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PRECLINICAL STUDY

Short telomeres are frequent in hereditary breast tumors and are associated with high tumor grade

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Abstract Telomere shortening is a common event involved in malignant transformation. Critically short telomeres may trigger chromosomal aberrations and produce genomic instability leading to cancer development. Therefore, telomere shortening is a frequent molecular alteration in early stages of many epithelial tumors and in breast cancer correlates with stage and prognosis. A better understanding of the involvement of short telomeres in tumors may have a significant impact on patient management and the design of more specific treatments. To understand the role of telomere length (TL) in breast cancer etiology we measured the length of individual telomere signals in single cells by using quantitative telomere in situ hybridization in paraffin-embedded tissue from hereditary and sporadic breast cancers. A total of 104 tumor tissue samples from 75 familial breast tumors (BRCA1, $n = 14$; BRCA2, $n = 13$; non-BRCA1/2, $n = 48$) and 29 sporadic tumors were analyzed. Assessment of telomere signal intensity allowed estimation of the mean TL and related variables, such as percentage of critically short telomeres and percentage of cells with short telomeres. These data were correlated with the immunohistochemical expression of molecular breast cancer markers. Hereditary BRCA1, BRCA2, and non-BRCA1/2 tumors were characterized by shorter TL comparing to sporadic tumors. Considering all tumors, tumor grade was a strong risk factor determining the proportion of short telomeres or short telomere cells. Moreover, some histopathological features appeared to be differentially associated to hereditary or sporadic subgroups. Short telomeres correlated with ER-negative tumors in sporadic cases but not in familial cases, whereas a high level of apoptosis was associated with shorter telomeres in hereditary BRCA1 and BRCA2 tumors. In addition, TL helped to define a subset of non-BRCA1/2 tumors with short telomeres associated with increased

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expression of antiapoptotic proteins. These findings highlight the potential interest of TL measurements as markers of aggressiveness in breast cancer.

Keywords Telomeres · Q-FISH · Hereditary breast cancer · BRCA1 · BRCA2 · IHC markers

Introduction

Telomere shortening may underlay chromosome instability in cancer cells. Multiple lines of evidence have led to the hypothesis that telomere dysfunction contributes to inducing genetic changes required for the development and progression of tumors [1–3]. It has been described that telomere shortening commonly occurs at initial steps of cancer transformation [4–9], and in addition may contribute to tumor progression in many cancer types including breast cancer [7, 10].

Based on specific gene expression signatures, breast cancer has been classified into different molecular subtypes with different biological features, clinical outcomes, and responses to therapy [11–14]. These subtypes can also be distinguished by several immunohistochemical markers, such as: Luminal A (ER+ and/or PR+, HER2−), Luminal B (ER+ and/or PR+, HER2+), Basal-like (ER−, PR−, HER2−, CK5/6+ and/or EGFR+), and HER2 (ER− and PR−, HER2+) [15–19], making it easier to further characterize these subgroups. Other studies reflected the importance of using proliferation index Ki-67 to differentiate between Luminal A and B subtypes [20, 21]. These molecular differences have been shown to correlate well with clinical features, such as survival, prognosis, and sensitivity to treatment.

Recently, telomere shortening has been reported to be associated with specific breast tumor subtypes, with more aggressive subtypes such as Luminal B, HER2+, and triple-negative tumors having shorter telomeres [22].

On the other hand, hereditary breast cancer represents around 5 % of all breast cancer cases. To date, two major high-risk (>10 fold) susceptibility genes, *BRCA1* and *BRCA2*, have been described but they only explain up to 20 % of families with breast cancer. Other moderate and low susceptibility genes have been described, but around 70 % of families still remain genetically unexplained [23–25]. These breast cancer families, referred to as BRCAX families, comprise a molecular and histopathologically heterogeneous group. A previous study by our group analyzing telomere length (TL) in peripheral blood lymphocytes of hereditary breast cancer patients showed that hereditary breast cancer was characterized by significantly shorter telomeres compared to sporadic breast cancer cases and the control population [26]. Not only carriers of *BRCA1* and *BRCA2* mutations, but also a subset of BRCAX cases presented very short telomeres. In addition to their DNA repair function, *BRCA1* and *BRCA2* are involved in telomere maintenance [27–29], and genetic defects of these genes would not only induce genetic instability but would also contribute to modifying TL.

In this study we evaluated TL at single-cell resolution by a quantitative telomere in situ hybridization (Q-FISH) assay in breast cancer tissues of both hereditary and sporadic origin, and correlated it with the expression of immunohistochemical markers. We found that hereditary breast tumor samples showed shorter telomeres compared to sporadic breast cancer, and that tumors with shorter telomeres had specific histological and immunohistochemical features as determined by the expression of established molecular markers.

Materials and methods

Tumor samples

A total of 104 breast tumor samples were analyzed to investigate TL in tumor tissue and its relation with histopathological and immunohistochemical parameters. Tumor samples included 75 familial breast tumors; 14 of them carried a *BRCA1* mutation, 13 a *BRCA2* mutation, and 48 were non-BRCA1/2 tumors. These breast cancer samples were collected in three centers in Spain: Centro Nacional Investigaciones Oncológicas, the Fundación Jimenez Díaz in Madrid, and the Hospital Sant Pau in Barcelona, and selected from high-risk families with at least three women affected with breast and/or ovarian cancer and at least one of them diagnosed before 50 years of age, or from families with female breast and/or ovarian cancer and at least one case of male breast cancer. All cases were studied for mutations in the *BRCA* genes using standard procedures [30, 31]. In addition, 29 sporadic breast cancer tumors were analyzed. Most cases were diagnosed as invasive ductal carcinomas, and among hereditary tumors seven cases corresponded to invasive lobular carcinoma.

