

Prognosticating 2-Year Survival Rate of Breast Cancer Patients Through Plasma miRNA-21 and Other Associating Factors

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Background: miRNA-21, one of breast cancer (BC) predictive markers, is now gaining cardinal attention from researchers worldwide to evaluate BC patients' survival rate. However, cancer staging, hormonal status, and other BC markers still have to be discussed. We aim to determine the relationship between miRNA-21 and associating factors such as BC staging, other tumor markers, and hormonal status to predict the 2-year survival rate of BC patients.

Methods: We conducted a prospective cohort study on 49 BC patients (26 early stage, 23 advanced stage). Apart from cancer staging, we also examined CEA, Ca15-3, and hormonal status (ER, PR, Her2) and correlated them with miRNA-21 to predict 2-year survival rate. We did bivariate, multivariate, and survival analyses to determine the link between miRNA-21 and those factors to prognosticate on 2-year survival rate.

Results: There are significances between advanced and loco-regional stage ($p < 0.001$); high and low miRNA-21 ($p = 0.002$) and CA 15-3 ($p = 0.001$), and low survival rate in patients with ER/PR-Her2- status ($p=0.0015$). Cox proportional hazard showed miRNA-21 (Adjusted HR 1.41; 95% CI = 1.205–1.632), cancer stage (Adjusted HR 9.5; 95% CI = 1.378–20.683), and CA15-3 (Adjusted HR 4.64; 95% CI = 1.548–13.931) affected patients' mortality within 2 years.

Conclusion: Low two-year survival rate depends on miRNA-21, cancer stage, CA15-3, and ER/PR-Her2-. Cancer stage is robustly associated with miRNA-21 in predicting 2-year survival rate.

Keywords: breast cancer, miRNA-21, survival, stage, health

Introduction

Breast cancer (BC) is the most prevalent malignancy in women and is the second leading malignancy-related cause of death.¹⁻⁴ Nearly 1.5 million new cases are diagnosed each year, and 460,000 of them die from chemoresistance and metastases.¹ Since the incidence rockets every year, early diagnosis and appropriate treatment remain the prime key to reduce the mortality. Clinical considerations involving prognostic factors are indispensable in determining appropriate therapy.⁵ BC's pathological and clinical characteristics including patients' age, histologic subtype, tumor size, lymph node enlargement, hormone receptor status, and human epidermal growth factor receptor-2 (Her-2) have been used as prognostic factors foretelling survival rate and post-therapy clinical outcome. In addition, BC patients with similar characteristics often have distinct clinical outcomes. Moreover, microscopic metastases have already occurred once they were initially diagnosed. This indicates that the clinicopathological picture of BC has limitations in determining the

diagnosis, prognosis, and prediction of clinical outcomes; therefore, more advanced diagnostic and prognostic techniques are required to determine the survival rate and appropriate treatment modalities for patients.^{1,6}

In recent years, examination of tumor markers using various molecular techniques, particularly gene expression panels, has been extensively used as an indicator of aggressiveness, invasiveness, and tumor extension.⁷ Tumor markers routinely observed in BC patients are miRNA, CEA, and CA15-3. MiRNA is an endogenous single-stranded RNA playing a prime role in regulating tumor's cells growth, proliferation, differentiation, and death. Data from recent studies have shown its ability to control tumorigenesis by functioning as oncogenes (oncomiRNA) or tumor suppressor genes, and by influencing other mechanisms such as migration and metastasis in BC.⁸ MiRNA can be easily detected through biopsy specimens or serum.³ The type of miRNA that is highly susceptible to modification and multiplying in BC is miRNA-21.⁸ A study from Pan et al stated that miRNA-21 was more associated with Asian BC population than non-Asian.⁹

