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Short Communication

Prognosis value of circulating telomere repeat binding factor 2 and leukocyte telomere length in breast cancer mortality

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Abstract

Telomere repeat binding factor 2 (TRF2) is currently a novel tumor marker, yet its clinical implication has not been investigated. The aim of this study was to investigate the prognostic value of circulating TRF2 and leukocyte telomere length in 5-year mortality in breast cancer patients. In this cohort retrospective study, breast cancer patients were included and the length of telomeres and circulating TRF2 were quantified. Receiver operating characteristics and the Youden index were used to determine the optimal cut-off. To analyze the overall survival rate in five years, Kaplan Meier analysis was used, while the prognostic value of both variables was analyzed in Cox proportional hazard regression on both univariate and multivariate models. Our data indicated that the optimal cut-off points for TRF2 and leukocyte telomere length were 598 pg/mL and 0.93 kb, respectively. Based on the optimal cut-off points, the participant's data was grouped, and our data indicated that the high TRF2 group had a poorer overall survival rate in comparison to the low group (91.3% vs 83.87%; log-rank test; $p<0.01$). The overall survival between short and long telomeres was comparable (88.24% vs 88.37%; log-rank test; $p=0.64$). TRF2 (hazard ratio (HR): 3.66; 95%CI: 1.45–9.29) and molecular subtype ($p=0.04$) were identified as independent factors to predict mortality. In conclusion, a high circulating TRF2 in breast cancer participants was associated with lower overall 5-year survival rates in comparison with the low TRF2 group. Moreover, high TRF2 could predict the mortality of the breast cancer population to be 3.66 times higher than the lower group. In contrast, telomere length was not associated with overall survival rate nor predicting mortality in five years.

Keywords: Breast cancer, TRF2, telomere length, mortality, survival rate

Introduction

Since the first time of its discovery, breast cancer has always been challenging. In 2019, World Health Organization (WHO) data indicated that breast cancer had the highest mortality rate worldwide, especially among women aged below 70 years old [1]. In Asia, one million cases were recorded in 2020, while in Indonesia, 19.2% of cancer cases were breast cancer, with 68–73% of the cases already at an advanced stage [2-4].

It has been known for long that telomere plays an important role in carcinogenesis and evasion of cell-death pathway in cancer cells [5]. Generally, cell division shortens the telomere

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with each division to a length where the cell cannot be divided, known as the Hayflick limit [6]. However, cancer cells evade this programmed cellular death by protecting their telomeres [6-8]. It is well-known that a short telomere is a risk factor for breast cancer development [9-12]. Some studies have also revealed the importance of short telomere as a prognostic value in cancer patients [13-16].

In healthy human cells, the telomere shortening is controlled by telomere-capping proteins called shelterin and telosome complexes [7,8]. Shelterin in vertebrates consists of six main proteins: telomeric repeat-binding factor 1 (TRF1), TRF2, RAP1, TERF1-interacting nuclear factor 2 (TIN2), TIN2-interacting protein 1 (TPP1) and protection of telomeres protein 1 (POT1) [7]. Since telomere length is associated with cancer development, shelterin and telosome complex may play a role in mortality and disease-free survival of breast cancer. Recent studies revealed that TRF1 and TRF2 are associated with disease-free survival [17,18]. Higher expression of these proteins may have prognostic value as well, especially in cancer cells with low telomerase activity. TRF2 is currently a novel tumor marker [18]; yet its clinical implications have not been investigated. The aim of this study was to investigate the prognostic value of TRF2 and leukocyte telomere length for predicting mortality in all variants of breast cancer.

Methods

Study design and setting

A cohort retrospective study was conducted at Dr. Sardjito General Hospital, Yogyakarta, Indonesia, incorporating newly diagnosed breast cancer patients between January 1, 2015, to December 31, 2022, who underwent tumor removal surgery. Before the treatment, venous blood samples were drawn prior to surgery, collected in EDTA tubes, and stored in a biobank at -80°C. These samples were later used to measure the TRF2 levels and leukocyte telomere length. The outcome (mortality) was then assessed. The patients were followed up until March 31, 2024.

