

## The Relationship of CCND1 RS614367 Polymorphism with Clinicopathological Features in Breast Cancer Subjects

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Recent studies have shown that the CCND1 rs614367 polymorphism increases the risk of breast cancer and its invasive nature. However, studies evaluating the relationship of the CCND1 rs614367 polymorphism based on the clinicopathology of breast cancer patients in Indonesia were still limited. This study is aimed to determine the CCND1 rs614367 polymorphism in breast cancer and its relationship with the patient's clinicopathology. **Methods:** This study was a cross-sectional study on 45 samples of breast cancer patients. After collecting demographic and clinical data, PCR and sequencing will be performed on all blood samples to determine the CCND1 rs614367 polymorphism. All variables that have been collected will be analyzed using SPSS version 25.0 to determine the relationship between the CCND1 rs614367 polymorphism and the clinicopathology of breast cancer patients. The CCND1 rs614367 gene polymorphism in breast cancer subjects showed that 25 (55.5%) and 20 (44.5%) subjects had C and T alleles. Subjects aged  $\geq$  50 years old had a significant 4.45 risk of having the T allele type ( $p=0.037$ ). In addition, subjects with metastases (M1) were also at a significant 4.89 times risk of having the T allele type ( $p=0.015$ ). Subjects with histological grade III also had a significantly 4.77 times risk of having the T allele type ( $p=0.013$ ). In conclusion, there was a significant relationship between CCND1 rs614367 polymorphism and breast cancer subjects' clinicopathology features (age, metastasis, and grade). More than half of the subjects with this polymorphism had the C allele.

**Keywords:** CCND1 rs614367, breast cancer, clinicopathological, polymorphism.

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Breast cancer is a malignancy in breast tissue cells, with the highest percentage of incidence (43.3%) and mortality (12.9%). In Indonesia, the incidence of breast is 42.1 per 100.000 population,

with an average mortality rate of 17 per 100.000. In addition, the average survival of subjects after the first relapse or survival from the first relapse is only 17 months.<sup>1</sup>

The high mortality rate of breast cancer and the many risk factors associated with breast cancer have resulted in the identification of a new prognostic marker, one of which is CCND1 rs614367. Cell-cycle regulator cyclin D1 (CCND1) encodes a cyclin protein that is important for the cell cycle. The CCL5/STAT/CCND1 signaling pathway plays an important role in the crosstalk between epithelial and immune cells.<sup>2</sup> The allele frequency of CCND1 rs614367-T was higher in subjects with breast cancer than for controls resulting in a significant positive association (OR 1.38,  $p=0.032$ ), restricted explicitly to the CCND1 rs614367-T/T homozygous genotype (OR 3.64,  $p=0.016$ ). CCND1 rs614367 is associated with a substantial and specific increase in risk for TNBC and one of the most potent breast cancer risk loci identified. CCND1 is a positive estrogen receptor (ER) breast cancer marker.<sup>3</sup> Studies in Indonesia examining the relationship between the CCND1 RS614367 polymorphism and the histopathological condition of breast cancer subjects are still very limited, especially in Bali. This study aimed to determine the CCND1 RS614367 polymorphism and its relationship with the clinicopathology of breast cancer subjects.

## MATERIAL AND METHODS

### Study design

This study was an analytical cross-sectional to examine the relationship between the CCND1 rs614367 polymorphism and clinicopathological characteristics in breast cancer subjects.

### Setting

This research was carried out at the Integrated Biomedical Laboratory and Biochemistry Laboratory, Faculty of Medicine, Udayana University, from May 2022 to November 2022.

### Participants

The population in this study were breast cancer subjects who had taken venous blood samples and stored them at the Department of Biochemistry, Faculty of Medicine, Udayana University as Stored Biological Materials (BBT). The inclusion criteria were subjects with blood venous samples stored in the Department of Biochemistry, Faculty of Medicine, Udayana University. The exclusion criteria were venous

blood samples that were not suitable for use, inadequate amount of serum when centrifuged, and contaminated with other samples.

