Research Article

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Telomere Length Is Predictive of Breast Cancer Risk in *BRCA2* Mutation Carriers

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Abstract

Background: Germline *BRCA2* mutations increase risk of breast cancer and other malignancies. BRCA2 has been shown to play a role in telomere protection and maintenance. Telomere length (TL) has been studied as a modifying factor for various diseases, including breast cancer. Previous research on TL in *BRCA* mutation carriers has produced contradicting results.

Methods: We measured blood TL, using a high-throughput monochrome multiplex qPCR method, in a well-defined Icelandic cohort of female *BRCA2* mutation carriers ($n = 169$), sporadic breast cancer patients ($n = 561$), and healthy controls ($n = 537$).

Results: Breast cancer cases had significantly shorter TL than unaffected women ($P < 0.0001$), both *BRCA2* mutation carriers ($P = 0.0097$) and noncarriers ($P = 0.00006$). Using exclusively samples acquired before breast cancer diagnosis, we found that shorter telomeres were significantly associated with increased breast cancer risk in *BRCA2* mutation carriers [HR, 3.60; 95% confidence interval (CI), 1.17–11.28; *P*, 0.025] but not in noncarriers (HR,1.40; 95% CI, 0.89–2.22; *P*, 0.15). We found no association between TL and breast cancer–specific survival.

Conclusions: Blood TL is predictive of breast cancer risk in *BRCA2* mutation carriers. Breast cancer cases have significantly shorter TL than unaffected women, regardless of *BRCA2* status, indicating that samples taken after breast cancer diagnosis should not be included in evaluations of TL and breast cancer risk.

Impact: Our study is built on a well-defined cohort, highly accurate methods, and long follow-up and can therefore help to clarify some previously published, contradictory results. Our findings also suggest that BRCA2 has an important role in telomere maintenance, even in normal blood cells. *Cancer Epidemiol Biomarkers Prev; 26(8); 1248–54. ©2017 AACR.*

Introduction

Germline mutations in the *BRCA2* gene are associated with highly increased risk of breast, ovarian, and other cancers. Great numbers of mutations with variable impact on cancer risk and progression are known in the gene worldwide. A single founder mutation, 999del5, has been detected in the *BRCA2* gene in the Icelandic population (1), making it feasible to study the influence of a single mutation at a population level. The *BRCA2 999del5* mutation can be found in approximately 6%–7% of female breast cancer patients in Iceland and is associated with poor prognosis, although mutation carriers seem to differ with respect to age of onset and severity of disease (1–3). BRCA2 was originally shown to play a role in homologous recombination repair of double-strand breaks in DNA (4, 5) and has more recently been shown to be involved in protecting and stabilizing stalled replication forks (6, 7) as well as playing a protective role at telomeres (8–10).

Telomeres are protein-bound repeated nucleotide sequences $(TTAGGG)_n$ that play a critical role in maintaining chromosomal stability by protecting chromosome ends from damage and degradation (11). Telomere sequences shorten progressively with each cell division as the replication machinery cannot fully replicate to the end of the chromosomes, commonly referred to as the end-replication problem. Under normal circumstances, telomere length reaches a certain threshold, which leads to replicative senescence in cells which has been associated with aging (12). Dysfunctional telomere maintenance and shortening can lead to genomic instability, a driving force behind cancer formation (13). Telomere shortening has been shown to be an early and common molecular alteration in epithelial cancers (14, 15), including breast cancers (16). Telomere dysfunction and chromosome instability of this kind are common in *BRCA*-mutated cancer cells (10, 17, 18).

A myriad of papers have reported a relationship between mean telomere length (TL) and risk of developing various diseases, including cancer (19–21). In the case of breast cancer, the results of association between TL and cancer incidence, progression, and mortality are inconclusive. Studies have reported both shorter (22–24) and longer TL (25, 26) to be associated with breast cancer risk and others have found no such association (27–30).

A few studies have examined blood TL in *BRCA1* and *BRCA2* mutation carriers. Most of these studies are retrospective and have yielded contradicting results. Martinez-Delgado and colleagues reported shorter mean leukocyte TL in breast cancer affected *BRCA* mutation carriers (pooled) than noncarrier relatives, sporadic breast cancer patients, and a healthy control group. Furthermore, they found telomere shortening to be associated with earlier age of breast cancer onset in successive generations of *BRCA* cancer patients, suggesting genetic anticipation (31). The same group has also described shorter TL in blood samples from both familial and sporadic ovarian cancer cases than in healthy controls (32).