Mean diagnosis ages from the hereditary breast cancer cases were: for BRCA1 37 years (range 28–54), for BRCA2 41 years (range 32–62), and for non-BRCA1/2 patients 48 (range 34–77). In addition the included 29 patients with sporadic breast cancer had a mean age of 58 (range 41–74).

Measurement of telomere length by Q-FISH

For Q-FISH analysis of breast cancer tissues, deparaffinized sections were hybridized with a PNA-tel Cy3-labeled

probe, and TL was determined as described [32–36]. DAPI and Cy3 signals were acquired simultaneously in separate channels using a confocal Leica TCS-SP5 microscope and maximum projections from image stacks were generated for image quantification. In all cases, background noise was subtracted from the image prior to quantification.

Quantitative image analysis of telomere fluorescence intensity was performed on confocal images using Definiens Developer Cell software (Definiens Developer XD). The DAPI image was used to define the nuclear areas that were separated using Definiens' Cellenger software. After defining the nuclear areas, a predefined ruleset was used for the quantification of telomere fluorescence intensity (Cy3 image). Cy3 fluorescence intensity was measured as "average gray value" (total gray value per nucleus) units (arbitrary units of fluorescence). These "average telomere fluorescence" values represent the average Cy3 pixel intensity for the total nuclear area, thus ruling out the possibility that differences in nuclear size affect TL measurements. Percentage of cells with short telomeres as well as percentage of short telomeres were calculated (in the latter case, TL values were analyzed assessing >5,000 individual telomere spots per sample). We define short telomeres as those with intensity values below the first quartile. The fluorescence values for each tumor section were exported to GraphPad Prism, and graphs were generated (only tumor samples with more than 100 analyzed nuclei were included in the analysis). Student's *t* test was used for statistical analysis.

To control for differences in probe accessibility, we used an Alexa488-labeled PNA probe directed against human alpha satellite type-I DNA. No significant differences in centromeric fluorescence were detected between different samples and arrays, ruling out the possibility that differences in probe accessibility were responsible for the observed differences in TL.

Immunohistochemical studies

The morphological subtype and grade of all tumors was assessed in complete sections stained with hematoxylin–eosin (HE). The Nottingham histological grading system was used to assess the grade of invasive ductal carcinomas.

Tumor samples were included in three different tissue microarrays (TMAs) by selecting two different 1 mm tissue cores representative of each tumor according to previous studies done by our group [37, 38]. For all markers analyzed at least two pathologists were evaluating the immunohistochemical staining.

Eleven antibodies were used for immunohistochemistry (IHC) to evaluate them in relation with the TL parameters. Briefly, IHC assays were performed by the Envision method (Dako, Glostrup, Denmark) with a heat-induced antigen retrieval step. Sections from the tissue array were immersed in 10 mM boiling sodium citrate at pH 6.5 for 2 min in a pressure cooker. Dilutions and suppliers of the 11 antibodies used in this study are listed in Table 1.

Between 150 and 200 cells per core were scored to determine the percentage of cells with positive nuclei or cytoplasm, depending upon the marker. We evaluated nuclear staining for estrogen receptor (ER), progesterone receptor (PR), p53, Ki-67, and survivin; evaluation of cytoplasmic staining was carried out for BCL2, vimentin, and CK5/6 as described previously [38]. Unequivocal membrane was considered as representing positivity for EGFR. HER-2 expression was evaluated according to the four-category (0 to 3+) Dako system proposed for the evaluation of the HercepTest, and HER-2 expression of 3+ was the only value considered positive [38]. Apoptotic cells were estimated by immunohistochemistry (IHC) with an antibody against activated (cleaved) caspase-3 [39]. Active caspase-3 staining was divided into three categories (1: <5 % positive cells; 2: 5–20 % positive cells; and 3: >20 % positive cells).

Expression of the histological and selected immunohistochemical markers in hereditary and sporadic breast tumors is summarized in Table 2.

Statistical analysis

For telomere length examination, different parameters were defined for each tumor sample, including mean TL, percentage of short telomere cells (STC), and percentage of short telomeres (ST). To measure the relationship between each of the telomere variables and some of the observed characteristics of the tumors (age of diagnosis, grade, ER, PR, Her2, Ki67, and hereditary or sporadic tumor types) binary logistic regression was used. TL was divided into two categories (short vs. long telomeres) using the median value from all tumor samples analyzed as the cutoff. Similarly two categories were established by using the median for the other telomere measurements (STC and ST) into groups of low percentage vs high percentage of short telomeres or STC. Multivariable logistic regression analysis was performed with variables being significant after univariate logistic regression.