Carcinoembryonic antigen (CEA) and cancer antigen 15-3 (CA15-3) are also linked to BC patients survival and have been inspected for more than 30 years. CEA, one of the cell-adhered glycoproteins and CA15-3, a glycoprotein from the mucin-1 (MUC-1) group, are extensively expressed in various types of cancer. Several studies have shown that elevated CEA and CA15-3 can predict survival in BC patients.^{10–15} CEA and CA15-3 also have been approved by the Food and Drug Administration (FDA) as tumor markers to evaluate BC. On the contrary, The American Society of Clinical Oncology (ASCO) does not recommend them as screening, diagnosis, staging, post-therapy monitoring tool. Therefore, the function of CEA and CA15-3 as prognostic markers remains controversial.⁵

In our preceding study, it was concluded that miRNA-21 was detected high in BC patients and was correlated with 1-year survival.^{16–18} Subsequently, we proceed with the evaluation of miRNA-21 and determine its association with CEA, CA15-3, and other factors regarding the 2-year survival rate of BC patients and their clinical and pathology features.

Materials and Methods

Data Collection

From November 2019 to November 2021, we conducted a cohort-prospective analysis at the Universitas Airlangga Hospital's Oncology-Hematology Outpatient Clinic in Surabaya. We follow through our previous research and observe 2-year survival rate of BC patients, as well as the link between CEA, CA15-3, ER/PR, and Her-2.¹⁶ The following are the requirements for inclusion: female; aged ≥ 18 ; no past chemotherapy or radiotherapy; and willing to sign an informed consent form (see Figure 1).

To assess the value of expression, we used the quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) to detect circulating plasma miRNA-21. As an endogenous control, the Hsa-miR-16-5p LNA PCR primer set was employed. The blood samples were run at the Surabaya Prodia laboratory and then transported to the Jakarta Prodia laboratory for storage and processing.

Ethical Clearance

- Ethical clearance was provided by the Research Ethics Committee of Universitas Airlangga Hospital (No: 164/KEP/2019). All patients signed informed consent before the study.
- Our research complies with the Declaration of Helsinki that each potential subject was well informed of the aims, methods, sources of funding, any possible conflicts of interest, institutional affiliations of the researcher, the anticipated benefits and potential risks, and any other relevant aspects of the study.

Statistical Analysis

The sample size for this investigation was determined using the correlative analytic sample formula. While 37 individuals were initially needed for the trial, we ended up with 49, 26 patients with early-stage breast cancer and 23 patients with advanced-stage breast cancer. We utilized SPSS version 24.0 for statistical analysis (Chicago, IL, USA). Depending on the data distribution, we expressed categorical variables as numbers (%) and continuous variables as mean (standard or