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These results are contradicted in more recent papers. Pooley and colleagues found no significant association between mean blood TL and breast or ovarian cancer risk in a much larger group of *BRCA* mutation carriers (EMBRACE study-set; ref. 33). Interestingly they found that *BRCA* mutation carriers, especially *BRCA2* mutation carriers, had longer mean TL than their noncarrier and cancer-free relatives. Killick and colleagues had previously reported no association between blood TL and cancer risk in *BRCA1/2* mutation carriers, neither in prostate cancer patients (IMPACT study) nor a smaller subset of breast cancer patients (RMH Carrier Clinic Set; ref. 34).

In the current study, we measured blood TL in a well-defined Icelandic cohort of female *BRCA2* mutation carriers, sporadic breast cancer patients, and healthy controls with the aim of determining if TL measurements could be used as a stratification method for breast cancer risk and/or prognosis in mutation carriers.

Materials and Methods

Study cohort

The study group consisted of Icelandic female *BRCA2 999del5* mutation carriers ($n = 169$), sporadic breast cancer patients ($n = 561$), and healthy controls ($n = 537$). Blood samples were collected between the years of 1998 and 2006 as part of a large-scale sample acquisition, focused on breast cancer cases and their relatives, at the University of Iceland Cancer Research Laboratory, in collaboration with the Icelandic Cancer Society and Landspitali University Hospital. In the current study, only samples that had gone through exactly the same blood handling and DNA isolation methods (phenol–chloroform) were included. All samples had been screened for the only two known local *BRCA* founder mutations, i.e., *BRCA2 999del5* (c.771_775del5) and a much less frequent *BRCA1 5193G→A* (2, 35). Carriers of this rare *BRCA1* founder mutation were excluded from the study. Sporadic breast cancer cases were defined as those who did not carry either of these two local *BRCA* founder mutations. Some participants had already been diagnosed with breast cancer at blood sampling and others were diagnosed during follow-up. Information on cancer diagnoses and survival was supplied by the Icelandic Cancer Registry as of May 31, 2016. Cohort/participant characteristics are further detailed in Table 1.

This work was carried out according to permits from the Icelandic Data Protection Commission (2006050307) and Bioethics Committee (VSNb2006050001/03-16). Informed consent was obtained from all participants.

Telomere length measurements

TL was measured in archived genomic DNA samples extracted from whole blood using standard phenol-chloroform DNA extraction and stored long term at 100 ng/μL in TE-4 buffer (10 mmol Tris–HCl, 0.1 mmol EDTA, pH 7.5) at 4°C.

Relative mean TL was measured using a modified version of a multiplex monochrome qPCR method, first described by Cawthon (36). TL was calculated as a ratio (T/S) between detected fluorescence from telomere repeat copy number (T) and a single copy reference sequence (S) from the β-globin gene. Primers used to amplify both telomere repeats and the single-copy gene were as described by Cawthon (36): telomere primers were telg, ACACTAAGGTTTGGGTTTGGGTTTG GGTTTGGGTTAGTGT and telc, TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA. β-globin primers were hbgu, CGGCGGCGGGCGGCGCGGGCTGGGCGGCTTCATCCACGTTCACCTTG and hbgd, GCCCGGCCCGCCGCGCCCGTCCCGCCGGAGGAGAAGTCTGCCGTT.

DNA samples were amplified in a total reaction volume of 5 μL containing 2.5 μL of PowerUpSYBR Green PCR Master Mix (2x; Applied Biosystems), 900 nmol of telc and telg telomere primers, 500 nmol of both β-globin primers (hbgu and hbgd; Sigma Aldrich) and 2.5 ng of DNA, with H_2O to final volume. DNA samples were diluted with Milli-Q H_2O prior to qPCR runs. A single lot of each reagent was used throughout the study to eliminate possible batch to batch variations. Runs were performed using the Bio-Rad CFX384 Touch Real-Time PCR Detection System, in 384-well plate format. The thermal cycling profile was as follows, stage 1: incubation at 50°C for 2 minutes and at 95°C for 5 minutes; stage 2: 2 cycles of 94°C for 15 seconds and 49°C for 15 seconds; stage 3: 35 cycles of 94°C for 15 seconds, 62°C for 10 seconds, 74°C for 15 seconds, 84°C for 10 seconds and 88°C for 15 seconds, with signal acquisition for telomere C_t values at 84°C and at 88°C for β-globin C_t values. For all samples, C_t values were determined for both telomere and β-globin on each plate using a predefined threshold on the exponential phase of the amplification curves. All samples were tested in triplicate and the C_t values averaged for a final value, sample triplicates with C_t SD higher than 0.5 were reanalyzed.