Additionally telomere variables were analyzed as continuous variables. Distribution of telomere length variables (TL, ST, and STC) in hereditary and sporadic tumors taken into account the status of different histopathological variables such as tumor grade, hormone receptors, HER2 expression, and proliferation and apoptosis markers, were estimated by either paired *t* tests or ANOVA as appropriated. Correlation between TL variables and age at breast

Table 1 List of antibodies used in immunohistochemical analysis

Antibody	Clone	Dilution	Supplier	Threshold (%)
ER	1D5	1:30	Novocastra	10
PR	1A6	1:30	Novocastra	10
HER-2	Herceptest	Prediluted	DAKO	3+
Ki-67	MIB1	1:30	DAKO	<5; 5–25; >25
P53	DO-7	1:50	Novocastra	25
EGFR	EGFR.113	1:10	Novocastra	*
CK 5/6	D5/16 B4	1:25	DAKO	*
Vimentin	V9D	1:500	DAKO	*
BCL2	124	1:80	DAKO	70
Survivin	Poly rabbit	1:1,000	RD systems	*
Cleaved caspase-3	Poly rabbit	1:20	Cell signaling	<5; 5–20; >20

* At least one cell positive

cancer diagnosis for hereditary and sporadic tumors was estimated by Pearson's coefficient.

Only p values below 0.05 were considered as statistically significant.

Quartiles of the percentage of STC were defined by taking into account all tumor samples, and named as (Q1: very low, Q2: low, Q3: high, Q4: very high number of cells with short telomeres). Differences in Quartile distribution of the percentage (STC) among the different tumor groups (BRCA1, BRCA2, BRCAX, Sporadic) were estimated by Pearson's Chi squared test.

The statistical program SPSS version 19 (SPSS Inc., Chicago, IL, USA) was used for these analyses.

Results

Telomere length distribution in hereditary and sporadic breast tumors

Significant differences in the mean TL between hereditary tumors and sporadic breast tumors were found, with shorter telomeres in hereditary breast cancer, either comparing each of the subgroups (Fig. 1a) or taking all hereditary breast cancer samples together (Fig. 1b). The lowest telomere intensity was found in the BRCAX group (Fig. 1a). Similarly, the percentage of STC was significantly higher in hereditary tumors than in sporadic tumors (hereditary mean STC = 13.7 % vs. sporadic mean STC = 5.9 %, $p = 0.006$) (Fig. 2a). In addition, analysis of the mean percentage of ST in tissue samples gave similar results with hereditary tumors showing a significantly higher percentage of ST than sporadic tumors ($p < 0.001$, data not shown).

The number of cells with critically short telomeres were further analyzed by dividing them into quartiles (Q1: very low, Q2: low, Q3: high, Q4: very high number of cells with short telomeres) (Fig. 2b). Quartile distribution of the percentage of STC showed significant differences when comparing all hereditary breast tumors versus sporadic tumors ($p = 0.0004$). The proportion of sporadic breast tumors with STC in Q3 and Q4 was around 30 %, while in BRCA1 tumors it was close to 80 %, and in BRCA2 tumors and in BRCAX tumors it was around 50 %. In BRCAX tumors the percentage of STC in Q4 was very high (40 %), while only 3 % of the sporadic tumors fell into Q4 (Fig. 2b). These results indicate that hereditary tumors have shorter telomeres than sporadic cases.

Histopathological variables and telomere length measurements

To study how tumor characteristics might be influencing TL variables, logistic regression was performed (Table 3). For TL measured as the mean intensity of fluorescence of telomere probes, none of the histopathological characteristics were significantly differentiating short versus long telomere groups. However, the hereditary or sporadic origin was significantly determining TL ($p = 0.029$).

The other two variables analyzing percentage of short telomeres (ST) or STC in the tissue samples were also significantly affected by the hereditary or sporadic condition. Moreover, tumor grade appeared as an important risk factor for both the percentage of STC or ST in the tumors (Table 3). In addition, other histopathological features also determined the percentage of STC. Younger age of diagnosis was significantly associated with higher percentage of STC, and in addition, expression of Caspase 3 was increased in the group of tumors with higher percentage of

Table 2 Histopathological features of hereditary and sporadic tumors

	BRCA1		BRCA2		BRCAX		Sporadic	
Mean age	37 (28–54)		41 (32–62)		48 (34–77)		58 (41–74)	
	N	%	*N*	%	*N*	%	*N*	%
Grade								
1	0	0	3	25	9	32	8	32
2	1	9	4	33	5	18	12	48
3	10	91	5	42	14	50	5	20
Total	11		12		28		25	
ER								
Positive	3	21	6	54	27	63	14	82
Negative	11	79	5	45	16	37	3	18
Total	14		11		43		17	
PR								
Positive	1	7	5	45	26	60	9	51
Negative	13	93	6	55	17	40	8	47
Total	14		11		43		17	
Her2								
Positive	0	0	0	0	9	27	2	15
Negative	14	100	13	100	24	73	11	85
Total	14		13		33		13	
Ki67								
1	3	23	5	45	26	63	7	44
2	4	31	2	18	9	22	4	25
3	6	46	4	36	6	15	5	31
Total	13		11		41		16	
Caspase 3								
0	1	9	6	43	32	64	10	40
1	2	18	3	21	14	28	12	48
2	7	64	2	14	4	8	3	12
3	1	9	3	21	0	0	0	0
Total	11		14		50		25	