### Research Flow

All stored biological material from venous blood samples, taken from breast cancer subjects visited Prof. dr. IGNG Ngoerah, were collected consecutively. The clinicopathology data was collected in a data collection sheet, such as age, cancer subtype, histological grade, tumor stage, distant metastases, tumor size, Ki67, ER, and PR. All research subject data is stored on the researcher's computer using a password that the research team can only access to maintain the confidentiality of the research subject's data

The serum from the blood sample will be PCR processed to determine the CCND1 rs614367 polymorphism. The PCR instruments used were a set of GoTaq® Green Master Mix, primer CCND1 rs614367, template DNA, nuclease-free aqua, thermocycler, PCR tube, micropipette 200-1000 $\mu$ l, 20-200 $\mu$ l, and 1-20  $\mu$ l, blue, yellow, white tip, electrophoresis instrument, agarose gel, TAE, microwave, ethidium bromide, Gel Box (Electrophoresis Unit).

DNA isolation from venous blood samples was started by placing 200 $\mu$ l of blood into a centrifugation tube and adding 200 $\mu$ l of BB Buffer. Add 20  $\mu$ l Proteinase X and incubate at 65 $^{\circ}$ C for 10 minutes. Add 200 $\mu$ l Absolute Alcohol, mix immediately, then transfer the mixture to a spin column. Centrifuge at 5000 x g for 1 minute, remove the excess liquid from the spin column and add 500 $\mu$ l Wash Buffer 1. Centrifuge at 5000 x g for 1 minute, remove the excess liquid from the spin column and add 500 $\mu$ l Wash Buffer 2. Centrifuge at 5000 x g for 1 minute minutes, discard the liquid that has passed from the spin column and add 500 $\mu$ l Wash Buffer 2. Next, centrifuge at 12000 rpm for 3 minutes and remove the liquid that has passed from the spin column. Then, transfer the spin column to a new centrifugation tube. Add 100 $\mu$ l of Elution Buffer previously warmed at 65 $^{\circ}$ C or H<sub>2</sub>O, leave for 2 minutes. Centrifuge at 5000 x g for 1 minute and store DNA at 4 $^{\circ}$ C or -20 $^{\circ}$ C.

DNA sample amplification by PCR by inserting a 25  $\mu$ L PCR reaction mixture into the Thermocycler machine. Carry out the primary denaturation process by incubating at 94 $^{\circ}$ C for 1 minute, followed by incubation at 72 $^{\circ}$ C for 7

minutes. Then do the PCR process for 40 cycles, with each rotation at 94°C for 30 seconds, 57°C for 30 seconds, and 72 degrees of Celcius for 30 seconds. Visualization of DNA samples by electrophoresis using a gel containing ethidium bromide and observation under ultraviolet (UV) lamps. DNA purification from gel electrophoresis consists of gel dissociation, DNA binding, washing, and DNA elution. The purified DNA is then sent to Genetics Science for sequencing.

### Statistical methods

Statistical analysis was performed using SPSS for Windows version 25.0 software.

**Table 1.** The Clinicopathological Characteristics of Subjects

Clinicopathological Data	N	%
Age		
<50 years old	14	31.1
≥50 years old	31	68.9
T stage		
T1	3	6.7
T2	7	15.6
T3	8	17.8
T4	27	60.0
N stage		
N0	13	28.9
N1	22	48.9
N2	7	15.6
N3	3	6.7
M stage		
M0	29	64.4
M1	16	35.6
ER status		
Negative	16	35.6
Positive	29	64.4
PR status		
Negative	23	51.1
Positive	22	48.9
HER2 status		
Negative	21	46.7
Positive	24	53.3
Histological type		
Luminal A	9	20.0
Luminal B	13	28.9
HER2	9	20.0
TNBC	6	13.3
Luminal-HER2	8	17.8
Grade		
I-II	25	55.6
III	20	44.4

Univariate analysis of proportions for characteristic variables such as histopathological grading, cancer stage (TNM system), occupation, and the presence or absence of the CCND1 rs614367 polymorphism. Bivariate inferential analysis, namely chi-square or fisher-exact, when the data does not meet the chi-square requirements to evaluate the relationship between polymorphism and the variables that have been determined. The p value is considered significant if  $P < 0.05$ .

## RESULTS

A total of 45 samples of breast cancer subjects had PCR and electrophoresis carried out at the Biochemistry Laboratory, Faculty of Medicine, Udayana University. The basic characteristics of the subjects are described in Table 1

The results of CCND1 rs614367 gene polymorphism sequencing in breast cancer subjects showed that 25 (55.5%) and 20 (44.5%) subjects had C and T alleles, which were then subjected to bivariate analysis. The frequency of polymorphism alleles was described in Table 2.