To allow for efficiency calculation of runs, a standard curve prepared by 2× serial dilution of DNA ranging from 12.5 ng to 0.39 ng was run in triplicate in every 384-well plate throughout the study. Amplification efficiencies were calculated separately for telomere and β-globin from the standard curve slopes according to $\text{Eff} = 10^{(-1/\text{slope})}$. All runs had standard curve efficiencies within 90%–110%, the mean efficiency was 102.7% for telomere runs and 96.2% for β-globin runs. All participant samples were required to have C_t values within the range of the standard curve.

Each plate included a reference DNA sample run in triplicate to monitor interplate variability as assessed by the coefficient of

Table 1. Summary characteristics for the study group

	Noncarriers		*BRCA2* mutation carriers	
	Unaffected	Affected	Unaffected	Affected
N	537	561	68	101
Age at blood draw (mean, SE)	49.9 (0.9)	59.8 (0.6)	42.4 (1.5)	53.8 (1.3)
Sample acquisition time (n)				
Before breast cancer diagnosis	—	76	—	18
After breast cancer diagnosis	—	485	—	83
Age at breast cancer diagnosis (mean, SE)	—	52.7 (0.5)	—	49.7 (1.1)
Follow-up time to breast cancer diagnosis in years (mean, SE)	13.3 (0.1)	7.5 (0.4)	13.7 (0.8)	7.7 (0.9)
Survival follow-up time in years (mean, SE)	—	19.2 (0.5)	—	15.5 (0.9)

variation (CV = 3.9%). The relative telomere length (T/S ratio) for each participant sample was calculated from the C_t values of both telomere (T) and β-globin (S) adjusted for the standard curve efficiency and calibration sample C_t values per reaction, thus eliminating plate to plate differences. The T/S ratio was calculated according to the following formula, as described in (21): $T/S = [(Eff_{tel})^{-Ct(tel,sample)})/(Eff_{\beta\text{-globin}})^{-Ct(\beta\text{-globin,sample})}]/[(Eff_{tel})^{-Ct(tel,reference\ sample)})/(Eff_{\beta\text{-globin}})^{-Ct(\beta\text{-globin,reference\ sample})}]$. The intraplate average CV, that is, concordance among triplicates, was 0.69% (±0.24%) for telomere runs and 0.72% (±0.21%) for β-globin runs.

Age adjustment

The association of TL with age at blood draw was evaluated in an evenly age-distributed control group ($n = 537$) of healthy, cancer-free women not carrying a *BRCA* mutation, using linear regression (Fig. 1). The expected negative association between age and TL was observed ($P = 1.27 \times 10^{-8}$). All TL measurements were subsequently adjusted for age at blood sampling using the line of best fit for controls.

Statistical analyses

The age adjusted TL (T/S) values were transformed using the natural logarithm to obtain a normally distributed parameter. The natural log transformed TL values (age adjusted) were used in all downstream statistical analyses.

To determine whether the distribution of TL differed among *BRCA2* mutation carriers and noncarriers we carried out Student *t*-test, stratifying by the time of blood sampling with regards to breast cancer diagnosis.

We determined whether TL could predict risk for developing breast cancer using multivariate Cox proportional hazard model. This was carried out by exclusively using samples taken from women who had not been diagnosed with breast cancer at the time of blood extraction. Clearly, a subset of these women later developed the disease whereas others did not (i.e., resembling a prospective study). The Cox proportional hazards regression included two adjustment variables, that is, year and age at blood sampling. Participants diagnosed with other types of cancer were censored at the time of that diagnosis.