Patients and criteria

All newly diagnosed breast cancer patients between January 1, 2015, and December 31, 2022, were considered eligible. Only female patients older than 30 years old with invasive breast carcinoma of no special type and who did not have distant metastasis prior to the surgery were included. The pathological diagnoses were based on the 2019 WHO classification of breast cancer: basal-like, HER2-enriched, luminal A, luminal B HER+, luminal B HER- [19]. Other types without clear immunohistochemistry profiles were classified as unspecified. The patients were excluded if (1) the date of diagnosis was missing; (2) the patient or the family could not be contacted during the follow-up period; (3) the presence of other chronic diseases such as chronic kidney disease, congestive heart failure and stroke; (4) the histopathological examination was based on fine-needle aspiration; (5) had a distant metastasis since the first day of diagnoses; and (6) the blood sample could not be analyzed. Patients who could not be contacted either by phone, in the daily practice, or home-visit session were excluded. A total sampling method was used, meaning all patients within the study period who met the inclusion criteria were eligible. Nevertheless, the minimal sample size was calculated using Lemeshow's formula yielding a minimum total of 70 patients [20].

Data collection and study variables

For all patients, information of age on the first day of diagnosis (year), age at surgery (year), affected side, tumor size (cm), number of infiltrated lymph nodes, staging based on the American Joint Committee on Cancer (AJCC) cancer staging system [21], histopathological grade, treatment history, and family history of cancer were collected. In addition, the expression of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor-2 (HER2), and Ki-67 were tested by immunohistochemistry (IHC). The expression of ER, PR, HER2, and Ki-67 was assessed using an anti-ER antibody (SP1, Ventana, Arizona, USA), anti-PR (1E2, Ventana, Arizona, USA), anti-HER2/neu (4B5, Ventana, Arizona, United States), and anti-Ki-67 (30-9, Ventana, Arizona, USA) monoclonal antibody and its clone antibody, respectively. The Ki-67 index was calculated as follows: ten sections in each field were selected under 400×

magnification, and 100 cells per field were counted until counts reached 1,000 cells; the percentage of Ki-67 positive cells in each section was then calculated. All procedures were conducted following the manufacturer's instructions. Based on the molecular expression of the IHC examination, the molecular subtypes of the cancer were classified based on the 2019 WHO classification of breast cancer [19].

For the TRF2 quantification, whole blood samples collected from patients before surgery were stored in the biobank. The telomere length was measured using quantitative polymerase chain reaction (qPCR). The DNA from the sample was extracted using gSYNC™ DNA Extraction Kit (Geneaid, New Taipei City, Taiwan). The following primer pairs were used in qPCR for telomere detection: 5'- GGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT - 3'(forward) and 5'- GGCTTGCCTTAC CCTTACCCTTACCCTTACCCTTACCCT - 3'(reverse). The amplification of DNA was done using CFX96 (Bio-rad, California, United States), while the measurement of telomere length used AmpliTaq DNA Polymerase (Applied Biosystem, Roskilde, Denmark) following the manufacturer's protocol. The DNA amplification was carried out with a mixture consisting of 6.25 µL master mix, 0.375 µL of each forward and reverse primer, 1 µL DNA template and 4.5 µL RNAse free water. The amplification consisted of pre-denaturation at 95°C for 15 minutes, annealing at 57°C for 1 minute, extension at 72°C for 1 minute, and conditioning at 40°C for 5 minutes. Finally, the result of the amplification process was measured and calculated. Using Cawthon's method, the relative ratio of the telomere (T) repeat copy number to a single gene (S) copy number (T/S ratio) was calculated using the $2^{-\Delta\Delta Ct}$ formula where $\Delta\Delta Ct = (Ct_{Telomere} - Ct_{Hbg})_{sample} - (Ct_{Telomere} - Ct_{Hbg})_{reference\ DNA}$ [22].

The TRF2 levels were measured using enzyme-linked immunosorbent assay (ELISA) method with Human TBPL1 / TRF2 Quant ELISA Kit (LSBio, Washington, USA, LF-F33945) according to the manufacturer's protocol.

Study outcomes

The primary outcome, mortality, was assessed by phone, in the daily practice, or in home visit sessions. Overall survival was defined as the duration from the date of surgery to the date of death. The T/S ratio was established for experimental samples using a standard curve as explained.

Statistical analysis

Age on the first day of diagnosis, circulating TRF2, and T/S ratio–treated as continuous variables–were tested with Shapiro Wilk test to determine the distribution of these variables. Skewed distribution data were reported as median with its interquartile range (IQR), while the normally distributed data were reported as mean and standard deviation (SD). Other variables were treated as categorical data and were reported in percentages (%).

By utilizing the receiver operating characteristic (ROC) curve and Youden index, the area under the curve (AUC) value, sensitivity, specificity, and optimal cut-off points of TRF2 and T/S ratio were determined to predict mortality in breast cancer. Subsequently, all baseline data were divided according to the determined optimal cut-off. The relationships between clinical characteristics with TRF2 and T/S ratio were analyzed using an independent Student's t-test or Mann-Whitney U test based on the distribution or χ^2 as appropriate. The Kaplan-Meier and Cox proportional hazard regression were used to determine the association between risk factors and overall survival rate in breast cancer patients. For variables with $p<0.25$ in univariate analysis, they were included in multivariate Cox proportional hazard regression. The significance of multivariate analysis was set at 0.05.

