Received: 24 June 2016 | Accepted: 18 September 2016

DOI: 10.1002/jcla.22083

RESEARCH ARTICLE

WILEY

Comparison of telomere length and insulin-like growth factor-binding protein 7 promoter methylation between breast cancer tissues and adjacent normal tissues in Turkish women

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Funding information
Research Foundation of Marmara University (BAPKO), Grant/Award Number: SAG-C-YLP-101011-0286; Scientific and Technological Research Council of Turkey (TUBITAK), Grant/Award Number: SBAG-111S161

Background: Both insulin-like growth factor-binding protein 7 (IGFBP7) and telomere length (TL) are associated with proliferation and senescence of human breast cancer. This study assessed the clinical significance of both TL and IGFBP7 methylation status in breast cancer tissues compared with adjacent normal tissues. We also investigated whether IGFBP7 methylation status could be affecting TL.

Methods: Telomere length was measured by quantitative PCR to compare tumors with their adjacent normal tissues. The IGFBP7 promoter methylation status was evaluated by methylation-specific PCR and its expression levels were determined by western blotting.

Results: Telomeres were shorter in tumor tissues compared to controls (P<.0001). The mean TL was higher in breast cancer with invasive ductal carcinoma (IDC; n=72; P=.014) compared with other histological type (n=29), and TL in IDC with HER2 negative (n=53; P=.017) was higher than TL in IDC with HER2 positive (n=19). However, telomeres were shortened in advanced stages and growing tumors. IGFBP7 methylation was observed in 90% of tumor tissues and 59% of controls (P=.0002). Its frequency was significantly higher in IDC compared with invasive mixed carcinoma (IMC; P=.002) and it was not correlated either with protein expression or the other clinicopathological parameters.

Conclusion: These results suggest that IGFBP7 promoter methylation and shorter TL in tumor compared with adjacent tissues may be predictive biomarkers for breast cancer. Telomere maintenance may be indicative of IDC and IDC with HER2 (−) of breast cancer. Further studies with larger number of cases are necessary to verify this association.

KEYWORDS
breast cancer, insulin-like growth factor-binding protein 7, methylation, telomere, telomere length

1 | INTRODUCTION

Breast cancer, the most common cancer in women, is highly heterogeneous in terms of its etiological and pathological characteristics.[1] Based on GLOBOCAN estimates, in 2012 approximately 1.7 million new women were diagnosed with breast cancer and 522 000 deaths have occurred worldwide. Telomere-induced chromosomal instability could drive the tumorigenic process by increasing mutation rates for oncogenes and tumor-suppressor (TS) genes.[2] Insulin-like growth factor-binding protein 7 (IGFBP7) acts as a TS gene, inhibiting the

proliferation of cells in breast and prostate cancer cell lines, as well as reducing tumor growth in breast and prostate cancers, and its proliferation control mechanisms in human cells are linked to telomere shortening.[3-5] Some studies suggest that cellular senescence is induced by the classical mechanism via the p53/p21 pathway through telomere shortening.[6] It was suggested that telomere shortening and promoter hypermethylation of related genes might serve as breast cancer biomarkers.[7] Both IGFBP7 and p21 are cellular senescence pathways.[8] Zuo et al.[9] have shown that IGFBP7-induced senescence is associated with enhanced expression of p21. Moreover, expression of IGFBP7 was shown to be down-regulated in various types of tumors associated with DNA methylation.[10,11]

Telomeres are the specialized structures at the ends of linear chromosomes that play an important role in the biology of eukaryotic cells.[12] The dynamics of telomere shortening differs from one type of cell to another and from one individual to another.[13] Mammalian telomeres contain specific hexanucleotide repeats, $(TTAGGG)_n$ and somatic human cells have telomeres of 5-15 kilobases (kb) in length.[14] These repeats shorten because of end-replication mispairing, loss of telomere-binding proteins, or oxidative damage.[15-17] Telomere shortening is an early genetic alteration and may contribute to tumor progression in breast cancer.[18] Therefore, the shortened telomeres may be a marker for susceptibility to cancer; however, this is not yet clear for all cancer types.[19,20] In the studies related to breast cancer, short telomeres were shown in peripheral blood [21,22] and telomere shortening was found to be associated with more aggressive subtypes (such as Luminal B, HER2+), triple negative tumors, and hereditary tumors.[23,24] In most human cancer cells, telomere length (TL) is maintained either by telomerase enzyme complex (the reverse transcriptase-TERT, a telomerase RNA component-TERC, and additional proteins such as dyskerin) or by a mechanism termed as "alternative lengthening of telomeres-ALT".[25] Genetic variants in genes of the telomerase complex have been also associated with breast cancer.[26] Despite the presence of normal telomerase activity, the majority of tumor cells possessing potential genetic instability have shorter telomeres than the corresponding normal tissues.[27] Chromosomal instability could increase mutation rates of TS genes and oncogenes. Conversely, a study has indicated longer telomeres in the blood of breast cancer patients compared with controls.[28]