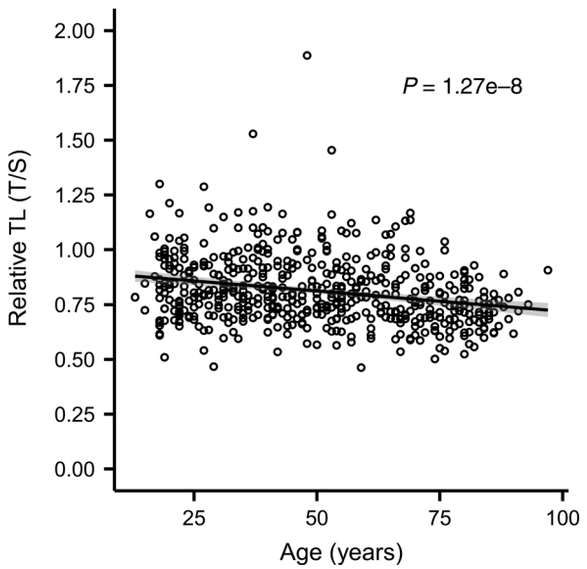

Figure 1.
Association between relative TL of the control group ($n = 537$) and age at blood draw.

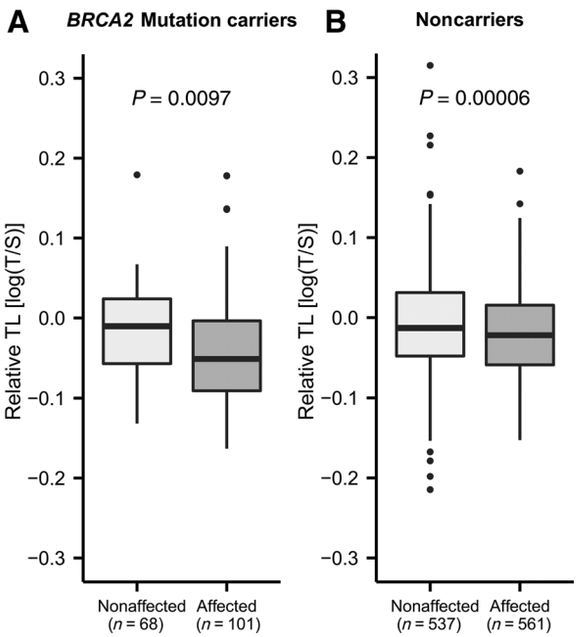

Figure 2.
Comparison of TL between breast cancer affected and nonaffected women in *BRCA2* mutation carriers (**A**) and noncarriers (**B**). Data represented through boxplots showing the median and interquartile distance for each group. All TL values have been age-adjusted. The *P* values shown on the figure were derived from the Student *t* test.

Cox proportional hazards models were used to perform breast cancer specific survival analyses, adjusting for possible confounding variables, such as year and age at breast cancer diagnosis, and breast cancer molecular subtype (estrogen receptor status and Ki67 status).

Cox proportional hazards models fulfilled the assumptions of constant proportional hazards over time based on established methods (cox.zph function in R) and a statistically significant *P* value at < 0.05 derived from log-rank hypothesis testing. All analyses were done separately for *BRCA2* mutation carriers and noncarriers as well as being stratified by breast cancer status and sample acquisition timing. All analyses were performed using R (CRAN).

Results

BRCA2 mutation status in association with TL

First, our results show that breast cancer affected women had significantly shorter TL than unaffected women ($P < 0.0001$), this held true for both *BRCA2* mutation carriers ($P = 0.0097$; Fig. 2A) and noncarriers ($P = 0.00006$; Fig. 2B). Second, no significant differences between carriers and noncarriers were found with respect to TL within the subset of women who were unaffected by breast cancer ($P = 0.22$; Fig. 3A). Third, by exclusively focusing on women who were