N number of cases, % percentage of cases

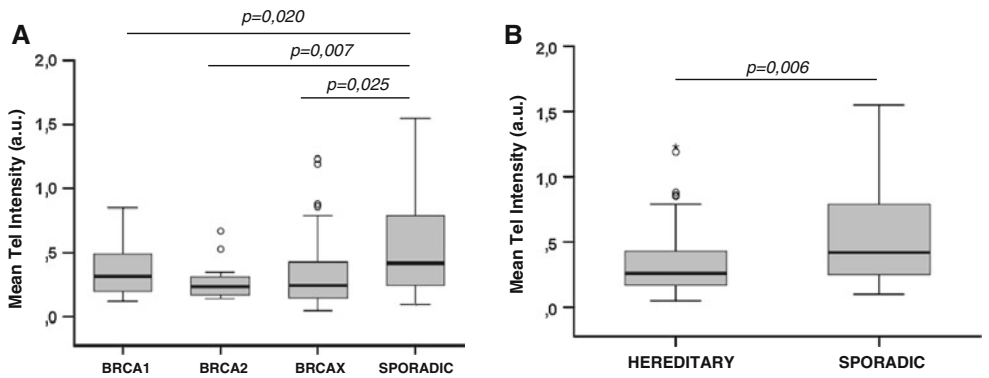

Fig. 1 Average telomere intensity in the different genetic groups of hereditary breast cancer and in sporadic breast tumors. **a** Differences in TL among BRCA1, BRCA2, BRCAX, and sporadic breast tumors indicate shorter telomeres in all familial tumors. **b** Significantly shorter telomeres in all hereditary tumors taken together than in sporadic breast tumors. Telomere intensity corresponds to the average value of all measured cells from all tumors included in each group. The *p* values of comparisons between the different groups are shown

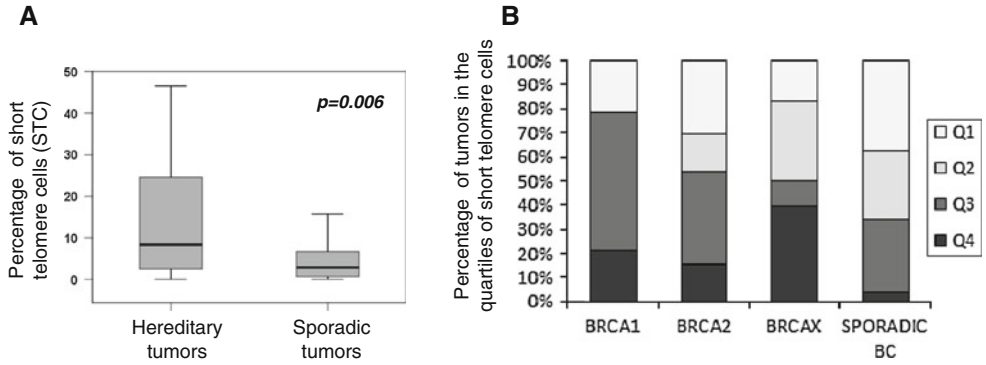

Fig. 2 Percentage of STC in hereditary and sporadic breast tumors. **a** Student's *t* test shows a significantly higher percentage of STC in hereditary tumors than in sporadic ones. The median percentage of each group is indicated by a *black bar*. **b** Quartile distribution of the percentages of STC. Quartiles were defined taking into account the measurement of the percentage of STC in all tumor samples. Then the distribution of tumors in each category (BRCA1, BRCA2, BRCAX, Sporadic) were established based on the quartiles in overall tumor samples (Q1: very low, Q2: medium, Q3: high, Q4: very high number of cells with short telomeres)

Table 3 Risk factor analysis of histopathological parameters on telomere variables

	Mean TL				% Cells with short telomeres (STC)				% Short telomeres (ST)			
		CI 95 %				CI 95 %				CI 95 %		
	OR	Lower	Upper	*p value*	OR	Lower	Upper	*p value*	OR	Lower	Upper	*p value*
Logistic regression												
Age	1.024	0.983	1.065	0.255	**0.949**	**0.908**	**0.991**	**0.018**	1.008	0.969	1.049	0.694
Grade (1/2/3)	1.384	0.74	2.588	0.308	**2.929**	**1.428**	**6.005**	**0.003**	**3.996**	**1.756**	**9.092**	**0.001**
ER (positive/negative)	0.996	0.983	1.008	0.511	0.545	0.226	1.318	0.178	0.524	0.218	1.259	0.148
PR (positive/negative)	0.997	0.985	1.01	0.681	1.057	0.451	2.48	0.898	0.542	0.229	1.282	0.163
Her2 (positive/negative)	1.641	0.436	6.176	0.464	1.012	0.279	3.668	0.986	3.238	0.784	13.37	0.104
Ki67 (<10 %/10–25 %/>25 %)	1.224	0.727	2.063	0.447	1.419	0.852	4.418	0.199	1.141	0.678	1.919	0.62
Caspase3 (<5 %/5–20 %/>20 %)	1.181	0.706	1.975	0.526	**1.997**	**1.131**	**3.528**	**0.017**	1.019	0.612	1.699	0.941
Type (Her/sporadic)	**0.371**	**0.152**	**0.906**	**0.029**	**2.553**	**1.046**	**6.23**	**0.039**	**2.697**	**1.104**	**6.587**	**0.029**
Multivariable logistic regression[a]												
Age	–	–	–	–	0.980	0.910	1.055	0.585	–	–	–	–
Grade(1/2/3)	–	–	–	–	**2.770**	**1.178**	**6.513**	**0.019**	**3.772**	**1.639**	**8.685**	**0.002**
Caspase3 (<5 %/5–20 %/>20 %)	–	–	–	–	1.745	0.720	4.230	0.218	–	–	–	–
Type (Her/Sporadic)	–	–	–	–	2.587	0.437	15.300	0.295	1.397	0.395	4.946	0.604

Bold values indicate statistically significant results

[a] For the multivariable logistic regression all the significant variables obtained in the univariate logistic regression were included

STC. Multiple logistic regression taken into account significant tumor features indicated that, for the STC and ST, tumor grade was the most important associated risk factor.