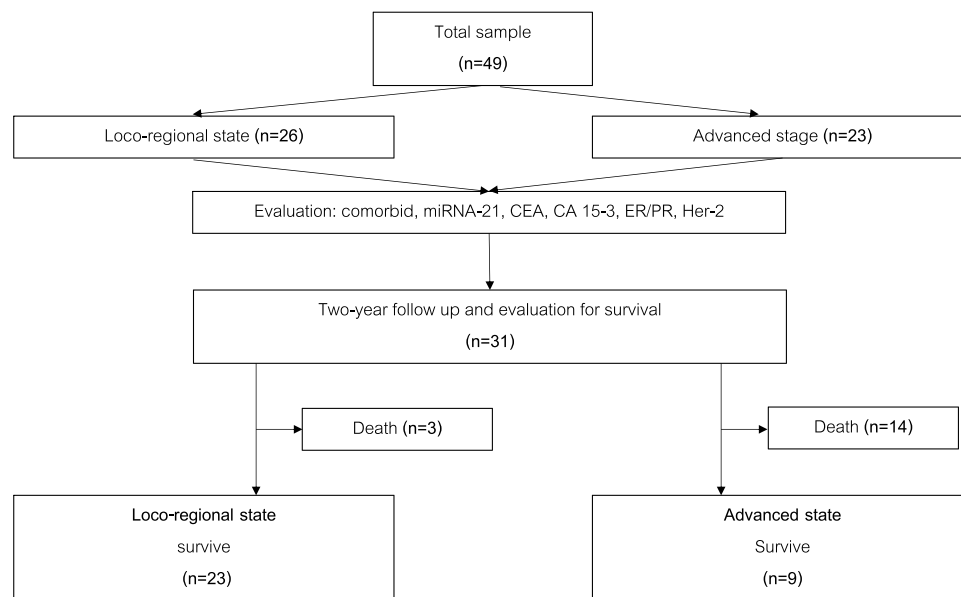


Figure 1 Two-year follow-up on breast cancer patients.

median (range). For missing data, we employed list-wise deletion or univariable and multivariable analysis. We used Mann–Whitney tests, *t*-tests, chi-square tests, or Fischer’s exact tests based the variable type. Chi-square test was used for dichotomous variable, whereas continuous variables were analyzed with the independent sample *t*-test or the Mann–Whitney test.

To compare the survival rates of each group, we use the Kaplan–Meier curve and the Log rank test. We also performed univariate and multivariate Cox proportional-hazard regression models to find prognostic factors for 2-year survival rates. The researchers calculated hazard ratios (HRs) and 95% confidence intervals (CIs). The “entry” approach was used to adjust multivariate analyses for the selected covariates in order to estimate HRs of the tested tests.

Result

Of 49 patients, 26 were in the early-stage, 23 advanced-stage. Most of the patients were housewives. Twenty-five patients were menopausal. Type 2 diabetes mellitus and hypertension were the most frequent comorbid. Twenty-three patients had ER positive, while 18 had PR positive. The mean value of miRNA-21 was 6.00, CEA 5.63, and CA15-3 73.73 (see Table 1).

Two years later, death cases reached 17 cases. Three cases occurred in the early stage, whereas 14 cases occurred in advanced-stage cases ($p = 0.001$). Four death cases were accompanied by DM ($p=0.043$). Patients with negative PR had the highest mortality rate (11 cases ($p=0.022$)), while patients with positive ER showed no statistical difference in mortality ($p=0.120$). There were significant differences among CEA, CA15-3, and miRNA-21 in those who died ($p=0.028$; $p=0.005$; $p=0.003$). Mean survival time in patients with advanced-stage, negative PR, and high CA 15-3 was 12.61 months, 14.47 months, and 13.00 months, respectively. Multivariate analysis showed that only cancer stage and miRNA-21 affected the 2-year survival rate ($p=0.024$; $p=0.05$) (see Tables 2 and 3).

Moreover, there was a significant difference between the loco-regional and advanced stage groups ($\chi^2 (1) = 15.491$ Log rank; $p < 0.001$) identified in the curve of survival function, as well as between high (≥ 4.4) and low miRNA (< 4.4) groups ($\chi^2 (1) = 9.571$ Log rank; $p = 0.002$) (see Figure 2).

A significant difference was also identified between high and low CA15-3 ($\chi^2 (1) = 11.285$ Log rank; $p = 0.001$). Conversely, the curve of survival function did not show any significant difference between low and high CEA ($\chi^2 (1) = 0.440$ Log rank; $p = 0.507$) (see Figure 3). The curve also exhibited a significant difference among ER, PR, and HER2. ($\chi^2 (1) = 10.509$ Log rank; $p = 0.015$) (see Figure 4.)

