Impact of chemotherapy on telomere length in sporadic and familial breast cancer patients

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Received: 6 October 2014 / Accepted: 15 December 2014 / Published online: 21 December 2014
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Abstract Recently, we observed that telomeres of BRCA1/2 mutation carriers were shorter than those of controls or sporadic breast cancer patients, suggesting that mutations in these genes might be responsible for this event. Given the contradictory results reported in the literature, we tested whether other parameters, such as chemotherapy, could be modifying telomere length (TL). We performed a cross-sectional study measuring leukocyte TL of 266 sporadic breast cancer patients treated with first-line chemotherapy, with a median follow-up of 240 days. Additionally, we performed both cross-sectional and longitudinal studies in a series of 236 familial breast cancer patients that included affected and non-affected BRCA1/2 mutation carriers. We have measured in leukocytes from peripheral blood: the TL, percentage of short telomeres (<3 kb), telomerase activity levels and the annual telomere shortening speed. In sporadic cases we found that chemotherapy exerts a transient telomere shortening effect (around 2 years) that varies depending on the drug combination. In familial cases, only patients receiving treatment were associated with telomere shortening but they recovered normal TL after a period of 2 years. Chemotherapy affects TL and should be considered in the studies that correlate TL with disease susceptibility.

Keywords Chemotherapy · Sporadic breast cancer · Familial breast cancer · Telomere length · Telomerase

Introduction

Telomeres are nucleoprotein structures that cap and protect the ends of chromosomes against chromosomal fusion, recombination and terminal DNA degradation [1].

Electronic supplementary material The online version of this article (doi:10.1007/s10549-014-3246-6) contains supplementary material, which is available to authorized users.

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In humans, the average telomere length (TL) typically ranges from 10 to 15 kb [2], but telomere DNA shortens with each cell replication. This leads to a progressive telomere shortening during life that ranges from 24.7 to 45.5 bp/year [3]. This shortening continues until the telomere reaches a critical length [4], which triggers cell-cycle arrest leading to senescence or apoptotic cell death.

Germ cells and stem cells can counteract telomere shortening through the action of an enzyme called telomerase, which can synthesize telomeric DNA de novo [5]. However, cancer cells present altered DNA damage response mechanisms and are able to divide even when they present critically short telomeres by up regulating telomerase activity or activating the ‘alternative lengthening of telomeres’ mechanism [5].

There are several retrospective case–control and longitudinal studies suggesting that short telomeres found in DNA from surrogate tissues may predispose to different diseases including cancer [6–11]. However, the largest prospective study in the general population to date (47,102 subjects with a 20-year follow-up) has recently suggested only a weak relationship between short telomeres and breast cancer risk among a large group of cancer types [12].

These results led to a contradiction in the literature regarding the association between TL and cancer or other diseases, and point to differences in the strategies used to perform these studies (prospective or retrospective), technical issues, and/or the existence of TL modifiers as sources of variability [13–16].

In a previous study, we found that patients with hereditary breast cancer (BRCA1/2 and non-BRCA1/2 carriers) presented shorter telomeres than sporadic breast cancer patients and the control population, suggesting a modifier effect of the BRCA1 and BRCA2 genes on TL [17]. Because our study was retrospective, like two other recent studies that presented contradictory results [18, 19], we decided to investigate the possible cause of these discrepancies.

We have focused our attention on the possible impact of chemotherapeutic cancer treatment, because some publications have already suggested a telomere shortening effect after the administration of chemotherapy in vivo (in different tumors) and in vitro (in normal human leukocytes and a mouse spermatogonial cell line) [20, 21].

We have explored this hypothesis in sporadic and familial breast cancer, and we have found that chemotherapy has a telomere shortening effect that is reversible after a period of time once the treatment is finished.

### Samples and methods

**Samples**

Two different series of patients and two different strategies were used. We took a cross-sectional approach in a set of sporadic breast cancer cases and both a cross-sectional and a longitudinal approach in a series of familial breast cancer cases.

The first series comprised 253 sporadic breast cancer patients (age range 30–81 years) who were undergoing or had already received chemotherapy and 13 patients recently diagnosed that had not received chemotherapy (age range 28–68 years). They were included in a clinical trial that involved chemotherapy based on taxane derivatives and were followed for a median of 240 days (range 5–1,855 days). We established two main categories based on their status: “During treatment” and “Post-treatment”. In addition, patients were classified according to the taxane combination they received (Table 1). Details regarding doses, number of cycles, duration, and patient follow-up after treatment are summarized in Table S1.