Histopathological variables specifically associated with shorter telomeres in hereditary or sporadic tumors

Since hereditary and sporadic breast tumors usually show different expression of typical breast cancer inmunohistochemical markers, hereditary, and sporadic tumors were independently analyzed to find differences in TL depending on the expression of any of the histopathological markers analyzed. Logistic regression in hereditary or sporadic subgroups independently analyzed did not show any histopathological variable influencing TL, probably due to the reduction in the number of cases. The only exception was tumor grade that was significantly affecting the percentage of STC and ST specifically in hereditary tumors. Thus, association between telomere variables taken as continuous variables, and histopathological parameters were also examined.

Since TL is known to decrease with age, this association was also analyzed in tumor samples. There was no significant association between short telomeres and age at

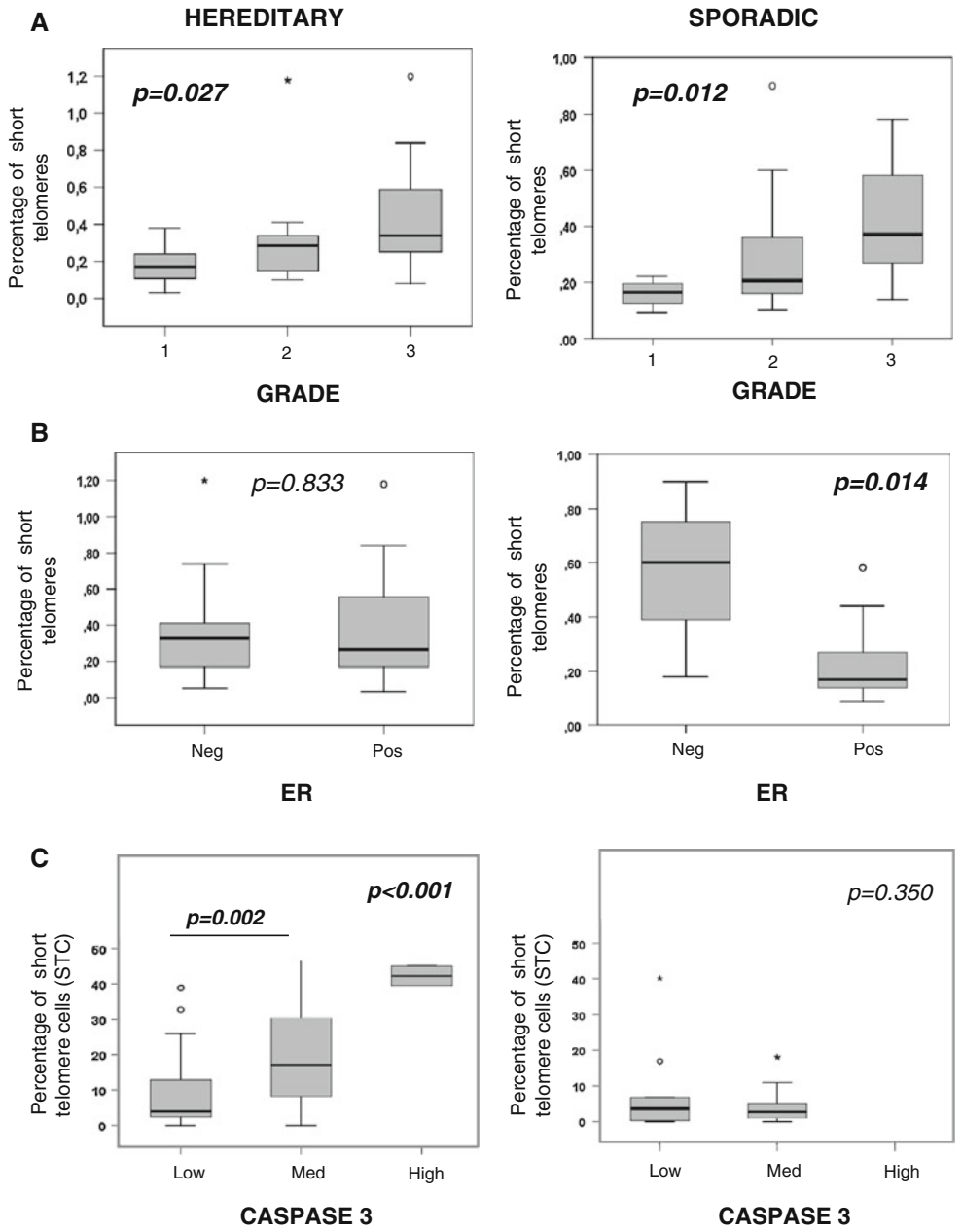

Fig. 3 Histopathological variables associated with shorter telomeres in hereditary and sporadic breast tumors. Association between shorter telomeres, defined by percentage of critically short telomeres (ST) or number of STC in tumors, and different histopathology markers; grade (**a**), estrogen receptor expression (**b**), and activated caspase-3 (**c**) are represented. The median percentage of each group is indicated by a *black bar*

tumor diagnosis, neither in hereditary nor in sporadic tumor samples.