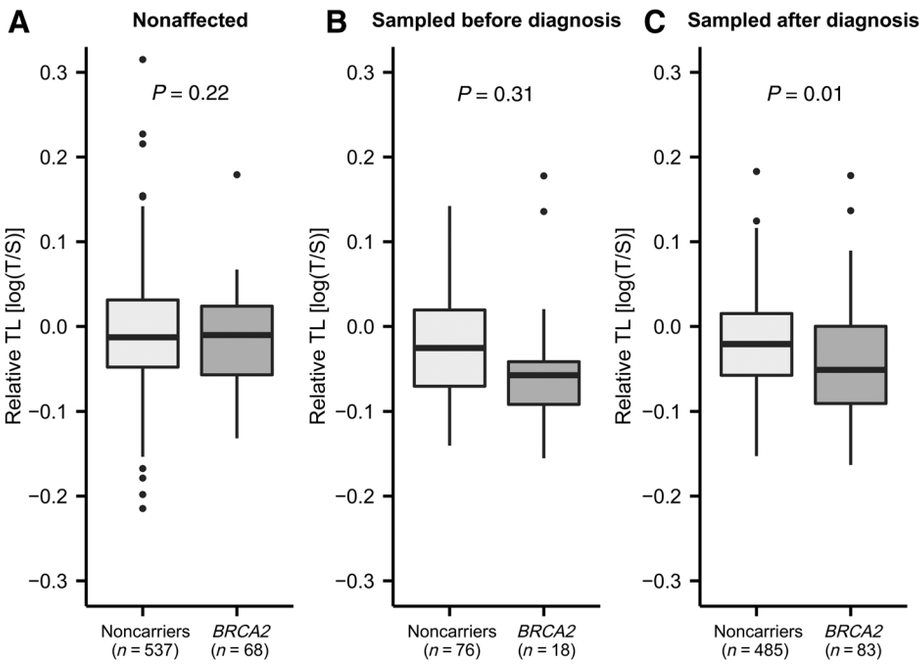

Figure 3.
Comparison of TL between *BRCA2* mutation carriers and noncarriers, stratified by time of blood sampling with regards to breast cancer diagnosis. Nonaffected women (**A**), women sampled before breast cancer diagnosis (**B**), and women sampled after breast cancer diagnosis (**C**). Data represented through box-plots showing the median and interquartile distance for each group. All TL values have been age-adjusted. The *P* values shown on the figure were derived from the Student *t*-test.

cancer-free at the time of blood extraction but were affected by breast cancer later on during the course of their lives, we did not find a statistically significant difference between *BRCA2* carriers and noncarriers with respect to TL ($P = 0.31$; Fig. 3B). Finally, we did find a statistically significant difference in TL when comparing women who had already been diagnosed with breast cancer at the time of blood extraction revealing shorter telomeres in *BRCA2* mutation carriers compared with noncarriers ($P = 0.01$; Fig. 3C).

TL is a modifier of breast cancer risk in *BRCA2* mutation carriers

In a multivariate Cox proportional hazards regression for time to breast cancer diagnosis analyzed with respect to TL, we find shorter telomeres to be significantly associated with increased risk for developing breast cancer in the *BRCA2* mutation carrier group [HR, 3.60; 95% confidence interval (CI), 1.17–11.28; $P = 0.025$] but not among the noncarriers (HR, 1.40; 95% CI, 0.89–2.22, *P*, 0.15; Fig. 4). In this analysis, TL was included as a two-group variable defined on the basis of the median among carriers

