inhibit heat-induced apoptosis in HCT 116 cells via ERK activation and JNK inactivation (Deng *et al*., 2007; Li *et al*., 2012).

Regarding the distribution of cancer stages in the research sample, the majority of the research samples were colorectal cancer patients with advanced stages (stages III and IV) totaling 24 samples (80.0%) and the remainder were early stage (stages 0, I, and II) as many as 6 samples (20.0%). This is in line with previous studies which found that advanced stages (stage III) were often found in patients with colorectal cancer (Savu *et al*., 2023). In terms of cancer grading, the research sample was dominated by 28 people with low grade (93.3%), and only 2 samples were found

with high grade (6.7%). In previous studies it was found that the majority of patients were grade 2 (59.3%). However, statistical analysis did not find any significant differences in the grading aspect between the groups of complicated and uncomplicated colorectal cancer patients (Savu *et al*., 2023).

### *4.2. Association between BMP4 rs4444235 gene polymorphism and stage of colorectal cancer at Prof. dr. I G.N.G. Ngoerah General Hospital*

Based on the analysis carried out to test the relationship of the BMP rs4444235 polymorphism to colorectal cancer stage in this study, no significant relationship was found with a p value of >0.05. A study before, showed that increased *BMP4* gene expression can influence Wnt signaling which can trigger B-catenin activation. B-catenin activation is one of the conditions that initiates the development of cancer cells. The presence of Wnt signaling will influence the differentiation of crypt basal cells in the colonic epithelium. If there is excessive expression of *BMP4*, it will result in a migratory phenotype and a more invasive tumor condition (Lubbe *et al*., 2012). Previous studies have analyzed the role of the BMP gene and other types of cancer, especially in aspects of cancer cell progression and its involvement in metastasis. Previous studies have explained that there is a role for BMP in the development of cancer cells, such as prostate cancer. This study states that there is a role for the BMP gene in stimulating bone metastasis in prostate cancer. The BMP gene is a gene that is expressed by several organs, such as the prostate and breast. In previous studies, it was found that the BMP-2 gene and the BMP-5 gene have the ability to inhibit proliferation, modulate steroidogenesis of human adrenocortical tumor cells in vitro through a BMP-dependent mechanism (Singh & Morris, 2010).

Previous studies also found that there is a role for the *BMP4* gene in hepatocellular cancer in the process of tumor cell migration and growth. These characteristics can be seen through *BMP4*-specific siRNA. Apart from that, other studies also show a role for *BMP4* in lumen formation, the growth of invasive cord-like structures, cells adhesion in mammary epithelial cells and inducing EMT in pancreatic cancer. This gene can also stimulate migration and invasion of various cancers including melanoma and breast (Maegdefrau *et al*., 2009; Montesano, 2007; Singh & Morris, 2010). In colorectal cancer, increased *BMP4* gene expression can influence Wnt signaling which can trigger B-catenin activation. B-catenin activation is one of the conditions that initiates the development of cancer cells. The presence of Wnt signaling will influence the differentiation of crypt basal cells in the colonic epithelium. If there is excessive expression of *BMP4*, it will result in a migratory phenotype and a more invasive tumor condition (Lubbe *et al*., 2012). Previous studies analyzing the role of BMP and Wnt signaling in colorectal cancer showed that activation of BMP signaling results in an increase in Wnt signaling. Furthermore, the study analyzed mutations that could affect BMP-Wnt interactions. Colorectal cell lines that had an inverse correlation in BMP and Wnt activity or low BMP signaling activity in line with increased Wnt signaling were shown to have SMAD4 and p53. This indicates that if there are mutations such as loss of SMAD4 and/or p53, the BMP-Wnt interaction can increase or decrease (Voorneveld *et al*., 2015).