Regarding tumor grade, significant association between higher grade and short telomeres was found among hereditary tumors analyzed separately ($p = 0.027$) (Fig. 3a). Sporadic breast tumors also showed significant association between high grade tumors and increased percentage of short telomeres ($p = 0.012$) (Fig. 3a).

Among IHC markers, in familial cases there was no significant association between ST and ER, PR, or HER2 overexpression. Additionally, proliferation rate of tumors, measured by KI67 expression, was not associated with ST or with the number of STC. However, in sporadic breast cancer, a higher percentage of ST was associated with ER-negative tumors than with ER-positive tumors ($p = 0.014$) (Fig. 3b). No association between any other histological or immunohistochemical variable and shorter telomeres in sporadic breast cancer was found.

When apoptosis markers were analyzed, we found that expression of activated caspase-3 was associated with STC

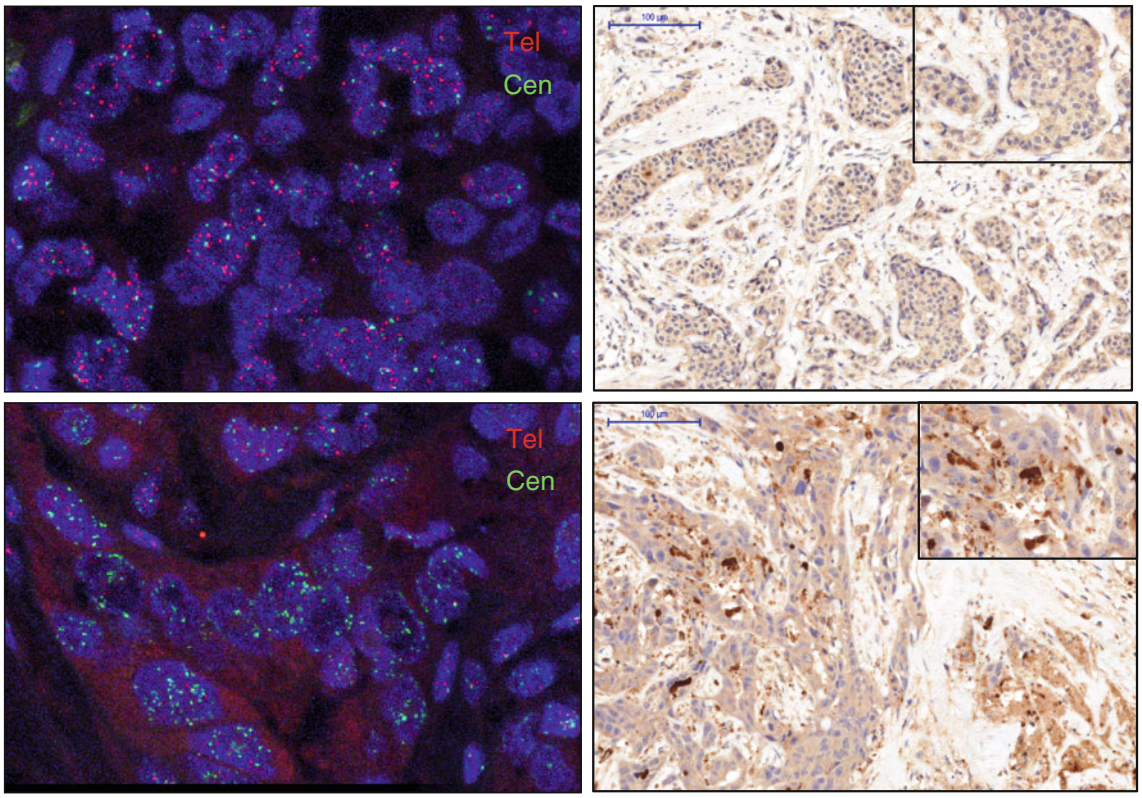

Fig. 4 Representative images of evaluation of apoptosis by immunostaining of active caspase-3 and telomere FISH. Hereditary breast tumors showing correlation between level of apoptotic cells and TL. In telomere FISH images (*left panels*), telomeres correspond to *red* fluorescence signals and centromeres are detected as *green* florescence signals. Active caspase-3 IHC images (*right panels*) were obtained at ×20 and ×70 magnification. *Top* images correspond to a hereditary tumor with a low percentage of STC (3 %) and low expression of active caspase-3. *Bottom* images correspond to a *BRCA2*-mutated tumor showing a high proportion of cells with short telomeres (39 %) and a high level of apoptotic cells

in hereditary ($p = 0.00016$), but not in sporadic tumors ($p = 0.350$) (Fig. 3c). Tumors with a higher percentage of STC also had higher expression of caspase-3, indicating a higher number of apoptotic cells.

Among hereditary tumors, we also considered possible differences specifically associated with the different genetic groups. In BRCA1 tumors, the increased number of STC was specifically correlated with increased proliferation rate ($p = 0.039$) as well as with increased activated caspase-3 expression ($p = 0.022$). Caspase-3 was also significantly associated with an increased percentage of STC in *BRCA2*-mutated tumors ($p = 0.027$) (Fig. 4). Moreover, p53 overexpression ($p = 0.059$), and CK5/6 ($p = 0.014$) and VIM ($p = 0.027$) expression in these tumors were also associated with a higher number of cells with short telomeres.