Table 1 Patients' Baseline Characteristics

	All (N = 49)	
	n/Mean (Min – Max)	%/SD
Age (mean/SD)	52 (29–73)	10.2
Occupation		
Housewife	39	79.6
Civil servants	5	10.2
Private employees	1	2.0
Others	4	8.2
Comorbid factors		
Yes	17	34.7
No	32	65.3
DM (n,%)	5	10.2
HT (n,%)	11	22.4
Menarche		
Pre-menopause	25	51
Menopause	24	49
Stage		
Loco-regional stage	26	53.1
Advanced stage	23	46.9
miRNA (mean/SD)	6.00 (1.12–32.22)	5.4
ER		
Positive	23	46.9
Negative	14	28.6
No data	12	24.5
PR		
Positive	18	36.7
Negative	19	38.8
No data	12	24.5
CEA		
Low	37	75.5
High	12	24.5
CEA (mean/SD)	5.6 (0.80–34.80)	6.7
CA153		
Low	29	59.2
High	20	40.8
CA15-3 (mean/SD)	73.7 (4.73–587.90)	150.1
Weight (mean/SD)	57.1 (32–78)	11.1
Height (mean/SD)	152.9 (142–163)	4.8
Hemoglobin (mean/SD)	12.3 (9.4–15.6)	1.3

Abbreviations: DM, diabetes mellitus; HT, hypertension; miRNA, microRNA; ER, estrogen receptor; PR, progesterone receptor; CEA, carcinoembryonic antigen; CA15-3, cancer antigen 15-3.

Cox-proportional hazard showed that miRNA-21 (Adjusted HR 1.67; 95% CI = 1.179–1.632), cancer stage (Adjusted HR 12.07; 95% CI = 2.38–61.21), and CEA (Adjusted HR 1.033; 95% CI = 0.981–1.088) were correlated with 2-year mortality, whereas the other CA15-3 (Adjusted HR 1.81; 95% CI = 1.171–19.112) did not show any significant association (see Table 4).

Discussion

MiRNA, a prognosis factor for the severity and survival of BC patients, is considerably associated with severity and mortality when detected in a higher level.^{2,4,8,19} There are many types of miRNAs detected in BC, one of which is miRNA-21 that mainly depicts the progression of BC.^{20,21} A meta-analysis study of Wang et al stated that miRNA-21

Table 2 The Comparison Among Associating Factors of BC 2-Year Survival Rate

	2-Year Survival Rate of BC Patients				
	Survived (n = 32)	Died (n = 17)	p value Bivariate Mean Survival Time (SD)	Mean Survival Time (Months) (SD)	p value Multivariate
Age (mean/SD)	50.9 (10.8)	53.94 (8.89)	0.327		
Occupation					
Housewife	25 (78.1)	14 (82.4)	0.774		
Civil servant	3 (9.4)	2 (11.8)			
Private employee	1 (3.1)	0			
Others	3 (9.4)	1 (5.9)			
Comorbid factors			1.000		
Yes	11 (34.4)	6 (35.3)	18.35 (7.48)		
No	21 (65.6)	11 (64.7)	17.84 (8.71)		
DM (n,%)	1 (3.1)	4 (23.5)	0.043 ^a		0.090
HT (n,%)	8 (25)	3 (17.6)	0.725		
Menarche			0.192		
Pre-menopause	19 (59.4)	6 (35.3)	19.12 (8.24)		
Menopause	13 (40.6)	11 (64.7)	16.88 (8.25)		
Cancer stage			0.001 ^a		0.024 ^a
Loco-regional stage	23 (71.9)	3 (17.6)		22.81 (3.71)	
Advanced stage	9 (28.1)	14 (82.4)		12.61 (8.64)	
miRNA (mean/SD)	4.19 (2.25)	9.42 (7.56)	0.003 ^a		0.05 ^a
ER			0.120		
Positive	17 (53.1)	6 (35.3)	18.78 (8.32)		
Negative	6 (18.8)	8 (47.1)	14.71 (8.65)		
No data	9 (28.1)	3 (17.6)			
PR			0.022 ^a		0.105
Positive	15 (46.9)	3 (17.6)		20.17 (7.54)	
Negative	8 (25)	11 (64.7)		14.47 (8.73)	
No data	9 (28.1)	3 (17.6)			
CEA			0.729		
Low (<3, 6)	25 (78.1)	12 (70.6)	18.68 (8.05)		
High (>3, 6)	7 (21.9)	5 (29.4)	16.00 (8.83)		
CEA (mean/SD)	4.69 (6.29)	7.41 (7.38)	0.028 ^a		0.300
CA15-3			0.005 ^a		0.740
Low (<21)	24 (75)	5 (29.4)		21.48 (6.05)	
High (>21)	8 (25)	12 (70.6)		13.00 (8.51)	
CA15-3 (mean/SD)	54.11 (124.14)	110.66 (188.52)	0.150		
Weight (mean/SD)	57.97 (11.45)	55.32 (10.38)	0.431		
Height (mean/SD)	152.28 (4.69)	154.15 (4.91)	0.199		
Hemoglobin (mean/SD)	12.41 (1.20)	11.96 (1.39)	0.243		