Results

Baseline characteristics

During the study period, from January 1, 2015, to December 31, 2022, 776 patients were recorded, and only 446 samples were available in the biobank. Due to distant metastasis on the examination day, 288 patients were excluded, leaving 158 patients. Out of these 158, 80 patients were able to be contacted (either the patients or their families). An additional three patients were excluded as one participant died due to non-cancer-associated reasons (coronavirus disease 2019), one

participant had mucinous breast cancer based on histopathological examination, and one had an unavailable T/S ratio. This resulted in 77 patients being included in the final analysis.

Out of a total of 77 patients included in the final analysis, the location of breast cancer was not recorded in six patients, and 41 patients had no histopathological grading (**Table 1**). The mean age of the patients was 52.69±9.75 years, and the mean follow-up was 3.92±2.12 years. Regarding the location of breast cancer, 29 (40.8%) cancers were affected on the right side, 36 (50.7%) were on the left side, and six (8.4%) were bilateral. Luminal A breast cancer (28.5%) was the highest molecular subtype that was observed in this study. More than half of the patients received radiotherapy (92.8%) and chemotherapy (70.0%). The detailed baseline characteristics of the included patients are presented in **Table 1**.

Table 1. Baseline characteristics of breast cancer patients included in the study (n=77)

Characteristic	Frequency (percentage)
Age (year), mean±SD	52.69±9.75
Telomeric repeat-binding factor 2 (TRF2) (pg/mL), median (min-max)	595.5 (123–4,270.5)
T/S ratio, median (min-max)	1.03 (0.25–2.54)
Mortality	30 (38.9)
Follow-up time (year), mean±SD	3.92±2.12
Cancer location (n=71)	
Right	29 (40.8)
Left	36 (50.7)
Bilateral	6 (8.4)
Tumor size (cm), median (range)	5 (2.8–20.9)
Affected lymph node, median (range)	2.5 (1.0–13.0)
Tumor size stage (n=54)	
I	10 (18.5)
II	11 (20.3)
III	26 (48.1)
IV	7 (12.9)
Lymph node status (total) (n=12)	
I	9 (75.0)
II	2 (16.6)
III	1 (8.3)
Estrogen receptor (ER) (n=57)	
Positive	19 (33.3)
Negative	38 (66.6)
Progesterone receptor (PR) (n=57)	
Positive	21 (36.8)
Negative	36 (63.1)
Human epidermal growth factor receptor-2 (HER2) (n=52)	
Positive	38 (73.1)
Negative	14 (26.9)
Ki67 index, mean±SD	0.25±0.19
Molecular subtype (n=77)	
Basal-like	6 (7.7)
HER-enriched	6 (7.7)
Luminal A	22 (28.5)
Luminal B HER-	8 (10.3)
Luminal B HER+	14 (18.1)
Unspecified	21 (27.2)
Histopathological grade (n=36)	
I	1 (2.7)
II	8 (22.2)
III	27 (75.0)
History of chemotherapy (n=70)	
No	5 (7.1)
Yes	65 (92.8)
History of radiotherapy (n=70)	
No	21 (30.0)
Yes	49 (70.0)
Family history of cancer (n=77)	
No	58 (75.3)
Yes	19 (24.6)

T/S ratio: telomere (T) repeat copy number to a single gene (S) copy number

Relationship between telomeric repeat-binding factor 2 (TRF2) and telomere (T) repeat copy number to a single gene (S) copy number (T/S ratio) with clinical variables

The optimal cut-off points for TRF2 and the T/S ratio, as determined by ROC analysis and the Youden index, were 648 pg/mL and 0.93 kb, respectively. The AUC for TRF2 was 0.69 (95% CI: 0.58–0.79) with a sensitivity of 0.63 and specificity of 0.74, while the AUC for the T/S ratio was 0.51 (95% CI: 0.40–0.66) with a sensitivity of 0.63 and specificity of 0.47 (**Figure 1**).