As described above, the BRCAX group of tumors showed great heterogeneity regarding TL, with an important subset of cases with very short telomeres and a high percentage of STC. We found that ST and STC were particularly associated with tumor grade ($p = 0.047$), with an increased number of STC as tumor grade increases (Fig. 5). In addition, BRCAX tumors with increased expression of the antiapoptotic markers BCL2 or survivin had a significantly higher percentage of STC ($p = 0.012$ and 0.013, respectively) (Fig. 5).

The association between TL and tumor subtypes was also analyzed. Familial tumors were categorized into the established breast cancer groups, Luminal A (27 cases), Luminal B (3 cases), HER2+ (3 cases), and triple-negative (22 cases), based on the expression of ER, PR, and HER2. In 20 tumors, subtype could not be determined due to lack of HER2 status data. We observed that among all familial breast tumors, those with the Luminal B phenotype had the highest percentage of short telomeres compared to the other subtypes, but the differences were not statistically significant ($p = 0.278$) (Supplementary Fig. 1).

Discussion

In this study, we evaluated TL in breast cancer tissues of both hereditary and sporadic origin, and evaluated the expression of histopathological and immunohistochemical

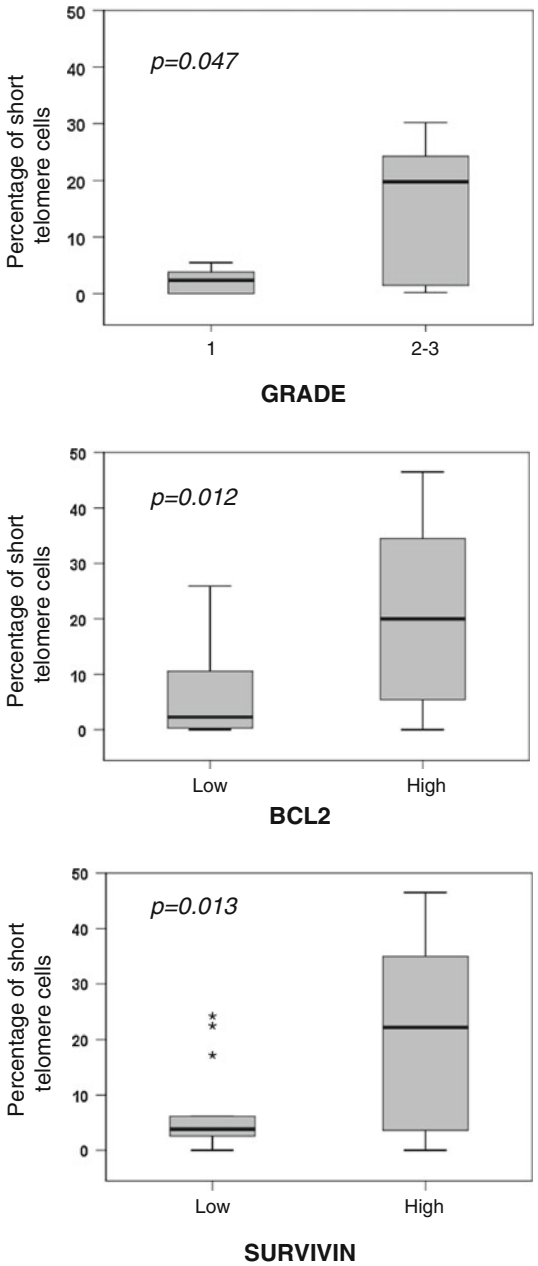

Fig. 5 Histopathological variables associated with shorter telomeres in hereditary BRCAX tumors. The percentage of cells with short telomeres was significantly associated with tumors of higher grade, and with tumors with higher expression of the antiapoptotic proteins BCL2 and survivin. The median percentage of each group is indicated by a *black bar*

markers in relation to TL. We found that hereditary breast tumor samples showed shorter TL compared to sporadic breast cancer samples. Tumor grade was found as an important risk factor determining tumors with higher percentage of STC or short telomeres.

Recently, a study on invasive breast cancer described an association between TL and established breast cancer prognostic markers such as ER, PR, HER2, p53, and Ki67 [22]. In this study, it was found that telomeres were shorter in the more aggressive Luminal B, HER2-positive, and triple-negative tumors. Here, by using a similar methodology, based on measurement of telomeres by FISH which allows estimation of TL at single cell resolution, we describe differences in TL between breast tumors arising in a familial context and sporadic tumors. BRCA1, BRCA2, and BRCAX tumors showed lower mean telomere intensity than sporadic cases, indicating that in general hereditary breast tumors are characterized by shortened telomeres compared to breast tumors of non-familial origin. This finding resembles what happens in blood samples from familial breast cancer patients [26]. Patients from familial breast cancer families carrying mutations in *BRCA1* and *BRCA2*, as well as BRCAX patients had shorter telomeres in peripheral blood lymphocytes than sporadic cases. Therefore, carrying a germline mutation may result in a faster induction of telomere shortening in cells, and predispose to developing tumors with shorter telomeres than those arising sporadically in somatic cells by accumulation of different genetic mutations [29]. In fact, hereditary tumors arise at a younger age than sporadically developed tumors, supporting this hypothesis. Unfortunately, it was not possible in this study to establish a correlation between TL in peripheral lymphocytes and matched tumor tissues, since blood samples from the patients were not available.