The IGF pathway plays a key role in regulating proliferation, differentiation, and apoptosis.[29] The interactions between the IGFs and their receptors are modulated by IGF-binding proteins (IGFBPs).[30] IGFBP-related proteins (IGFBP-rPs) and IGFBPs have structural and functional similarities, but IGFBP-rPs bind with a 100 times lower affinity for IGF-I.[31] IGFBP7, which is also known as IGFBP-rP1, is one of the recently identified low affinity IGFBPs shown to be secreted, which shares an N-terminal-conserved IB domain with IGFBPs. Moreover, it can bind strongly to insulin as different from other low affinity IGFBP-rPs, suggesting that IGFBP7 has the same special biological functions with other IGFBPs.[32] IGFBP7 has diverse biological functions in many cellular processes such as proliferation, apoptosis, and senescence.[4,5,33] The expression of IGFBP7 is frequently decreased as a consequence of methylation in tumors.[34,35] Most of the studies have indicated that promoter methylation of IGFBP7 in breast cancer cell lines is associated with silencing of the gene.[36] In contrary, another study has shown that promoter methylation of this gene in colon cancer cell lines is not associated with the levels of gene expression.[37]

Thus far, the TL in breast cancer has been reported, but the comparison of TL between breast cancer tissues and adjacent normal tissues has been studied far less. In some of the previous studies, different methods were used, such as Southern blot, slot blot, and Q-FISH analysis; while some others have used different tumor materials. Also, any association between TL and clinicopathological parameters of tumors has not been fully clarified yet. Similarly, the IGFBP7 methylation status between breast cancer tissues and adjacent normal tissues in association with clinicopathological parameters of IGFBP7 methylation has not been reported. In this study, we used a quantitative polymerase chain reaction (qPCR) to determine the TL of tumors and adjacent normal tissues and to evaluate the impact of TL on clinicopathological factors. We also used methylation-specific PCR (MSP) to study the methylation status of IGFBP7 promoter in tumors and adjacent normal tissues to evaluate its impact on clinicopathological parameters and its association with ethnicity, followed with IGFBP7 expression analyses. Furthermore, we also investigated the association between the TL and the IGFBP7 methylation in breast cancer.

2 | MATERIALS AND METHODS

2.1 | Clinical samples and characterization

Tumor and adjacent normal tissue samples of the 102 women (mean age, 54), surgically resected at the Marmara University Pendik Training and Research Hospital from 2010 to 2012, were included in this study. A total of 101 tumors and the 101 adjacent tissues were evaluated for TL, among which 61 tumors and 61 adjacent tissues were evaluated by MSP, and 28 tumors and 28 adjacent tissues by western blotting. The clinicopathological and histological characteristics, such as molecular subtypes, age at diagnosis, hormone receptor status (estrogen receptor-ER, progesterone receptor-PR, human epidermal growth factor receptor 2-HER2), menopausal status, and Ki-67 expression were analyzed as shown in Table 1.

Tumor histology and tumor grade were evaluated at primary diagnosis. A significant number of the patients were diagnosed with invasive ductal carcinomas. Tumors were graded according to the Bloom-Richardson grading modified system.[38] Appropriate positive and negative controls were included with each immunohistochemical staining. Adjacent normal tissue samples served as an additional internal control. HER2, PR, ER, and Ki-67 statuses were evaluated by immunohistochemical analysis using specific monoclonal antibody. The Dako HercepTest kit (Dako, Carpentaria, CA, USA) for HER2 status was used. HER2-positive cases were considered positive when scored 3+, whereas cases with 0 to 2+ were regarded as negative for HER2 in the absence of FISH analysis.[39] The PR receptor monoclonal antibody PgR 636 (Dako, Wiesentheid, Germany) for PR status; the ER receptor monoclonal antibody clone SP1 (NeoMarkers, Fremont, CA, USA) for ER status, were used.

TABLE 1 Summary of clinicopathological parameters in patients with breast cancer

Characteristics	Group	N	%
Age (y)	<50	41	41
	≥50	60	59
	Unknown	1	
Menopausal status	Premenopausal	35	40
	Others[b]	66	59
	Uncertain	1	
Histological type	IDC	72	71
	ILC	2	2
	IMC	14	14
	Others[a]	13	13
	Unknown	1	
Molecular subtype	LumA	27	27
	LumB	31	30
	HER2	7	7
	LumHER2	25	25
	Basal-like	10	10
	Unknown	2	
Grade	Grade1	10	10
	Grade2	44	43
	Grade3	47	46
	Unknown	1	
TNM stage	I	31	30
	II	57	55
	III	7	7
	V	5	5
	Unknown	2	
Breast cancer in family	Positive	26	26
	Negative	75	74
	Unknown	1	
ER	Positive	81	81
	Negative	19	19
	Unknown	2	
PR	Positive	78	77
	Negative	23	23
	Unknown	1	
HER2	Positive (+++)	23	23
	Negative (0,1,2)	77	76
	Unknown	2	
Ki-67	Positive (≥%14)	63	62
	Negative (<%14)	38	35
	Unknown	1	

ER, Estrogen receptor; PR, Progesterone receptor; HER2, Human epidermal growth factor receptor; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; IMC, invasive mixed carcinoma.

[a]Pleomorphic lobular carcinoma, metaplastic carcinoma, malignant phyllodes tumor, atypical medullar carcinoma, invasive micropapillary carcinoma, invasive apocrine carcinoma, medullar carcinoma, invasive cribriform carcinoma.

[b]perimenopausal and postmenopausal.