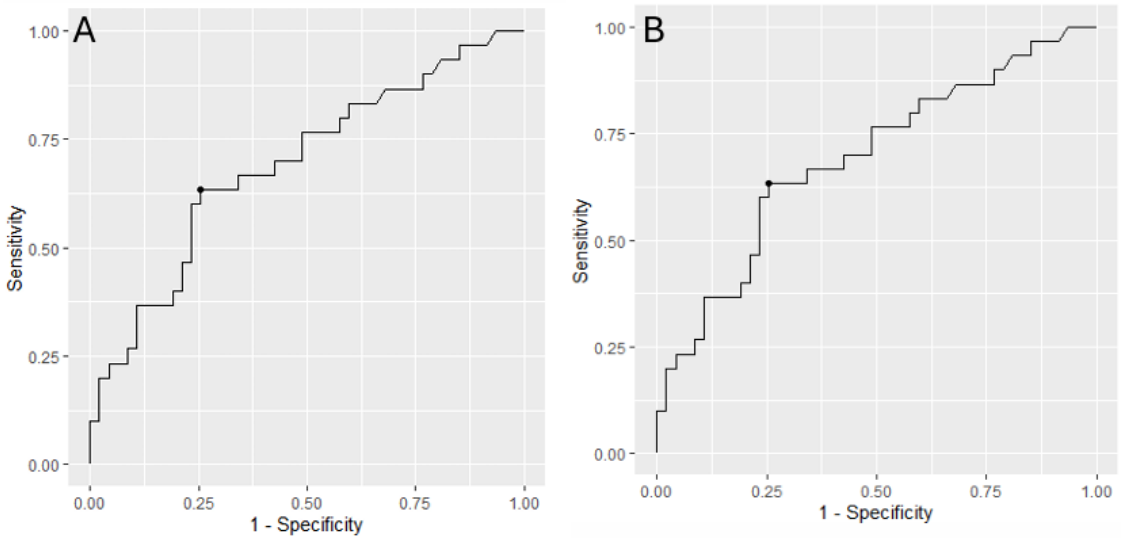

Figure 1. Receiver operating characteristic (ROC) plot of (A) telomeric repeat-binding factor 2 (TRF2) and (B) telomere (T) repeat copy number to a single gene (S) copy number (T/S ratio) to predict the mortality in breast cancer.

Using the optimal cut-off points from the ROC analysis (648 pg/mL for TRF2 and 0.93 kb for T/S ratio), the patients were then divided into two groups and the relationships of clinical characteristics with these classification groups were assessed. The association between demographic data, tumor size, number of infiltrated lymph nodes, staging based on the AJCC system [21], histopathological grade, treatment history, family history of cancer, expression of ER, PR, HER2, Ki-67, molecular and histopathological subtypes with TRF2 and T/S ratio classification are presented in **Table 2**. There were no significant associations observed with either TRF2 or T/S ratio groups.

Prognostic value of TRF2 and T/S ratio

Overall survival was significantly different between the TRF2 groups, with the low TRF2 group showing lower survival than the high TRF2 group (91.3% vs 83.87%; $p<0.01$) (**Figure 2**). However, there was no significant difference in overall survival between the T/S ratio groups (88.24% vs 88.37%; $p=0.640$) (**Figure 2**).

Factors associated with mortality

Univariate Cox proportional hazard regression indicated a high TRF2 was associated with lower overall survival (HR: 3.85; 95%CI: 1.79–8.28) compared to low TRF2, while the T/S ratio did not show any significance with overall survival (HR: 1.19; 95%CI: 0.57–2.48) (**Table 3**). Age, tumor size, primary tumor and regional lymph node stage, expression of HER2, the molecular subtype of breast cancer, history of radiotherapy, and history of cancer in the family were associated with overall mortality (**Table 3**). In multivariate Cox proportional hazard regression, however, only TRF2, age, molecular subtype, history of radiotherapy, and history of cancer in the family were included since the other variable had missing data of more than 20%. Multivariate Cox proportional hazard regression found that only TRF2 (HR: 3.66; 95%CI: 1.45–9.29, $p=0.004$) was independently associated with lower overall mortality (**Table 3**). Detailed results of Cox proportional hazard regression analysis are presented in **Table 3**.