Note: ^ap < 0.05.

Abbreviations: DM, diabetes mellitus; HT, hypertension; miRNA, micro RNA; ER, estrogen receptor; PR, progesterone receptor; CEA, carcinoembryonic antigen; CA15-3, cancer antigen 15-3.

was correlated with cancer proliferation and metastasis.²¹ Furthermore, other studies suggested that increased miRNA-21 was related to poor survival of BC patients.^{9,22,23} Subgroup studies in a meta-analysis by Pan et al stated that miRNA-21 was strongly associated with BC patients from Asia.⁹ In our previous study, the increase in miRNA-21 values was not associated with BC mortality in the earlier stages ($r=0.25$; $p=0.218$), otherwise, we found that the increase in miRNA-21 had a correlation with mortality in the later stage ($r=0.866$; $p<0.05$).¹⁶ However, our study revealed the different values of miRNA-21 in the early and advanced stages. Higher 2-year survival rate was identified in patients with advanced BC and high miRNA-21.

Table 3 Survival Time Based on the BC Subtypes

Subtype	Mean for Survival Time (95% CI)	Median for Survival Time (95% CI)
ER/PR+, HER2+	18.429 (13.778–23.079)	–
ER/PR+, HER2-	21.750 (17.625–25.875)	–
ER and PR+, HER2+	21.400 (16.842–25.958)	8.000 (2.156–13.844)
ER and PR-, HER2-	11.667 (15.115–20.731)	–

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth-2.

Estrogen receptor (ER), progesterone receptor (PR), and Her2 are markedly associated with the prognosis and treatment of BC. A retrospective-7-year survival study concluded that the triple-negative subtype (ER/PR-, Her2-) had the worst progression and disease-free survival. Like triple-negative subtype, ER/PR-, Her2+ subtype also showed low disease-free survival.²⁴ A cohort study demonstrated that PR, dependent on the tumor proliferation, functioned as an important prognostic value in all BC lesions with ER+/Her2-.²⁵ Meanwhile, PR status independently could be a prognostic indicator within 5 years after the diagnosis of BC is established.²⁶ Positive ER status was not independently associated with BC survival rate after adjustment to other influencing factors, but quantitative ER examination might have prognostic value.²⁷

Our present study concluded that patients with negative PR had a higher mortality rate within 2 years. Meanwhile, analysis of the ER status showed no significant difference. Our survival analysis of ER/PR Her2 showed similar results to previous studies in which ER/PR-Her2- subtype had a low 2-year survival rate. Moreover, there was a significant difference in mortality within 2 years in the advanced stage and metastases.