2.2 | DNA isolation and measurement of telomere length by qPCR ($2^{-\Delta\Delta Ct}$)

DNA was extracted using DNA Isolation Kit for Cells and Tissues (Roche, Munich, Germany) from breast cancer tissues and adjacent normal tissues. Quantification of DNA was carried out with NanoDrop after vortexing to ensure accurate and identical concentration. Telomere length was assessed by quantitative polymerase chain reaction (PCR) as previously described.[40,41] This technique calculates the TL as a ratio of telomere amount relative to 36B4 reference gene (encodes acidic ribosomal phosphoprotein PO, internal control) amount. The target and reference are amplified in separate wells of the same 96-well plate. The measurement unit used in this technology is the T/S value. Primers used to perform the quantitative PCR have been described previously.[42] Polymerase chain reaction assays were performed in Roche LightCycler® 480 (LC480) Real Time PCR System (Roche).

The PCR (20 µL) for telomere and 36B4 amplification consisted of 1×LightCycler 480 SYBR Green I Master kit (Germany) 0.1 µmol/L primers and 10 ng of genomic DNA. For standard curve, one randomly selected normal tissue DNA sample (the same DNA sample for all runs) was diluted twofold serially to produce a 5-point standard curve between 200 ng and 0.02 ng of DNA in reaction. The coefficient of determination (R2) for standard curve was >0.99. Also, PCR program and reaction mix were optimized with melting curve. If the result was within the acceptable range, then the sample was repeated.

In gene expression experiments, the calibrator for the $2^{-\Delta\Delta Ct}$ method is the expression of the same mRNA in the untreated control.[43] Therefore, we chose TL of the adjacent normal tissue as the calibrator. The data in the $2^{-\Delta\Delta Ct}$ method are presented as the fold change. For the adjacent normal tissue, ΔΔCt equals zero and 2^0 equals one, so that the fold change in TL relative to the adjacent control equals one, by definition. For the cancer samples, evaluation of the $2^{-\Delta\Delta Ct}$ indicates the fold change in TL relative to the adjacent normal control. Telomere length was expressed as the relative T:S ratio, which was normalized to the average T:S ratio of calibrator sample ($\Delta Ct_{sample}=Ct_{telomere} - Ct_{single\ gene}$, $2^{-(\Delta Ctsample - \Delta Ctcalibrator)}=2^{-\Delta\Delta Ct}$). Samples with a T/S>1.0 have an average TL greater than that of the calibrator; DNA samples with a T/S<1.0 have an average TL smaller than that of the calibrator DNA. As each sample was assayed in duplicate, two T/S results were obtained for each sample; the final reported result for a sample in a given run is the average of the two T/S values.

2.3 | Bisulfite modification and methylation-specific PCR

Bisulfite conversion of genomic DNA was carried out using the EpiTect Bisulfite Kit (Qiagen, Valencia, CA, USA). This process converts unmethylated cytosine residues to uracil, whereas methylated cytosine residues remain unchanged. Methylation-specific PCR (MSP) was then carried out to determine the methylation status of IGFBP7. Bisulfite modified DNA was used as a template for PCRs with primers specific for methylated or unmethylated alleles. CpGenome Universal

Methylated DNA and normal human unmethylated DNA (EpiTect PCR control DNA, Qiagen, USA) were used as positive and negative controls, respectively. Primers and PCR conditions used for MSP of IGFBP7 are from published study and have been standardized in our laboratory.[44] After amplification, the amplified 173-bp products for both methylated and unmethylated signals were visualized by UV-illumination on 2% agarose gel containing ethidium bromide (Sigma, St Louis, MO, USA).

2.4 | Protein isolation and western blotting analysis

Proteins were extracted from 10 to 20 mg tissue in 200-400 mL tissue protein extraction reagent (T-PER; Pierce, Rockford, IL, USA) containing protease inhibitors (Thermo Scientific Halt Protease Inhibitor Cocktail; Pierce, USA) on ice. Tissues were disintegrated with a scalpel on glass slides and fragmented tissues were transferred to tubes. The specimens were then homogenized and centrifuged at 10 000 g for 6 min at 14°C. The supernatants were stored at −20°C to be used for protein quantification and western analysis. The protein concentration was measured by a Bradford colorimetric assay (Bio-Rad, Hercules, CA, USA).

Equal amounts (20 µg) of proteins were subjected to sodium dodecyl sulfate 10%, polyacrylamide gel electrophoresis, and fractionated proteins were transferred to nitrocellulose transfer membranes (Thermo, 88014, Rockford, IL, USA). These membranes were blocked in TBS-T containing 5% nonfat milk, 0.05% Tween 20, and then incubated with the primary antibody overnight at 4°C. After washing with TBS-T, blots were probed with a secondary IgG HRP-linked antibody (anti-mouse IgG, Santa Cruz Biotechnology, Dallas, TX, USA) for 1 hour at RT. The antibodies to IGFBP7 (sc-133689) and beta-actin (sc-47778) were purchased from Santa Cruz Biotechnology. The blots were washed and then developed using an enhanced chemiluminescence detection system (Super Signal West Pico Chemiluminescent Substrate, Pierce) and Kodak X-ray film for autoradiography.