Table 2. Relationship between clinical characteristics with TRF2 and T/S ratio

Characteristics	TRF2		*p*-value	T/S ratio		*p*-value
	≤648, n (%)	>648, n (%)		≤0.93, n (%)	>0.93, n (%)	
Age (year), mean±SD	49.85±9.82	54.09±10.74	0.774[a]	51.86±8.91	53.75±11.25	0.391[a]
Side (n=71)	42	29	0.713[c]	34	43	0.609[c]
Right	16 (38.1)	13 (44.8)		17 (50.0)	19 (44.1)	
Left	23 (54.7)	13 (44.8)		13 (38.2)	16 (37.2)	
Bilateral	3 (7.1)	3 (10.3)		1 (2.9)	5 (11.6)	
Tumor size (cm), median (min-max)	5 (2.8–18.7)	7.5 (5.7–20.9)	0.661[b]	3.25 (3.1–10.8)	6.1 (2.8–20.9)	0.262[b]
Affected lymph node, median (min-max)	2.75 (1.00–8.00)	3.5 (1.00–13.00)	0.082[b]	2.50 (1.00–13.00)	3.00 (1.00–10.00)	0.213[b]
Tumor size stage (n=54)	33	21	0.293[c]	25	29	0.058[c]
I	6 (18.2)	4 (19.0)		5 (20.0)	5 (17.2)	
II	7 (21.2)	4 (19.0)		1 (4.0)	10 (34.5)	
III	18 (54.5)	8 (38.1)		14 (56.0)	12 (4.14)	
IV	2 (6.1)	5 (23.8)		5 (20.0)	2 (6.9)	
Lymph node status (n=12)	9	3	0.131[c]	6	6	0.451[c]
I	8 (88.9)	1 (33.3)		4 (66.7)	5 (83.3)	
II	1 (11.1)	1 (33.3)		2 (33.3)	0 (0.0)	
III	0 (0.0)	1 (33.3)		0 (0.0)	1 (16.7)	
Estrogen receptor (ER) (n=57)	36	21	0.769[c]	25	32	0.781[c]
Positive	13 (36.1)	6 (28.6)		16 (64.0)	22 (68.8)	
Negative	23 (63.8)	15 (71.4)		9 (36.0)	10 (31.2)	
Progesterone receptor (PR) (n=57)	36	21	1.000[c]	25	32	0.413[c]
Positive	13 (36.1)	8 (38.1)		14 (56.0)	22 (68.8)	
Negative	23 (63.8)	13 (61.9)		11 (44.0)	20 (31.2)	
Human epidermal growth factor receptor-2 (HER2) (n=52)	34	18	0.191[c]	23	29	0.355[c]
Positive	27 (79.4)	11 (61.1)		8 (34.8)	6 (20.9)	
Negative	7 (20.6)	7 (38.9)		15 (65.2)	23 (79.1)	
Ki67 (%), mean±SD	0.24±0.19	0.28±0.17	0.468[a]	0.29±0.20	0.19±0.18	0.549[b]
Molecular subtype (n=77)	46	31	0.569[c]	34	43	0.192[c]
Basal-like	3 (6.5)	3 (9.7)		5 (14.7)	1 (2.3)	
HER-enriched	4 (8.7)	2 (6.5)		3 8.8)	3 (6.9)	
Luminal A	15 (32.6)	7 (22.6)		6 (17.6)	16 (37.2)	
Luminal B HER-	3 6.5)	5 (16.1)		5 (14.7)	3 (6.9)	
Luminal B HER+	10 (21.7)	4 (12.9)		6 (17.6)	8 (18.6)	
Unspecified	11 23.9)	10 (32.3)		9 (26.5)	12 (27.9)	
Histopathological grade (n=36)	22	14	1.000[c]	16	19	0.913[c]
I	1 (4.5)	0 (0.)		1 (6.3)	0 (0.0)	
II	5 (22.7)	3 (21.4)		3 (18.8)	4 (21.1)	
III	16 (72.7)	11 (78.6)		12 (75.0)	15 (78.9)	
History of chemotherapy (n=77)	43	27	1.000[c]	29	41	0.791[c]
No	3 (75.0)	2 (7.4)		21 (72.4)	28 (68.3)	
Yes	40 (25.0)	25 (92.6)		8 (27.6)	13 (31.7)	
History of radiotherapy (n=77)	43	27	0.603[c]	2	68	0.514[c]
No	14 (32.5)	7 (25.9)		1 (50.0)	20 (29.4)	
Yes	29 (67.4)	20 (74.1)		1 (50.0)	48 (70.6)	

Characteristics	TRF2		*p*-value	T/S ratio		*p*-value
	≤648, n (%)	>648, n (%)		≤0.93, n (%)	>0.93, n (%)	
Family history of cancer (n=77)	46	31	0.187[c]	34	43	0.595[c]
No	32 (69.6)	26 (83.9)		7 (20.6)	12 (27.9)	
Yes	14 (30.4)	5 (16.1)		27 (79.4)	31 (72.1)	

[a] Analyzed with independent Student t-test
[b] Analyzed with Mann-Whitney U test
[c] Analyzed with Fischer-exact test

Table 3. Univariate and multivariate Cox proportional hazard regression on mortality