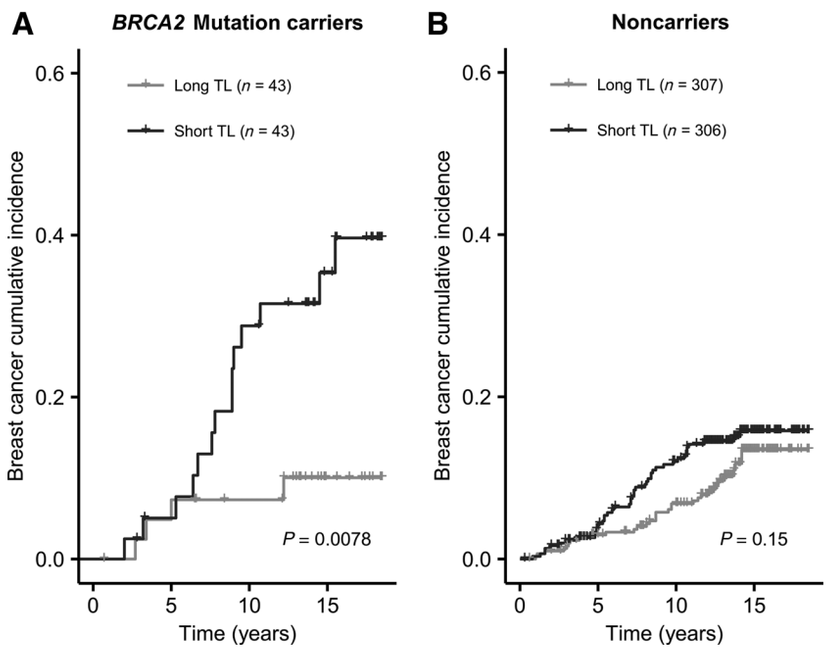

Figure 4.
Breast cancer–specific cumulative incidence according to analysis of TL. *BRCA2* mutation carriers ($n = 86$) (**A**) and noncarriers ($n = 613$) (**B**). The *P* values shown on the figure were derived from log-rank hypothesis testing for differences in times to breast cancer diagnosis between groups; that is, long and short TL (divided around the median). Cox proportional hazards model corrected for the year and age at blood sampling; *BRCA2* mutation carriers (HR, 3.60; 95% CI, 1.17–11.28; *P*, 0.025) and noncarriers (HR, 1.40; 95% CI, 0.89–2.22; *P*, 0.15).

Table. 2. Breast cancer–specific survival analysis according to blood TL in *BRCA2* mutation carriers and noncarriers

	Univariate			Multivariate[a]		
	HR (95% CI)	***P***	***n***	**HR (95% CI)**	***P***	***n***
BRCA2	1.02 (0.44–2.36)	0.96	101	1.03 (0.38–2.81)	0.95	83
Sporadic	0.92 (0.60–1.43)	0.72	561	0.96 (0.57–1.62)	0.88	400

NOTE: Breast cancer–specific survival analyses using Cox proportional hazard models according to blood TL divided into groups of shorter and longer TL around the median. Associations are presented as HR for short TL with 95% CI.

[a]Analyses adjusted for age and year at breast cancer diagnosis, sample acquisition timing, ER and Ki67 receptor status.

($TL_M = -0.030$) and noncarriers ($TL_M = -0.0146$) to then obtain a class label for each individual, that is, long or short TL. The adjustment variables included in both analyses were year and age at blood sampling.

Blood TL shows no association with breast cancer–specific survival

No significant associations were found between TL and breast cancer–specific survival in our study group, independent of *BRCA2* mutation status and time of blood sampling with regards to breast cancer diagnosis (Table 2).

Discussion

In this study we show that blood TL is a modifier of breast cancer risk in *BRCA2* mutation carriers. However, no association was seen between TL and prognosis in our breast cancer group. These results were based on TL measures in blood samples from a well-defined cohort of female *BRCA2* mutation carriers and noncarriers, using a highly accurate multiplex monochrome qPCR method.

Telomere dysfunction and the subsequent genomic instability may promote cancer development. Not surprisingly, numerous studies have reported a relationship between mean leukocyte TL and cancer incidence and mortality, but for many cancers the results have been inconsistent (20, 21, 37). In most cases the relationship is inverse, that is, shorter TL is associated with increased disease risk and progression. An emerging topic of debate is the impact of study design on the end results of TL measurement studies. Retrospective studies, where the samples for TL measurements have been acquired after cancer diagnosis, have mostly shown cancer patients to have shorter telomeres than controls. This is indeed reflected in our study group where TL is shorter in breast cancer affected women than in unaffected women. Radiotherapy and chemotherapy have both been shown to affect leukocyte TL (38–40), yet most studies do not account for treatment as a confounding factor, most likely due to incomplete information. Prospective studies with samples taken before diagnosis have, contrary to retrospective studies, generally shown no significant difference in TL between cases and controls (27, 41).

Previous studies on TL in breast cancer and *BRCA* mutation carriers have similarly yielded contradicting results (31–34), which can partly be explained by differences in methodology and study design. A weakness of all the aforementioned studies is that they were carried out retrospectively. It is therefore not clear whether the results were affected by cancer status or cancer treatment. An ongoing longitudinal prospective study is recruiting unaffected *BRCA* mutation carriers to examine potential relationships between blood TL and breast cancer risk in addition to various modifiable lifestyle factors (42). Results from such prospective studies are better suited for risk assessment as the bias of retrospective studies is avoided.