2.5 | Statistical analysis

Data analysis was carried out using GraphPad Prism-6 (La Jolla, CA, USA). The Chi-square test and Fisher's exact test were used to investigate the difference in the TL and the methylation status of the groups. The Mann-Whitney *U* test was used to compare the telomere length and IGFBP7 methylation status and clinicopathological parameters in the cancer tissues and adjacent normal tissues. The mean $2^{-\Delta\Delta Ct}$ values between tumors and adjacent normal tissues were compared by both the paired and unpaired *t* test. $P<.05$ was considered to indicate a statistically significant result and all tests were two-tailed. Odds ratios with 95% confidence intervals were given wherever appropriate.

3 | RESULTS

3.1 | Clinicopathological classification of breast cancer samples

We analyzed 102 specimens of breast cancer tissues and adjacent specimens of normal breast tissues. According to pathological tumor type and immunohistochemical staining, we separated our patient's samples into subgroups: invasive ductal carcinoma (IDC), invasive lobular carcinoma, invasive mixed and other invasive groups. Most of the patients were diagnosed as IDC (70%) and other clinicopathological factors such as staging, histological grading, hormone receptor status, and HER2 expression are listed in Table 1. The mean age and mean tumor diameter were 54 and 26 mm, respectively. Eighty-one cases (81%) were positive for ER; 78 (77%) were positive for PR; 63 (62%) were positive for Ki-67, and 77 (76%) were negative for HER2.

3.2 | The shorter TL in breast cancer tissues compared with adjacent normal tissues

In this study, telomere length was determined from the breast cancer tissues and adjacent normal tissues of 100 of 102 breast cancer women. Measurement of relative TL (RTL) was achieved by comparing products amplified from telomere-specific primers and single copy reference gene primers in a ratio (T/S).

Our data revealed that the median RTL was 0.721 among cancer tissues and 1.141 among normal tissues (calibrator: median ΔCt of normal tissues, Table 2, $P<.0001$ when used Mann-Whitney *U* test; $P=.0059$ when Wilcoxon matched-pairs signed-rank test). The TL in cancer tissues was significantly shorter than that in adjacent normal tissues. Similar trend was also observed in women over ($P<.0001$) and under ($P=.003$) the age of 50 when Mann-Whitney *U* test (unpaired)

TABLE 2 Telomere length (TL) between tumors and adjacent normal tissues

Characteristics (n=100)	Tumors TL (median, $2^{-\Delta\Delta Ct}$)	Healthy tissues TL (median, $2^{-\Delta\Delta Ct}$)	*P*	95% CI
Total samples	.721	1.141	**<.0001***	0.98-1.65
			.0059**	
<50 age (n=41)	.695	1.153	**.003***	0.81-1.73
			.144**	
≥50 age (n=59)	.723	1.141	**<.0001***	0.87-1.83
			.0154**	

Calibrator: median ΔCt of normal tissues. Statistically significant values are marked in bold.
*GraphPad-Mann-Whitney *U* test (unpaired *t* test, two-tailed, $P < 0.005$).
**Wilcoxon matched-pairs signed-rank test (paired *t* test, two-tailed, $P < 0.05$).

was used. However, TL in cancer tissues was significantly shorter than adjacent normal tissues in women only over the age of 50 (P=.015) using Wilcoxon matched-pairs signed-rank test (paired).

In this study, for each cancer sample, its own adjacent normal sample was chosen as the calibrator. When TL in tumor was investigated in comparison to adjacent normal samples, telomeres were shorter in 59 (58%) tumors compared to adjacent normal samples, whereas telomeres of 41 tumors (40%) were longer than their adjacent normal samples.

3.3 | Association between TL and clinicopathological parameters in breast cancer

We examined the relationship of TL with factors influencing telomere length such as age, tumor type, tumor size, lymph node involvement, extent of metastasis, stage, histological grading, receptor status, and pathological biomarkers (Table 3). Seventy-two tumors of total breast carcinoma samples were IDC type and 29 tumors of these samples were

TABLE 3 Association between clinicopathological characteristics and TL

			Telomere length ($2^{-\Delta\Delta Ct}$)		
Characteristics	**Group**	**N (%)**	**Short**	**Long**	***P***
Age (y)	<50	41 (41)	24	17	.843
	≥50	60 (59)	34	24	
	Unknown	1	2		
Menopausal status	Premenopausal	35 (34)	18	17	.392
	Others	66 (66)	35	23	
	Unknown	1	2		
Histological type	IDC	72 (71)	35	35	**.014**
	Others	30 (29)	23	6	
	Unknown		3		
Molecular subtype	Basal-like	10 (10)	7	3	.521
	Others	90 (90)	51	37	
	Unknown	2	2		
Grade	Grade-3	47 (47)	30	16	.297
	Others	54 (53)	28	25	
	Unknown	1	1		
TNM stage	IV	6 (6)	5	1	.396
	II	95 (94)	53	40	
	Unknown	1	2		
Breast cancer in family	Positive	26 (26)	16	10	.901
	Negative	75 (74)	42	31	
	Unknown	1	2		
ER	Positive	81 (79)	47	33	.849
	Negative	19 (21)	11	8	
	Unknown	2	1		
PR	Positive	78 (77)	45	31	.99
	Negative	23 (23)	13	10	
	Unknown	1	2		
HER2	Positive (+++)	23 (23)	16	6	.2
	Negative	77 (77)	42	35	
	Unknown	2	1		
Ki-67	Positive (≥%14)	63 (62)	38	24	.62
	Negative (<%14)	38 (38)	20	17	
	Unknown	1	2		