Variable	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% confidence interval	*p*-value	Adjusted hazard ratio	95% confidence interval	*p*-value
Telomeric repeat-binding factor 2 (TRF2) level	3.85	1.79–8.28	<0.001	3.66	1.45–9.29	0.004
T/S ratio	1.19	0.57–2.48	0.631			
Age	1.03	0.99–1.07	0.103	1.04	0.98–1.09	0.211
Tumor size	1.05	0.99–1.12	0.101[a]			
Affected lymph node	1.08	0.94–1.24	0.312			
Tumor size stage			0.132[a]			
I	Reference					
II	0.58	0.10–3.47				
III	1.79	0.50–6.41				
IV	3.61	0.78–16.7				
Lymph node status			0.056[a]			
I	Reference					
II	7.47	1.04–53.5				
III	19.9	1.01–391				
Estrogen receptor (ER)	0.84	0.34–2.06	0.713			
Progesterone receptor (PR)	1.22	0.49–3.05	0.672			
Human epidermal growth factor receptor-2 (HER2)	3.03	1.19–7.70	0.026[a]			
Ki67	2.62	0.23–30.3	0.441			
Molecular subtype			0.005			0.036
Basal-Like	Reference			Reference		
HER-enriched	1.36	0.23–8.24		1.68	0.25–11.2	
Luminal A	0.36	0.06–2.19		0.53	0.08–3.48	
Luminal B HER-	2.39	0.49–11.6		4.23	0.80–22.4	
Luminal B HER+	5.13	0.97–27.1		3.34	0.56–20.0	
Unspecified	2.13	0.46–9.78		2.99	0.57–15.8	
Histopathological grade			0.563			
I	Reference					
II	—	0.00–Inf				
III	—	0.00–Inf				
History of chemotherapy	0.45	0.13–1.53	0.252			
History of radiotherapy	3.14	1.09–9.10	0.017	2.29	0.70–7.48	0.151
Family history of cancer	0.57	0.22–1.50	0.234	0.53	0.17–1.66	0.303

T/S ratio: telomere (T) repeat copy number to a single gene (S) copy number
[a] Total of missing data was>20%