Other related tumor markers such as CEA and CA15-3 have been widely deliberated regarding prognosis and survival. The study of Gaglia et al in the late 1980s showed that CEA, as a single prognostic factor, was capable of predicting outcome and the need for adjuvant therapy.²⁸ Numerous studies have also looked at the relationship among existing prognostic factors. Ebeling et al investigated CEA and CA15-3 as BC prognostic tools. Lower post-operative CEA was associated with disease-free survival and mortality in BC patients, while an increase in CA15-3 was consistent with relapse of BC.²⁹ The progression of BC was also in line with elevated CEA and CA15-3.³⁰ According to previous

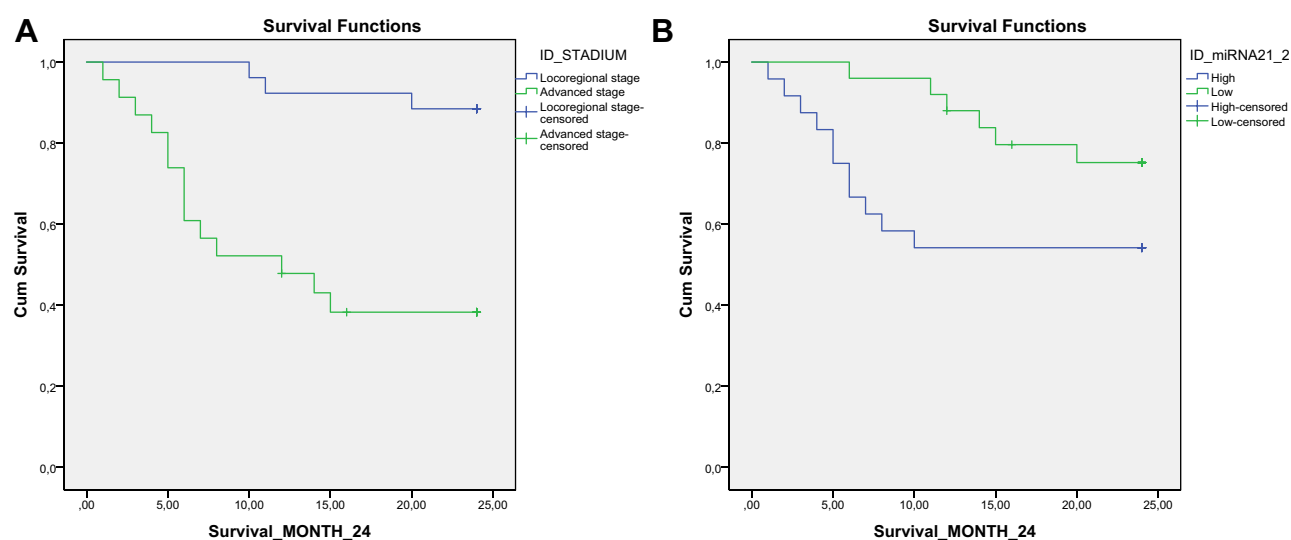


Figure 2 (A) Two-year survival rate based on cancer stage. (B) Two-year survival rate based on miRNA-21.