ER, Estrogen receptor; PR, Progesterone receptor; HER2, Human epidermal growth factor receptor; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; IMC, invasive mixed carcinoma.
Short: Telomere is shorter in tumor than its own adjacent normal tissue, Long: Telomere is longer in tumor than its own adjacent normal tissue. Each Fisher's exact test (column value <5), chi-square test for Yate's correction. TL in cancer tissue compared to adjacent normal tissue. Statistically significant values are marked in bold.

the other histological type. The mean level of the $2^{-\Delta\Delta Ct}$ values in IDC was 1.417 and it was significantly higher than the mean level of the $2^{-\Delta\Delta Ct}$ values in the other types (0.764, *P*=.014, calibrator: ΔCt of own adjacent normal tissue, Figure 1A). The other invasive tumors compared with IDC type were significantly more likely to have a greater fraction of shorter telomere when compared with adjacent normal tissues (*P*=.0135, Figure 1B). The IDC tumors have the same fraction of both shorter and longer TL. In addition, the mean level of the $2^{-\Delta\Delta Ct}$ values in IDC with HER2 (−) was 1.595 and it was significantly higher than mean level of IDC with HER2 (+) (0.943, *P*=.017, Figure 1C). Longer telomere length was more prevalent in IDC tumors with HER2 negative, and shorter telomere length was also more prevalent in tumors with HER2 positive, but it did not reach statistical significance (*P*=.056, Figure 1D).

We also found that telomeres were longer in cancer tissues than their own adjacent samples for tumor stage I and II (*P*>.05, 1.306), while they were longer in their own adjacent samples for tumor stage III and IV (*P*>.05, .677), but none of them reached statistical significance. As shown in Table 3, shorter telomere length was more prevalent in tumors with ER positive, PR positive, Ki-67 positive, HER2 positive, grade-III, and ≥50 age. TL variation between breast cancer samples and adjacent normal samples was not associated with the other clinicopathological parameters.

3.4 | IGFBP7 methylation status in breast cancer and adjacent normal tissues

The MSP assay was performed for IGFBP7 in tumor and adjacent normal tissues of 61 patients. We predicted the promoter using ExPASy program. The predicted result of the IGFBP7 promoter sequence was from −499 to +100 in the positions relative to the transcription start site (TSS) +1. PCR products of IGFBP7 promoter was from −312 to −139 (173 bp) in the positions relative to the TSS (+1). Representative band profiles of the MSP of the IGFBP7 are illustrated in Figure 2. Methylated (M) and unmethylated (U) signals of the IGFBP7 were detected in tumor and adjacent tissues (Figure 2). Biallelic methylation (M/M) signals and monoallelic methylation (M/U) signals were evaluated as methylated result (M); only biallelic unmethylation (U/U) signals were evaluated as unmethylated result (U).

In the cancerous tissues of patients, methylation in the IGFBP7 gene was detected in 90.2% (55/61) of the samples. The methylation rate of the IGFBP7 gene in adjacent normal tissues of patients was 59% (36/61) of the samples. The difference in IGFBP7 methylation frequency between tumors and adjacent normal tissues was statistically significant (*P*=.0002, Figure 3). When we studied the individual methylation in cancer relative to the adjacent normal tissue, IGFBP7