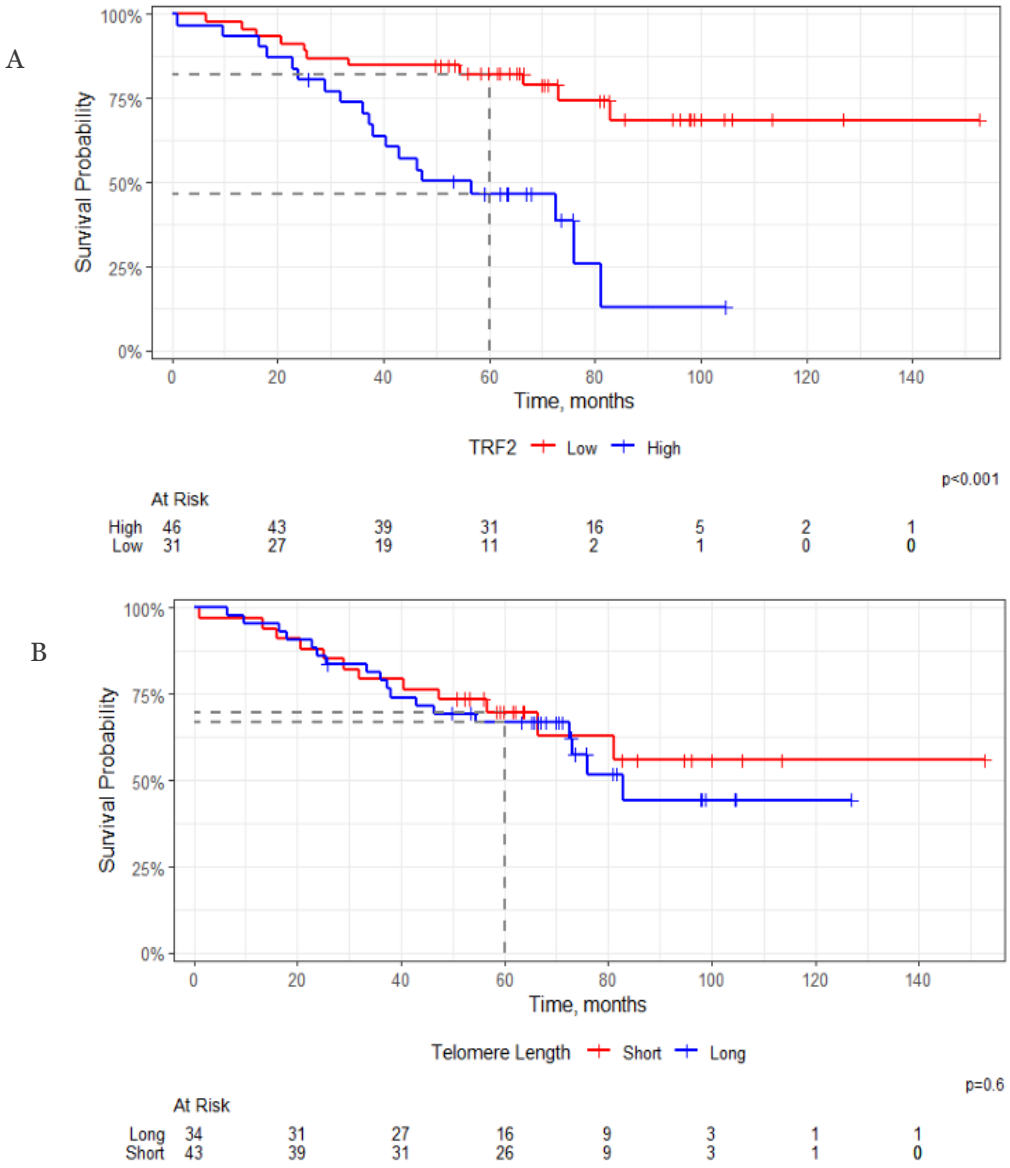

Figure 2. Kaplan-Meier plot of (A) telomeric repeat-binding factor 2 (TRF2) and (B) telomere (T) repeat copy number to a single gene (S) copy number (T/S ratio). A significant difference was observed in the TRF2 group, where the lower TRF2 group had a better survival probability than the higher one (p<0.001). On the other hand, the T/S ratio group did not show any significant difference in survival probability (p=0.640).

Discussion

This study shows the prognosis value of TRF2 on 5-year mortality in breast cancer. Using 648 pg/mL as the optimal cut-off, the low TRF2 shows a better overall survival rate in comparison with the higher one (91.3% vs 83.87%; log-rank test, p<0.01). Moreover, high TRF2 predicts the mortality of breast cancer patients 3.66 times higher than the lower one (HR: 3.66; 95%CI: 1.45–9.29). Unlike TRF2, telomere length is not associated with mortality in 5 years (HR: 1.19; 95%CI: 0.57–2.48).

Cancer cells are unique since they have a deviated characteristic compared to normal cells. According to Hanahan *et al*. review, there are 14 hallmarks of cancer: (1) sustaining proliferative signaling, (2) evading growth suppressors, (3) avoiding immune destruction, (4) enabling replicative immortality, (5) tumor-promoting inflammation, (6) activating invasion and metastasis, (7) inducing or accessing vasculature, (8) genome instability and mutation, (9) resisting cell death, (10) deregulating cellular metabolism, (11) unlocking phenotypic plasticity, (12) non-mutational epigenetic reprogramming, (13) polymorphic microbiomes, and (14)

unlocking phenotypic plasticity [23]. Among these hallmarks, telomere length is associated with replicative immortality mechanisms and resisting cell death [24-26]. A telomere is a structure composed of numerous repeats of TTAGGG and is organized in a complex 3-dimensional structure located at the very end of all chromosomes [24,26,27]. Similar to the DNA, telomere is double-stranded for most of its length, except at the very end of the leading strand, which is single-stranded. This single-stranded overhang of the telomere exposed the telomere to shortening either in every mitotic division or the activation of DNA damage repair (DDR) mechanism [24-27]. This mechanism is crucial to prevent damaged DNA from being inherited by the new cell.

Telomere function and maintenance are tightly linked to the shelterin complex, as mentioned earlier. This complex forces the double-stranded DNA to fold back, forming a T-loop and D-loop structure that hide the single-stranded telomere and prevent telomere shortening [24,26,27]. When the telomere becomes critically short, T-loop formation is no longer possible, resulting in the activation of the DDR mechanism. Thus, the cells arrest their proliferation cycle and gradually become senescence [26,27].

Aside from protecting the single-stranded telomere from the DDR, telomere can be elongated through telomerase activity. Telomerase is a multi-unit ribonucleoprotein complex, with telomerase reverse transcriptase (TERT) and telomerase RNA component (TERC) as its core [26,28]. Other units, including Pontin, Reptin, Gar1, Nhp2, and Tcab1 are required for proper telomerase unit assembly and recruitment to chromosomes [27]. TERC serves as the template for the TERT unit, which is the catalytic protein crucial for telomere elongation. Interestingly, shelterin is involved in the recruitment of telomerase to the telomeric DNA, suggesting that synchroneity between these complexes retains the telomere length and acquires replicative immortality [25,27]. TERT has been a variant of interest in oncology research, whilst TRF2 has become popular recently. Owing to its main function to bypass the DDR mechanism, TRF-2 plays an important role in cancer. TRF2 and telomere length are inversely correlated according to Diehl *et al*. findings, indicating the short telomere is protected by ubiquitous TRF2 evading DDR [18].