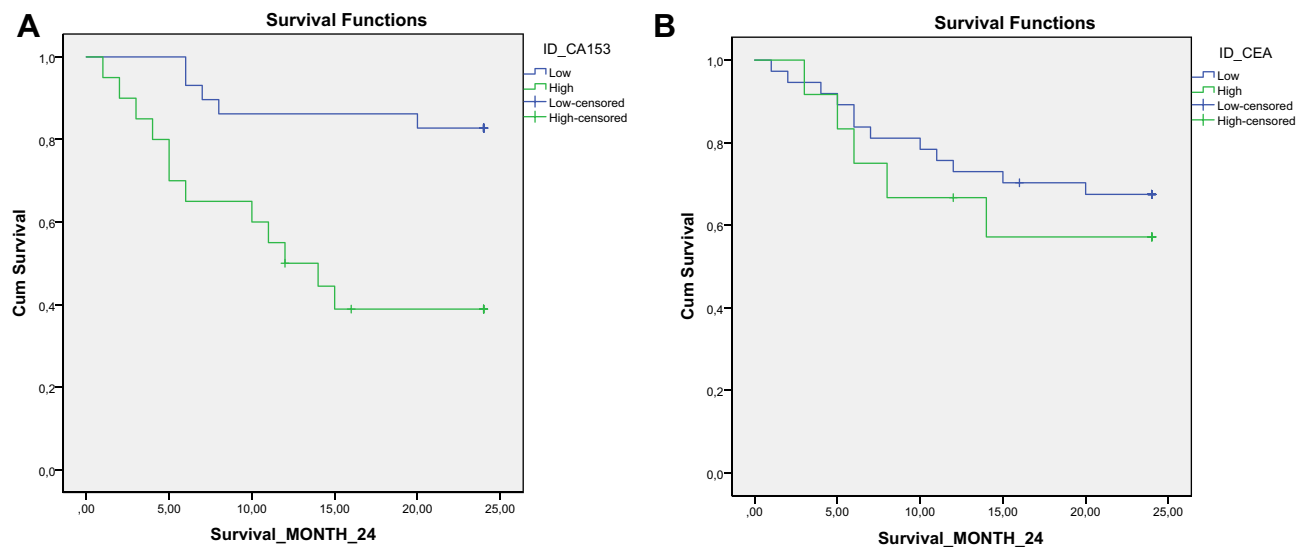


Figure 3 (A) Two-year survival rate based on CA15-3. (B) Two-year survival rate based on CEA.

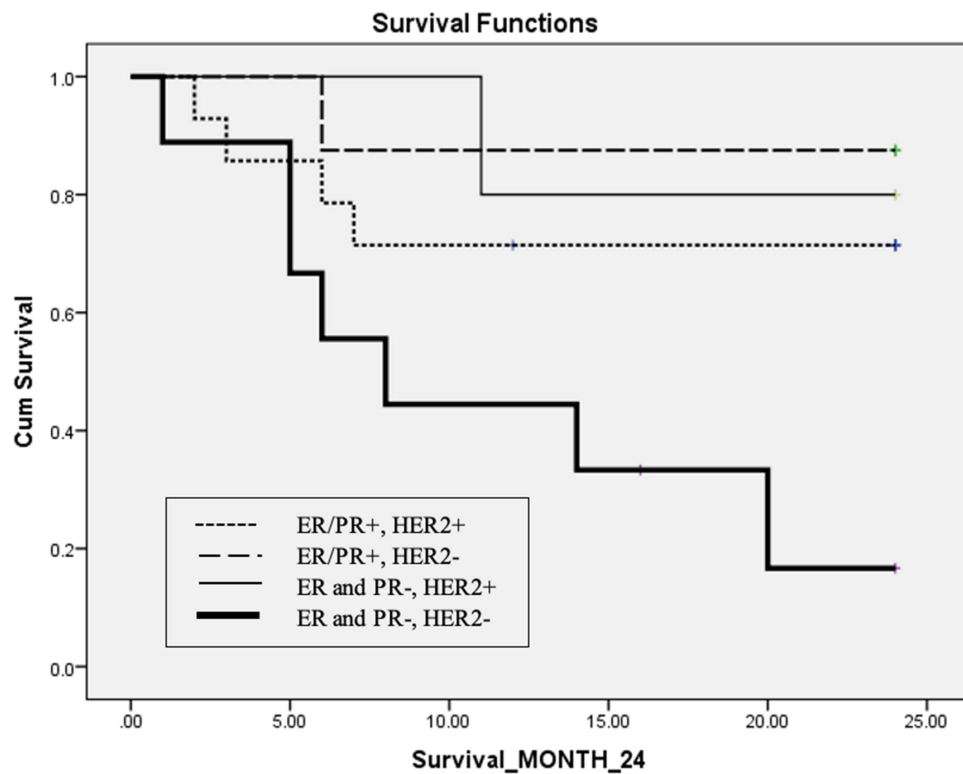


Figure 4 Two-year survival rate based on ER, PR, and HER2 status.

studies on preoperative, treatment outcome, and survival, elevated CEA and CA15-3 values were highly associated with poor treatment outcomes and mortality.^{10–12,14,15,31}