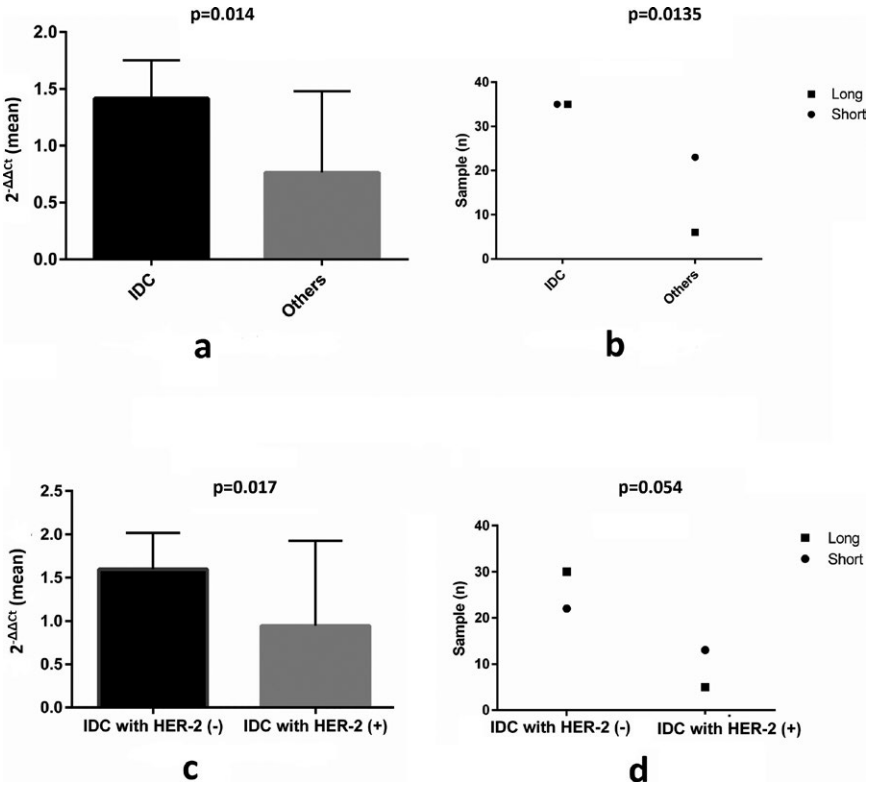

FIGURE 1 Association between clinicopathological characteristics and TL in IDC compared with adjacent normal tissues. IDC, invasive ductal carcinoma; Others, invasive ductal carcinoma, invasive lobular carcinoma, invasive mixed carcinoma, pleomorphic lobular carcinoma, metaplastic carcinoma, malignant phyllodes tumor, atypical medullary carcinoma, invasive micropapillary carcinoma, invasive apocrine carcinoma, medullar carcinoma, invasive cribriform carcinoma. For each cancer sample, its own adjacent normal sample was chosen as the calibrator. (A) The relative telomere length in histological tumor types (IDC and others). *P* value (unpaired *t* test) represents level of significance. (B) Distrubition of tumors with short and long telomeres in histological tumor types (IDC and others). *P* value (Chi-square test) represents level of significance. (C) The relative telomere length in IDC with HER2 (−) and IDC with HER2 (+). *P* value (unpaired *t* test) represents level of significance. (D) Distrubition of tumors with short and long telomeres in IDC with HER2 (−) and IDC with HER2 (+). *P* value (Fisher's exact test) represents level of significance

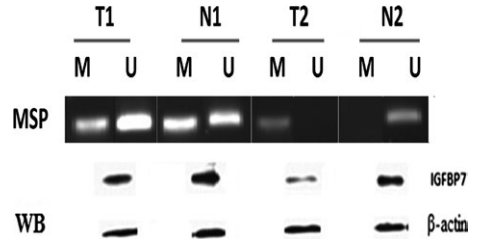

FIGURE 2 MSP and western blot results in the breast cancer tissues and adjacent normal tissues. MSP, Methylation-specific PCR; WB, western blotting; M, methylated primer-specific PCR; U, unmethylated primer-specific PCR; T, breast cancer tissue; N, adjacent normal tissue; M/M, biallelic methylation; M/U, monoallelic methylation; U/U, biallelic unmethylation;T1, N1, and T2 samples were evaluated as methylated result, only N2 sample was evaluated as unmethylated result

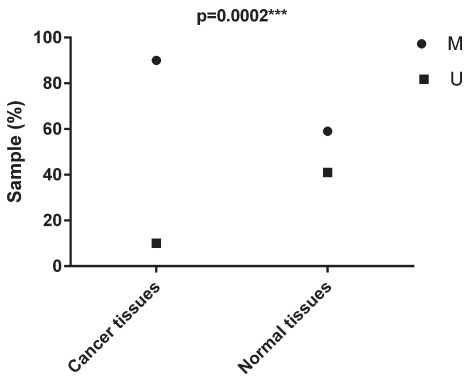

FIGURE 3 Prevalence of IGFBP7 promoter methylation (M) and unmethylation (U) in tumors and adjacent normal tissues of patients. M, Methylated; U, Unmethylated. *P* value (Yate's Chi-square test) represents level of significance

promoter methylation status was identical for both tumor and normal tissues in 59% (n=36/61) of patients and methylation was confirmed to tumor only in 36.1% (n=22/61) of patients (Table 4). We also found that 23 patients had a higher degree of methylation in tumor than the adjacent normal tissue and three patients (4.9%) had methylation of the IGFBP7 gene in only adjacent normal tissue (Table 4). On the other hand, when we checked IGFBP7 protein expression levels by western blotting, we did not detect complete matching between methylation status and protein expression levels of the gene, but there was a tendency for positive correlation.

3.5 | Correlation between clinicopathological characteristics of patients and IGFBP7 promoter methylation in tumors compared with adjacent normal tissues

The rate of IGFBP7 promoter methylation in IDC compared with IMC was significantly higher (*P*=.002, Figure 4). We could not find any significant IGFBP7 methylation changes between the samples and their other clinicopathological parameters (*P*>.05, data not shown).

TABLE 4 Distribution of methylation and unmethylation of IGFBP7 genes

	Adjacent normal tissues		
Patients (n=61)	M/M	M/U	U/U
Cancer tissues			
M/M (n=4)	0	1	3
M/U (n=51)	2	30	19
U/U (n=6)	0	3	3

M, methylation; U, unmethylation; M/M, biallelic methylation; M/U, monoallelic methylation; U/U, biallelic unmethylation.

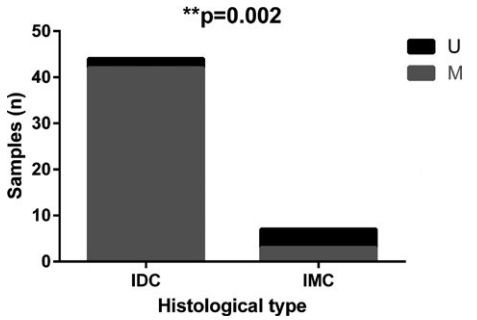

FIGURE 4 Distribution of IGFBP7 promoter methylation in IDC and IMC. M, methylated; U, Unmethylated. IDC, invasive ductal carcinoma; IMC, invasive mixed carcinoma. *P* value (Chi-square test) represents level of significance

Insulin-like growth factor-binding protein 7 methylation status was classified into two distinct groups according to the presence of methylation in only tumor tissue (Group-I) or the presence of the same methylation pattern in both tumor and adjacent normal tissues (Group-II). There was no statistically significant association between the two groups and the clinicopathological parameters (Table 5).