Until now there has been no full explanation regarding the relationship between Wnt and BMP in colorectal cancer. However, previous studies have shown that the paradoxical B-catenin (resulting from constitutive activation of Wnt signaling) can at least partly be explained by the effects of SMAD4 loss or p53 mutations on the ability of BMP signaling to suppress Wnt signaling in colorectal cancer cells in in front of an invasive tumor. Furthermore, the findings of previous studies indicating the possible involvement of the BMP pathway in determining the level of Wnt signaling in invasive tumor cells is that there is a combination of extensive stroma in the tumor section and loss of SMAD4 which causes a worse tumor prognosis. High levels of Wnt signaling are seen in cells that have the ability to invade and metastasize. This condition occurs during the transition from advanced adenoma to invasive carcinoma and the two mutations most frequently found at this stage are SMAD4 and p53 (Voorneveld *et al*., 2015). The results of this study are also in line with previous studies showing that loss of SMAD4 is associated with increased levels of Wnt activity in colorectal cancer cell lines and the presence of p53 mutations was found to increase Wnt signaling activity in vitro (Kim *et al*., 2011; Freeman *et al*., 2012).

### *4.3. Association between BMP4 rs4444235 gene polymorphism and grade of colorectal cancer at Prof. dr. I G.N.G. Ngoerah General Hospital*

Based on the analysis carried out to test the relationship of the BMP rs4444235 polymorphism variant to colorectal cancer grading in this study, no significant relationship was found with a p-value > 0.05. The relationship between

BMP and cancer grading is currently limited to other types of cancer and nothing specific to colorectal cancer. Li *et al*'s meta-analysis study was conducted to examine the association between the *BMP4* rs4444235 polymorphism and the risk of colorectal cancer. Odds ratios (ORs) with 95% CIs were pooled as indicators of effect. A comprehensive search of relevant publications was conducted and publications that met the inclusion criteria were included. Heterogeneity tests, meta-regression, subgroup analysis, cumulative meta-analysis, assessment of publication bias, and sensitivity tests were performed using Stata 11.0. 8 articles on rs4444235 and obtained 19,893 cases and 22,106 controls used in the study. The study found little heterogeneity that may stem from ethnicity and source of control. The pooled results for all five genetic models were statistically significant. In the Caucasian population, carriers of the C allele, CC genotype, and CT genotype had an increased risk of developing CRC, with ORs of 1.079 (95% CI = (1.044, 1.114)), 1.095 (95% CI = (1.034, 1.159))), and 1.199 (95% CI = (1.117,1.287)) respectively. Cumulative meta-analysis showed that the pooled OR approached 1.1 as the year of publication progressed. The sensitivity test showed stable results. The study confirmed that the *BMP4*-rs4444235 polymorphism plays a role in CRC risk, and the C allele is a possible risk factor in the population as a whole. The same conclusion was also drawn in Caucasians, but no significant results were obtained in other ethnic populations, which is likely due to the limited sample size (Li *et al*., 2012).

A study by Liu *et al.* (2014) used a comprehensive strategy of logistic regression and a mode-free approach to more precisely evaluate the role of *BMP4* rs4444235 in susceptibility to colorectal cancer (CRC). A total of 19 studies with 28770 cases and 28234 controls were included. Metagene analysis found that *BMP4* rs4444235 was the best fit for the additive model. In assessing the additive model, heterogeneity was observed (P=0.059, I2=36.1), and the pooled OR per allele was 1.08 (95% CI=1.05–1.11). Based on the model-free approach, the pooled ORG was 1.09 (95% CI=1.05–1.14) based on the random effects model. Stratified analysis showed that heterogeneity could be partially explained by population ethnicity, study design, control source, and sample size. Sensitivity analyzes further supported the strong stability of the current results, by showing a similar set of estimates before and after sequential removal of each study. This meta-analysis provided a strong estimate of the positive association between rs4444235 and CRC risk and further emphasized the importance of *BMP4* rs4444235 in CRC risk prediction.

A study by Geng *et al.* (2015) aimed to investigate the association between colorectal cancer (CRC) genetic susceptibility variants in the Han population in China. The study was a case-control study conducted on 360 esophageal cancer patients and 310 healthy controls. Thirty-one single nucleotide polymorphisms (SNPs) associated with CRC risk from previous genome-wide association studies were analyzed. The study found that the minor allele of rs4444235 was associated with a 1.28-fold increased risk of cancer (95%CI: 1.03-1.60; P = 0.028). Green and Pisano's study states that bone morphogenetic protein (BMP) is part of the TGF-β signaling pathway. Genetic variations in this gene may be involved in colorectal cancer. In this study, we evaluated the relationship between genetic variations in *BMP4* and the risk of CRC cancer, as well as the molecular phenotype of the tumor. The study used data from a population-based case-control study (colon cancer n=1574 cases, 1970 controls; rectal cancer n=791 cases, 999 controls). The study observed that *BMP4* genetic variations were associated with the risk of developing colorectal cancer, with a 20% to 30% increased risk in most high-risk genotypes. The high-risk genotype showed a twofold increased risk of colon cancer compared to the upper risk category (OR 2.49 95% CI 1.95, 3.18). Genes in the BMP signaling pathway were consistently associated with CIMP+ status in combination with KRAS -mutated tumors and MSI tumors. The *BMP4* gene interacts statistically significantly with other genes in the TGF-β signaling pathway, including TGFβ1, TGFβR1, Smad 3, Smad 4, and Smad 7 (Greene and Pisano, 2012).