The strength of the current study lies in the cohort characteristics and the quality of clinical data available through national databases in Iceland. Most studies combine various mutations in the *BRCA2* gene, potentially biasing risk assessments as it is known that different mutations in the gene convey variable cancer risk. An advantage of our Icelandic cohort is that all mutation carriers have the same *BRCA2 999del5* early truncation mutation and the population is demographically homogenous. The samples included in this study were treated in a controlled and identical manner from collection to storage, eliminating bias due to difference in blood handling and DNA processing. Notably, as the analysis of TL is sensitive to batch effects, the experimental setup was carefully designed to minimize this effect. As a result, our assessment of technical variability indicates only minor effects (see details in Materials and Methods).

Through the Icelandic Cancer Registry, we have complete information on cancer incidence, type, and mortality. The Icelandic Cancer Registry has been internally validated and has >99% completeness (43). With some participants already diagnosed with cancer at blood sampling and others diagnosed during follow-up, a part of our dataset could be viewed as prospective. This gave us a chance to subset the data according to the time of blood sampling relative to cancer diagnosis, which is very important for predicting potential effects on cancer incidence and survival with greater accuracy. Using only samples from women who were cancer-free at the time of blood sampling, we show that shorter TL is associated with increased risk of breast cancer in *BRCA2* mutation carriers but not in noncarriers.

In our study, there is a trend toward shorter TL in breast cancer affected *BRCA2* mutation carriers compared with sporadic cases, although not reaching significance for the group sampled before diagnosis, possibly attributable to low sample numbers.

We do not see a connection between TL and breast cancer survival when adjusting for possible confounding factors, independent of *BRCA2* mutation status and time of blood sampling. For samples that were obtained after breast cancer diagnosis, effects of different cancer treatments cannot be ruled out. In agreement with our results, three other studies have reported breast cancer specific survival data from blood TL measurements and showed no such association (26, 30, 44). In a prospective study by Ennour-Idrissi and colleagues, no association between TL and traditional prognostic factors in breast cancer patients was detected, but they found TL being positively associated with physical activity in breast cancer patients (45).

The frequency of critically short telomeres, rather than mean TL, has been shown to constitute telomere dysfunction and be critical for chromosome stability (46). Thus, it has been rightfully debated whether measuring the mean TL in a cell or population of cells is the most dependable way of linking TL to disease risk or other factors. This is of course the case in blood, where many cell types are present. Telomere length has been shown to be cell-type specific (47), so the percentage of each cell type in a blood sample

can affect the final mean TL measurement. Blood cell type composition can be influenced by many factors, including the time of day at sampling and even minor factors such as having a cold. Information of this kind is rarely noted at the time of sampling nor available to researchers. Which cell types are in the mix can also be affected by blood handling before DNA extraction, for example, using whole blood versus Histopaque, which is commonly used to isolate peripheral blood mononuclear cells (PBMC) from whole blood. It is therefore of paramount importance to standardize methods of blood sample handling and DNA isolation for having comparable measurements of telomere length. Pooling of research data from different laboratories and meta-analyses must also be performed with caution.

Conclusion

Looking exclusively at blood samples from unaffected *BRCA2* mutation carriers and noncarriers, we show that blood TL has a predictive value for breast cancer susceptibility in *BRCA2* mutation carriers. We also show that breast cancer cases have significantly shorter telomeres than unaffected women, and this is true both for *BRCA2* mutation carriers and noncarriers. Measurements of TL in samples taken after diagnosis of breast cancer should therefore not be included in evaluations of TL associated breast cancer risk.

Considering the sensitivity with respect to sample variation and treatment, it is our opinion that the qPCR analysis of TL would not be the method of choice for use in a clinical setting as a predictive and/or prognostic biomarker for cancer. Our results nonetheless show the importance of telomeres in *BRCA2* mutation carriers. Reduced amounts of the *BRCA2* protein are likely to lead to telomere dysfunction and genomic instability, as has been seen in breast epithelial and tumor cells from *BRCA2* mutation carriers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: B. Thorvaldsdottir, S.K. Bodvarsdottir, J.E. Eyfjörd
Development of methodology: B. Thorvaldsdottir, M. Aradottir, S.K. Bodvarsdottir
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.E. Eyfjörd
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B. Thorvaldsdottir, O.A. Stefansson, J.E. Eyfjörd
Writing, review, and/or revision of the manuscript: B. Thorvaldsdottir, O.A. Stefansson, S.K. Bodvarsdottir, J.E. Eyfjörd
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.E. Eyfjörd
Study supervision: J.E. Eyfjörd

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