3.6 | Association between TL and IGFBP7 methylation in breast cancer

The promoter methylation status of IGFBP7 was compared with TL in breast cancer and adjacent normal tissues. We found that TL was not significantly different in IGFBP7 methylated and unmethylated samples (Table 6). Similarly, TL was not significantly different among the IGFBP7 high-expression and IGFBP7 low-expression groups (Table 6). The $2^{-\Delta\Delta Ct}$ median level values of Group-I (methylation presence in only tumor) were higher than group-II (the same methylation pattern in both tumor and adjacent normal tissue), but it did not reach statistical significance (Group-I TL, 0.895; Group-II TL, 0.5873; *P*=.236).

3.7 | Survival analysis

An overall survival analysis was performed on 102 patients with long-term follow-up information (4-year overall breast cancer survival), but no statistically significant association was found between survival and investigated parameters. Because of the last follow-up visit, 100

TABLE 5 Association between clinicopathological characteristics and IGFBP7 promoter methylation

Characteristics	N (%)	Group-I % (n=23)	Group-II % (n=35)	*P*
Age (y)				
<50	23 (100)	8 (35)	15 (65)	.496*
≥50	34 (100)	15 (44)	19 (56)	
Uncertain	1	—	1	
Histological Type				
IDC	43 (100)	20 (47)	23 (53)	.07
Others	15 (100)	3 (20)	12 (80)	
Molecular type				
LumB	20 (100)	9 (45)	11 (55)	.497*
Others	39 (100)	14 (36)	25 (64)	
Grade				
G1	6 (100)	2 (33)	4 (67)	.173
G2	26 (100)	13 (50)	13 (50)	
G3	25 (100)	8 (32)	17 (68)	
Uncertain	1(100)	—	1	
ER				
Positive	43 (100)	19 (44)	24 (56)	.301
Negative	14 (100)	4 (29)	10 (71)	
Uncertain	1 (100)	—	1	
PR				
Positive	42 (100)	18 (43)	24 (57)	.518*
Negative	15 (100)	5 (33)	10 (67)	
Uncertain	1	—	1	
HER2				
Positive	14 (100)	7 (50)	7 (50)	.396*
Negative	43 (100)	16 (37)	27 (63)	
Uncertain	1	—	1	
Ki-67				
Positive	38 (100)	17 (45)	21 (55)	.417*
Negative	18 (100)	6 (33)	12 (67)	
Uncertain	2	—	2	

Group-I: IGFBP7 promoter methylated in tumor, but it unmethylated in adjacent normal tissue. Group-II: IGFBP7 promoter methylation status was the same both tumor and adjacent normal tissue.
*Chi-square test and Fisher's exact.

TABLE 6 Association between IGBP7 status and TL

IGFBP7 status (in tumors)	Telomere length		*P* value
	Short	Equal/long	
Methylated	34	19	1
Unmethylated	4	2	
High expression	15	9	.529
Low expression	3	0	

Short: Telomere is shorter in tumor than its own adjacent normal tissue, Long: Telomere is longer in tumor than its own adjacent normal tissue.

(98.04%) patients were alive. Two (1.96%) patients died; one from cirrhosis, and the other (0.98%) patient with IGFBP7 promoter methylation and shorter TL compared with adjacent normal tissue died from breast cancer. This patient was diagnosed at age 73 as IDC, ER positive, PR positive, Ki-67 negative, HER2 negative, and histopathological grade-II.

4 | DISCUSSION

In this study, telomere length and promoter methylation status of the IGFBP7 gene were examined in tumor and adjacent normal tissues from 102 patients with breast cancer. Although TL has been studied in breast cancer tissues,[7,23,45,46] TL variation between cancer tissues and its own adjacent normal tissues has not been thoroughly clarified. Our results show that the median RTLs were significantly shorter in tumor tissues compared to adjacent normal tissues (calibrator: median ΔCt of normal tissues, $P<.0001$). Telomeres were shorter in 59 (58%) tumors and were longer in 41 tumors (40%) compared to its own adjacent normal samples. TL variation between with breast cancer samples and adjacent normal samples was not associated with the other clinicopathological parameters except IDC type and IDC with HER2 negative. The IGFBP7 methylation frequency was significantly higher in tumors than the adjacent normal tissues ($P=.0002$). Moreover, methylation of IGFBP7 promoter was not significantly correlated with TL in the cancer tissues ($P>.05$).