Bivariate analysis showed a significant relationship between the CCND1 rs614367 gene polymorphism and the clinicopathological features, as described in Table 3. Based on the analysis results, subjects aged  $\geq 50$  had a significant 4.45 risk of having the T allele type ( $p=0.037$ ). In addition, subjects with metastases (M1) were also at a significant 4.89 times risk of having the T allele type ( $p=0.015$ ). Subjects with histological grade III also had a significant 4.77 times risk of having the T allele type ( $p=0.013$ ) (Table 3).

## DISCUSSION

The CCND1 gene plays an important role in the cell cycle and cell viability against mutagens, primarily through CCL5 signaling. CCND1, a gene with a length of 13,388 base pairs and codes for 295

**Table 2.** Polymorphism of CCND1 rs614367 in Breast Cancer Subjects

Target All	n	%
C	25	55,5 %
T	20	44,5 %

**Table 3.** Relationship between CCND1 rs614367 polymorphism and Clinicopathology Features in Breast Cancer Subjects

Clinicopathology	Allele type		OR (95%CI)	p
	C	T		
Age				
<50 years old	11	3	4.45(1.03-19.16)	0.037
≥50 years old	14	17		
Metastasis				
M0	20	9	4.89(1.31-18.23)	0.015
M1	5	11		
Grade				
I-II	18	7	4.77(1.35-16.96)	0.013
III	7	13		

amino acids, has several polymorphisms, some of which are related to breast cancer, namely CCND1 rs9344 and CCND rs614367.<sup>4</sup>

This study showed that the distribution of subjects with the CCND1 rs614367 mutation showed a significant relationship with older age. The distribution at an older age also suggested the interaction of polymorphism with degenerative processes.<sup>5</sup> Here, it also showed that there was a lower incidence of metastases in subjects with the C allele, similar with the analysis by Shan *et al.*, who found that C alleles were mostly found in the CCND rs614367 mutation as well as a lower number of metastatic events in the population (3). The lower incidence of metastases may be due to the effect of the CCND rs614367 mutation on CCL5 signaling activity. CCL5 protein or commonly called RANTES (regulated on activation, normal T cell expressed and secreted), physiologically plays a role in leukocyte migration in the event of infection. However, in chronic inflammatory processes such as tumorigenesis, CCL5 plays an essential role in the recruitment and maintenance of CD8+ T cell function, thereby maintaining the cytotoxic function of the cells.<sup>6</sup>

In general, the mutation in the CCND1 gene impairs CCL5 signaling and reduces the recruitment of CD8+ T cells, thereby increasing the risk of malignancy. The low incidence of metastases in this study may be due to the specific mutation CCND rs614367 differently affecting CCL5, but the exact mechanism for this is still unknown.<sup>7</sup> Recent research by Bekampyte *et al.* got different

results. Namely, the incidence of metastases was higher in the CCND1 rs9344 mutation. This can happen because the rs9344 mutation CCND1b protein synthesis undergoes alternative splicing, resulting in an abnormal protein in the C-terminal domain and overexpression of CCND1b.<sup>8</sup> Both of these phenomena are related to metastasis.<sup>9</sup>

Subjects with the C allele mutation on CCND rs614367 tend to have a lower tumor stage than the T allele. This phenomenon is also in line with the study by Shan *et al.* This can be attributed to the role of the CCND1 protein, which encodes a central protein for the cell cycle and carcinogenesis, cyclin D1. The activity of cyclin D can be influenced by estrogen. However, previous studies have shown that the degree of overexpression of CCND1 does not have a significant relationship with the clinicopathological characteristics of breast cancer.<sup>10</sup>

## CONCLUSION

So far, this study is the first study in Indonesia to analyze the clinicopathological relationship of breast cancer subjects with the CCND rs614367 polymorphism. The limitation of this study is that only samples from a single center were taken. The CCND rs614367 mutation in this study showed more distribution of the C allele and a lower incidence of metastases and tumor grading compared to the T allele. Further research is needed to ensure the validity and consistency of the results of this study.

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**Conflict of Interest**

Authors stated that this study had no conflict of interest.

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