Telomere length is known as a variable associated with the prognosis of numerous cancers [5]. In breast cancer, short leukocyte telomere length increased the mortality in 30 months (HR: 3.03; 95%CI: 1.11–8.18) [29]. A systematic review of 33 articles that investigated the telomere length in tumor specimens reported a trend of better prognosis in breast cancer with longer telomere length [30]. In contrast, a Mendelian randomization study found the poor prognosis was associated with longer breast cancer cell telomere length, especially in subtype ER-negative (OR: 1.84; 95%CI: 1.08–3.14) [31]. Moreover, there is a significant association between advanced-stage breast cancer and long telomere length, while early-stage breast cancer had a shorter telomere length [32]. Meanwhile, in this study, the significance of leukocyte telomere length in predicting mortality was not observed (HR: 1.19; 95%CI: 0.57–2.48). Altogether, the role of telomere length in breast cancer prognosis remains unresolved since both classic long telomere and alternative short telomere indicate a significantly worse prognosis in breast cancer, although this study does not support either variable [33]. Further research is needed to confirm the association between telomere length and overall survival of breast cancer patients.

On the other hand, TRF2 is a relatively novel marker in breast cancer and has not been meticulously studied. Fortunately, some studies have been conducted on other cancer types. In advanced colorectal cancer, co-expression of TRF2 and VEGF-A is associated with poor prognosis in colorectal cancer grade I-III [34]. The overexpression of TRF2 is associated with poor overall survival in non-small cell lung cancer [35], but not statistically significant in adenocarcinoma of the lung [36]. In parallel with these studies, circulating TRF2 is associated with 5-year mortality (HR: 3.66; 95%CI 1.45–9.29). In contrast, Ozden *et al.* observed that higher TRF2 expression in the cervical cancer tissue correlates with better overall survival [37]. Meanwhile, Chen *et al*. did not find any association of TRF2 expression with progression of prostatic cancer [38].

This present study verified the prognostic value of circulating TRF2 in breast cancer to predict mortality in five years, however, more meticulous research is needed to validate the association between circulating TRF2 and mortality in breast cancer. Despite its strength, this study has limitations. First, the subgroup analysis based on molecular subtype has not been done as the included participants are relatively small although it is important. Thus, the significant

finding of TRF2 as a prognosis factor is not evaluated using an estimated method, such as Akaike information criterion or C-index statistic, as the data was limited to build numerous multivariable analyses. Moreover, the advantage of TRF2 to predict mortality in breast cancer cases was only compared to the population with lower TRF2 concentration, rather than to healthy participants.

The association of TRF2 with mortality in breast cancer cases should be investigated. As this study showed the significance of circulating TRF2 as a prognostic factor, the expression of TRF2 in cancer tissue should be explored, along with its association with mortality in breast cancer. In cancer research, disease-free survival is also an important outcome that should be measured. Hence, the value of TRF2 in predicting disease-free survival should be studied well. Finally, the exploration of TRF2 in contributing to the mortality of breast cancer based on its subtype should be evaluated as well. To minimize the selection bias in future research, propensity score matching and inverse probability weighting should be utilized during the selection of participants [39-41].

Conclusion

A high circulating TRF2 in breast cancer participants is associated with lower overall survival rates in five years in comparison with the lower TRF2 groups. Moreover, high TRF2 predicts the mortality of the breast cancer population 3.66 times higher than the lower group. On the other hand, telomere length is not associated with overall survival rate or 5-year mortality prediction.

Ethics approval

This study had been approved by the Ethical Committee of the Faculty of Medicine, Public Health, and Nursing Universitas Gadjah Mada and conducted according to the principle of bioethics expressed in the Declaration of Helsinki (ethical number: KE/FK/0435/EC/2023). All patients or families has been informed and signed their informed consent to use their data for research purposes.

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Competing interests

The authors declare to have no conflict of interest during data retrieval, procession, analysis, and manuscript publishing.

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Underlying data

Derived data supporting the findings of this study are available from the corresponding author on request.

Declaration of artificial intelligence use

We hereby confirm that no artificial intelligence (AI) tools or methodologies were utilized at any stage of this study, including during data collection, analysis, visualization, or manuscript preparation. All work presented in this study was conducted manually by the authors without the assistance of AI-based tools or systems.

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