*BMP4* has diverse functions, and abnormalities in the BMP signaling pathway are involved in cancer development. *BMP4* activates the proliferation of certain cancer cells. Malignant cancer cell phenotypes, such as increased motility, invasion, and stemness, are enhanced by BMPs. Simultaneously, *BMP4* acts on various cellular components and regulates angiogenesis in the tumor microenvironment. Thus, *BMP4* functions as a pro-tumorigenic factor in various types of cancer. However, similar to TGF-β, which shows both positive and negative effects on tumorigenesis, *BMP4* also acts as a tumor suppressor in other types of cancer. In a previous study we reviewed important findings published in the last decade and summarized the pro-oncogenic function of *BMP4*, down to the underlying mechanisms (Ehata and Miyazono, 2022).

## 5. CONCLUSION

There was no association between *BMP4* rs4444235 gene polymorphism and stage and grade of colorectal cancer at Prof. dr. I G.N.G. Ngoerah General Hospital. Further research is needed to evaluate and validate the findings in this study with a larger sample size and with other research methods, so that they can better represent the population. Other confounding variables need to be considered in the study, to obtain an independent relationship between the BMP4 rs4444235 gene polymorphism and the stage and grade of colorectal cancer.

## References

Bach, D. H., Park, H. J., & Lee, S. K. 2018. The Dual Role of Bone Morphogenetic Proteins in Cancer. Molecular Therapy. Oncolytics 8(3), p. 1–13. Available from: https://doi.org/10.1016/j.omto.2017.10.002

Deng, H., Ravikumar, T. S. & Tang, W. L. 2007. Bone Morphogenetic Protein-4 Inhibits Heat-Induced Apoptosis by Modulating MAPK Pathways in Human Colon Cancer HCT116 cells. Cancer Letters 256, p. 207-17.

Dwijayanthi, N. K. A., *et al*. 2020. Karakteristik Pasien Kanker Kolorektal di Rumah Sakit Umum Pusat (RSUP) Sanglah Berdasarkan Data Demografi, Temuan Klinis dan Gaya Hidup (article in Indonesia language). Jurnal Medika Udayana 9(6), pp. 55-62.

Ehata, S. and Miyazono, K. 2022. Bone Morphogenetic Protein Signaling in Cancer; Some Topics in the Recent 10 Years. Frontiers in Cell and Developmental Biology 10(5), p. 1–14. Available from: https://doi.org/10.3389/fcell.2022.883523

Freeman, T. J., Smith, J. J., Chen, X., Washington, M. K., Roland, J. T., Means, A. L., *et al*., 2012. Smad4-Mediated Signaling Inhibits Intestinal Neoplasia by Inhibiting Expression of β-Catenin. Gastroenterology 142(3), pp. 562-71.

Geng, T. T., Xun, X. J., Li, S., Feng, T., Wang, L. P., Jin, T. B., *et al*. 2015. Association of Colorectal Cancer Susceptibility Variants with Esophageal Cancer in a Chinese Population 21 (22), pp. 6898-904.

Greene, R. and Pisano, M. M. 2012. Genetic Variation in Bone Morphogenetic Protein (BMP) and Colon and Rectal Cancer. Birth Defects Res C Embryo Today 90(2), p. 133–54. Available from: https://doi.org/10.1002/ijc.26047.Genetic

Kim, N. H., Kim, H. S., Kim, N. G., Lee, I., Choi, H. S., Li, X. Y., *et al*. 2012. P53 and miRNa-34 are Suppressors of Canonical Wnt Signaling. Sci Signal 4 (197), pp. 1-29.