In addition, our present study also declared that patients who died within 2 years had a higher mean baseline CEA and CA15-3 and lower mean survival. Comparing patients with high to low CA15-3 resulted in significantly different 2-year survival rates. In contrast, no significant difference was identified when comparing CEA. Nonetheless, this is not relevant

Table 4 Cox Proportional Hazard

	Unadjusted Model		Adjusted Model ^a	
	HR (95% CI)	p value	HR (95% CI)	p value
miRNA	1.332 (1.178–1.507)	<0.001 ^b	1.406 (1.205–1.640)	<0.001 ^b
Stadium (I)	8.294 (2.362–29.126)	0.001 ^b	9.497 (1.378–20.683)	0.001 ^b
CEA	1.419 (0.499–4.032)	0.512	1.026 (0.962–1.093)	0.441
CA15-3	5.040 (1.762–14.418)	0.003 ^b	4.643 (1.548–13.931)	0.006 ^b

Note: ^aAdjusted to history of diabetes mellitus and PR ^bp<0.05.

Abbreviations: miRNA, microRNA; CEA, carcinoembryonic antigen; CA15-3, cancer antigen 15-3.

enough since we only observed the initial CEA status; hence, we ought to examine post-treatment CEA to see how it is related to outcome and prognosis.

Following our multivariate analysis, it appears that miRNA-21 was significantly associated with BC stage but not with CEA and CA15-3. The study of Pan et al disclosed that miRNA-21 was strongly correlated with the expression of ER, PR, Her2, lymph node metastases, histological grade, and Her2/neu. Each of these parameters was associated with BC severity. The study also declared a strong association among miRNA-21, lymph node metastases, and histologic grading used to determine BC stage.⁹

Based on Cox proportional hazard test, the unadjusted model showed that increasing miRNA-21, cancer stage, CA15-3, and CEA were still associated with a low 2-year survival rate in BC patients. Only miRNA-21, cancer stage, and CA15-3 were significantly associated in the adjusted model. A cohort study in China showed that elevated CEA and CA15-3 were closely in line with decreased breast cancer-specific survival (BCSS) and disease-free survival (DFS).¹⁰ In BC patients with low CEA levels, levels of CA15-3 can completely predict overall prognosis.³² However, another study observing CEA and CA15-3 proclaimed no significant difference in the clinicopathological features of BC, while the observation of elevated miRNA-21 showed otherwise.⁸

To the best of our knowledge, studies explaining miRNA-21 in BC patients and associating factors have not been extensively discussed and published in Indonesia, although they started to be recognized worldwide. Our study provided an overview of BC patients with similar clinical features to patients presented in other studies, especially around Asia. Our study has several limitations. Regardless of petite sample size, we managed to examine other factors associated with miRNA-21, such as comorbidities, hormonal status, menarche status, and other tumor markers. Furthermore, CEA and CA15-3 were not evaluated periodically after therapy, so that the correlation between these tumor markers and miRNA-21 could not be obtained. It is necessary to examine in a more extended period of time; eg, 3–5 years, and regularly evaluate other markers such as CEA, CA15-3, CA125 to understand the association of miRNA-21 with the survival of BC patients.

Conclusion

Significant differences among cancer stage, DM, and PR were observed while predicting the 2-year mortality rate in BC patients. The low 2-year survival rate was determined by miRNA-21, cancer stage, CA15-3, and ER/PR-Her2-. MiRNA-21, a prognostic marker, is significantly related to cancer stage and several other factors, such as BC's clinical picture and pathology. No correlation was found among other three markers. More comprehensive studies with larger sample sizes and longer survival time are essentially needed to comprehend the association between miRNA-21 and other existing factors.

Data Sharing Statement

The dataset is available on 10.6084/m9.figshare.19583815.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

All of the authors declare no conflicts of interest in relation to this work.

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