Our study also reflects that close to 80 % of BRCA1 tumors and 50 % of BRCA2 and BRCAX tumors had higher percentage of cells with short telomeres (percentage of STC in quartiles Q3 and Q4). It has been described that BRCA1 and BRCA2 tumors are typically genetically unstable tumors displaying high genomic instability [40–42], and telomere dysfunction is likely one of the mechanisms leading to genomic instability by promoting critical telomere shortening. Moreover, a portion of BRCAX tumors also show high genomic instability and a pattern of genomic aberration similar to that observed in BRCA1/2 tumors [41]. This suggests similarities in the mechanisms promoting genomic instability in BRCA1/2-mutated tumors and in a subset of non-BRCA1/2 tumors.

It is noteworthy that tumors with shorter telomeres significantly corresponded to those with the highest tumor grade in both familial and sporadic breast tumors, and tumor grade clearly determined the percentage of short telomeres and STC. In this regard, a correlation between increased genomic instability and high grade of breast tumors has been described [43, 44]. There is other evidence demonstrating telomere shortening associated with high grade tumors in breast cancer [22, 45–47] as well as in other types of cancer [48, 49]. In addition, TL has been associated with tumor size, nodal involvement, TNM stage, and prognosis of breast cancer [22, 50]. In the present study, we show that shorter telomeres were more

frequently found in familial breast tumors than in sporadic ones and that this was associated with high grade tumors. Thus, it is possible that the proportion of short telomeres may reflect the level of underlying genetic instability of the tumors.

In addition to the association with tumor grade, increased telomere dysfunction in ER-negative tumors was also observed before [22, 47]. Our results add evidence of association between shorter telomeres and ER-negative tumors, but only in sporadic tumors.

It has been described that telomere dysfunction initiates chromosomal instability. Cells with extensive chromosomal instability eventually succumb to crisis which is characterized by wide-spread cell death [51]. However, some cancer cells survive crisis by activating telomerase to stabilize the existing telomeres and alleviate chromosome instability [52]. Our results show that in BRCA1 and BRCA2 tumors there exists a positive correlation between an increase in the percentage of cells with critically short telomeres and the number of apoptotic cells, measured by activated caspase-3 (Fig. 4), which could be indicative of the high level of genomic instability that characterizes these tumors, probably induced by DNA repair defects triggered by *BRCA1/2* mutations. In this regard, several studies have demonstrated that in breast carcinoma apoptotic cell death is increased in tumors with adverse histopathological features such as high-grade malignancy, aneuploidy, or p53 expression [53–55].

With regard to BRCAX, our study of TL in breast tumor tissues contributes to the large amount of data confirming the molecular heterogeneity that characterizes this group. Thus, to define the molecular alterations in these tumors is of great importance to better sub-classify them in order to establish improved ways to treat these tumors more efficiently. Here, we describe that a subset of BRCAX tumors is characterized by a high proportion of cells with critically short telomeres which significantly correlates with high tumor grade. In addition, in BRCAX tumors there are significant differences in the proportions of STC between tumors with high or low expression of two antiapoptotic proteins, BCL2 and survivin. These antiapoptotic proteins have been found overexpressed in different tumors including breast cancer [56–58]. Survivin expression in breast tumors is an unfavorable prognostic marker correlated with decreased overall survival [59]. Among BRCAX tumors, BCL2 and survivin expression were highly correlated and associated with shorter TL, likely identifying a subset of BRCAX tumors with a more aggressive phenotype. This result is opposite to the apoptotic behavior in BRCA1/2 tumors (high level of caspase-3 positive cells), and suggests underlying differences in mechanisms regulating apoptosis.

The relation between TL and the different molecular phenotypes was also examined. Tumor phenotypes were established by expression analysis of ER, PR, and HER2 and classified as Luminal A, Luminal B, HER2+, and triple-negative. Typically, BRCA1 tumors are triple-negative tumors with a basal-like phenotype because they share expression profiles, IHC markers and clinical features with basal-like cancers [60, 61]. On the other hand, BRCA2 and especially BRCAX tumors are heterogeneous and have different IHC profiles that resemble the typical molecular subtypes defined in breast cancer [62, 63]. In our study, familial breast cancer showing Luminal B and triple-negative phenotypes tended to have shorter telomeres than the other subtypes, similar to what was described in sporadic breast tumors [22]. However, in contrast to sporadic cases, we did not find an association between HER2+ hereditary tumors and ST or STC due to the limited number of cases with HER2 overexpression. Additionally, since familial tumors are characterized by short telomeres it is possible that differences in TL among molecular subtypes would be more difficult to demonstrate in this group of tumors.

In summary, our study shows that measurement of telomeres by Q-FISH is a useful technique that in combination with immunohistochemistry may differentiate specific subsets of tumors with short telomeres. Short TL was more frequent in hereditary tumors than in sporadic ones. The fact that tumor grade was the most important risk factor for an increased percentage of short telomeres and STC is probably related with the higher genetic instability of high grade tumors. In hereditary BRCA1 and BRCA2 a high level of apoptosis, measured as activated caspase 3, was associated with shorter telomeres. However, in BRCAX tumors shorter telomeres were associated with high expression of the antiapoptotic proteins BCL2 and survivin. In conclusion, telomere dysfunction is frequently found in hereditary tumors and may be a marker of tumor aggressiveness in breast cancer patients.

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Conflict of interest The authors declare that they have no conflict of interest.

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