Few studies have examined associations between tumor TL and adjacent normal tissue in the breast cancer and the results have been conflicting. One of the studies found no association between RTL and adjacent normal epithelial cells.[45] Another study found that breast carcinomas had shorter telomeres than normal breast tissue, and high grade invasive carcinomas had shorter telomeres than low grade (grade I) invasive carcinomas. However, Q-FISH assay and Southern blotting were used in these studies, respectively.[46] In another study, breast cancer tissues were also reported to have shorter telomeres compared to paired adjacent normal tissues, but the used tissues were noninvasive cancer tissues.[7] We analyzed TL with qPCR method in invasive cancer tissues and found that median RTLs in breast cancer were significantly shorter than those of the adjacent normal tissues, which is consistent with the aforementioned previous reports. Radpour et al.[7] have shown that shorter TLs were correlated with higher histological grading, but were not associated with other clinicopathological parameters such as under age of 50 and tumor size. We did not observe any significant association between TL and histological grading. Different than the previous study, our results showed that it was significantly associated in women both over and under the age of 50. However, we also noted that TL in cancer tissues was significantly shorter than that in adjacent normal tissues in women only over the age of 50 ($P=.015$).

Most of the previous studies investigating breast cancer risk and TL in peripheral blood have also found conflicting results, with two studies showing a significant association between longer telomeres and breast cancer risk,[28,47] but other studies demonstrated either no association, or an association only in subgroups.[48,49] Our results

showed that other invasive tumors, when compared with IDC type, had a significantly greater fraction of shorter telomeres when compared with adjacent normal tissues (P=.014, Figure 1A). The IDC tumors had the same fraction of both shorter and longer TL (Figure 1B) and short TL was more frequent in other invasive type tumors than in IDC ones (Figure 1B, Table 2). We also found that longer TL was correlated with HER2-negative patients with IDC tumors, while the shorter TL was correlated with HER2-positive patients with IDC tumors (P=.017, Figure 1C). The association between the longer TL and HER2 negative is similar to that which was reported by one study,[50] although the tissue type and population were different; investigators have found that longer blood TL was associated with worse prognosis for HER2/neu-negative cases. The association between the shorter TL and HER2-positive status in our study is in accordance with those of Heaphy et al.[23] who studied telomere length in breast cancer tissues.

Cancer cells had longer telomeres than its own adjacent normal tissue cells in 41 cases (%40). This can be explained by a multitude of telomere elongating and shortening factors.[28] Telomerase and estrogen can also influence telomere dynamics.[28,47] Other genetic and nongenetic factors may also play important roles in this pathway. A study has reported that postmenopausal women with hormone replacement therapy had longer telomere than postmenopausal women without hormone replacement therapy.[51] In this report, we did not find any significant associations between menopausal status and TL. Because data on hormonal replacement was not available in our study population, we did not evaluate the association between estrogen exposure and TL.

Although IGFBP7 has shown tumor-suppressor functions in a number of cancers, little is known about its methylation status in breast cancer. A study showed that promoter of IGFBP7 gene is aberrantly methylated in breast cancer cell lines with high IGFBP7 expression and promoter methylation of this gene was detected in 35% of primary breast carcinoma series; but investigators have not used adjacent normal tissues as the control group.[36] In our study, we demonstrated the promoter methylation of IGFBP7 in 90% of tumor tissues and in 59% of adjacent normal tissues. We also evaluated the promoter methylation of this gene in breast cancer tissues compared with adjacent normal tissues, and IGFBP7 promoter methylation was detected in the tumor tissues of only 37.7% of the patients (unmethylated for adjacent tissues). Previous studies have indicated that the IGFBP7 gene is inactivated by DNA methylation in human breast, colon, and lung cancer.[36,37,52] We did not find any significant difference between methylation and expression. It is possible that the result is due to the small sample size or that promoter methylation is not the only mechanism of silencing of IGFBP7 gene.

Telomere shortening in cells leads to senescence, which is regulated in part by effectors in the p16/Rb pathway, p14-p53-p21 pathway, IGFBP7 pathway, and FBXO31 pathway.[8] In our study, IGFBP7 methylation was not associated with TL in breast cancer tissues. One possible reason is that tumors in breast tissues with IGFBP7 methylation may develop from telomere-independent mechanisms. However, there are some limitations in our study: measurement of IGFBP7 methylation and expression was performed with DNA from small sample size, which may not reflect association between methylation and TL. Thus, future larger studies are needed to confirm our findings.

In summary, telomere length and promoter methylation status of the IGFBP7 gene were examined in breast cancer tissues and adjacent normal tissues. Although several studies have found that there is a TL variation in breast cancers, the relationship between TL and tumor progression is still controversial. Using quantitative real-time PCR, we measured the TL and showed that tumors have shorter telomeres with respect to adjacent normal tissues. Additionally, tumors with IDC type had significantly longer TLs as compared to other histological types. Moreover, short TL was more frequent in other invasive type tumors than IDC ones. This is the first study that compares IGFBP7 methylation status in breast tumors and adjacent normal tissues. Promoter methylation of IGFBP7 showed significant association with breast cancer, especially with IDC. In conclusion, IGFBP7 promoter methylation or the shorter TL in tumors compared to adjacent normal tissues may be a predictive biomarker for breast cancer, especially for IDC. Telomere maintenance may be also indicative of IDC with HER2 (−). Further studies with larger number of cases are necessary to verify this association.

ACKNOWLEDGMENTS

The authors thank Dr. Can Erzik for his critical revision of the manuscript. This work was partly supported by a grant (SAG-C-YLP-101011-0286 to AO) from the Research Foundation of Marmara University (BAPKO) and a grant (SBAG-111S161 to MA) from the Scientific and Technological Research Council of Turkey (TUBITAK).

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