Lan, Y. T., *et al*. Clinicopathological and Molecular Features of Colorectal Cancer Patients With Mucinous and Non-Mucinous Adenocarcinoma. Frontiers in Oncology 11(3), pp. 1-8.

Li, J., Sun, C., Yuan, Y., Liu, L., Xiong, G., & Wu, J. 2012. Bone Morphogenetic Protein-4 Polymorphism and Colorectal Cancer Risk: A Meta Analysis. Molecular Biology Reports 39(5), p. 5239–51. Availble from: https://doi.org/10.1007/s11033-011-1322-0

Liu, L., Su, Q. J., Li, L. X., Lin, X. H., Gan, Y., Chen, S. D. 2014. The Common Variant rs4444235 near *BMP4* Confers Genetic Susceptibility of Colorectal Cancer: An Updated Meta-Analysis Based on a Comprehensive Statistical Strategy. PLOS One 9(6), pp. 1-8.

Lubbe, S. J., Pittman, A. M., Olver, B., Lloyd, A., Vijayakrishnan, J., Naranjo, S., *et al*. 2012. The 14q22.2 Colorectal Cancer Variant rs4444235 Shows *Cis*-acting Regulation of *BMP4*. Oncogene 31, p. 3777-84.

Maegdefrau, U., Amann, T., Winklmeier, A., Braig, S., Schubert, T., Weiss, T. S. *et al*. 2009. Bone Morphogenetic Protein 4 is Induced in Hepatocellular Carcinoma by Hypoxia and Promotes Tumour Progression. Journal of Pathology 218, p. 520-9.

Mármol, I., Sánchez-de-Diego, C., Dieste, A. P., Cerrada, E., & Yoldi, M. J. R. 2017. Colorectal Carcinoma: A General Overview and Future
Perspectives in Colorectal Cancer. International Journal of Molecular Sciences 18(1). Available from: https://doi.org/10.3390/ijms18010197 Meyers

Montesano, R. 2007. Bone Morphogenetic Protein-4 Abrogates Lumen Formation by Mammary Epithelial Cells and Promotes Invasive Growth. Biochemical and Biophysical Research Communication 353, p. 817-22.

Savu, E., *et al*. 2023. Clinicopathological Analysis of Complicated Colorectal Cancer: A Five-Year Retrospective Study from a Single Surgery Unit. Diagnostics 13(12), pp. 2016.

Sayuti, M., and Nouva, N. 2019. Kanker Kolorektal. AVERROUS: Jurnal Kedokteran Dan Kesehatan Malikussaleh 5(2), p. 76. Available from: https://doi.org/10.29103/averrous.v5i2.2082

Singh, A and Morris, R. J. 2010. The Yin and Yang of Bone Morphogenetic Proteins in Cancer. Cytokine Growth Factor Rev 21(4), p. 299-313. Available from: https://doi:10.1016/j.cytogfr.2010.06.003

Valle, L *et al*. 2019. Genetic Predisposition to Colorectal Cancer: Syndromes, Genes, Classification of Genetic Variants and Implications for Precision Medicine. J Pathol 247(5), p. 574-88.

Voorneveld, P. W., Kodach, L. L., Jacobs, R. J., Noessel, C. J. M., Peppelenbosch, M. P., Korkmaz, K. S., *et al*. the BMP Pathway Either Enhances or Inhibits the Wnt Pathway Depending on the SMAD4 and p53 Status in CRC. British Journal of Cancer 112, pp. 122-30.

Yang, C. Y., *et al*. 2016. Single Nucleotide Polymorphisms Associated with Colorectal Cancer Susceptibility and Loss of Heterozygosity in a Taiwanese Population. PLOS One 9(6).

Yusuf, I *et al*. 2021. Genetic Risk Factors for Colorectal Cancer in Multiethnic Indonesians. Scientific Reports 11(1), p. 1-9.

Zannah, S. J., Murti, I. S, Sulistiawati, S. 2021. Hubungan Usia dengan Stadium Saat Diagnosis Penderita Kanker Kolorektal di RSUD Abdul Wahab Sjahranie Samarinda (article in Indonesia language). Jurnal Sains dan Kesehatan 3(5), p. 701-5.