The second series consisted of 236 familial breast cancer patients belonging to 104 families [23 harboring deleterious mutations in BRCA1, 28 in BRCA2, and 53 not associated to any of the known genes (BRCAX)]. Most of the families were selected from the registry of the Familial Cancer Consultation of the CNIO and had been attended between 2011 and 2013. Individuals from these families met high risk criteria and [22] had been previously screened for mutations in BRCA1 and BRCA2 by a combination of denaturing high-performance liquid chromatography and direct sequencing as previously reported [23]. Due to the lack of a complete knowledge concerning the specific chemotherapy treatment regimens and the follow-up of the patients after finishing the treatment, we established two general groups of patients. Only patients that were known to have been treated with any kind of chemotherapy were included in this analysis. Those, whose sample was extracted less than 2 years after diagnosis were considered as “During treatment”; while those whose sample was extracted more than 2 years after diagnosis were considered as “Post-treatment”.

We measured leukocyte TL by quantitative PCR, in the DNA of all individuals from this series, and whenever possible we measured the TL and the percentage of short telomeres by High Throughput Q-FISH, and the telomerase activity levels in peripheral blood. In the FBOC series, we had the opportunity to compare two TL measurements that corresponded to samples obtained at two different time
points, with an average of 6 years between both extraction points. Using the two measurements we were able to calculate the telomere shortening rate per year.

A summary of all pertinent information (sample size, median age, age range, and the experiments performed) of both cohorts of patients is shown in Table 2.

A set of 330 healthy Spanish women previously described [24], without personal or familial antecedents of cancer (age range 19–77 years) was used as a control population (Table 2-controls1) in order to adjust the TL of both sporadic and familial breast cancer cases according to age.

The study was performed in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from all patients prior to sample collection, and the study was approved by the Ethics Committee of Clinical Investigation from Centro Integral Oncologico Clara Campal, Madrid.

Telomere length quantification

DNA from peripheral blood was extracted using MagNA Pure LC 2.0 System (Roche Diagnostics, Indianapolis, Indiana). TL was measured using quantitative PCR-based technique. This technique calculates the TL as a ratio of telomere amount (t) relative to 36B4 reference gene amount (s) [25]. The measurement unit used in this technology is the t/s value. Primers used to perform the quantitative PCR have been described previously [17]. DNA samples were

<table>
<thead>
<tr>
<th>Drug/combination</th>
<th>During treatment</th>
<th>After treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Me-days treatment</td>
</tr>
<tr>
<td>AC + T/T + AC</td>
<td>24</td>
<td>88</td>
</tr>
<tr>
<td>FEC + T/T + FEC</td>
<td>63</td>
<td>85.5</td>
</tr>
</tbody>
</table>

Me-days treatment median number of treatment days, Me-days post-treatment median post-treatment follows up in days, A doxorubicin, C cyclophosphamide, F 5-fluouracil, E epirubicin, T paclitaxel

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Non-treated</th>
<th>Treated</th>
<th>Post-treated</th>
<th>Age median (years)</th>
<th>Age (range)</th>
<th>Gap (years)</th>
<th>Telomere length</th>
<th>Telomere shortening rate</th>
<th>Telomerase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls$_1$</td>
<td>330</td>
<td>330</td>
<td>–</td>
<td>–</td>
<td>43</td>
<td>19–77</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Sporadic</td>
<td>266</td>
<td>13</td>
<td>88</td>
<td>165</td>
<td>50</td>
<td>30–81</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Familial</td>
<td>236</td>
<td>54</td>
<td>45</td>
<td>68</td>
<td>50</td>
<td>20–87</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Controls$_2$</td>
<td>60/</td>
<td>–</td>
<td>–</td>
<td>50</td>
<td>19–87</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Healthy-BRCA1/2</td>
<td>54</td>
<td>54</td>
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<td>–</td>
<td>42</td>
<td>20–84</td>
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<td>+</td>
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<td>+</td>
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<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
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<td>–</td>
<td>33</td>
<td>51</td>
<td>32–72</td>
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<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>During treatment- BRCA1/2/X</td>
<td>45</td>
<td>–</td>
<td>–</td>
<td>45</td>
<td>26–69</td>
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<td>+</td>
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<tr>
<td>Familial (two time points)</td>
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<td>7</td>
<td>9</td>
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<td>–</td>
<td>6</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<tr>
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<tr>
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<tr>
<td>During treatment- BRCA1/2</td>
<td>7</td>
<td>–</td>
<td>7</td>
<td>–</td>
<td>46</td>
<td>29–61</td>
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</table>

Table 1 Details regarding the two main Taxane subgroup schedules

<table>
<thead>
<tr>
<th>Drug/combination</th>
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<th>After treatment</th>
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</table>

Table 2 General table with relevant information of the sporadic and familial breast cancer series

These controls have been used to calculate the telomere shortening rate of controls and to perform the comparative analysis

Controls$_1$ set of control used for obtain the adjustment line for the age, Controls$_2$ comprise the set of healthy family members without mutations in BRCA1/2 used as controls for the telomerase activity (n = 69) and High Throughput Q-FISH (n = 62), comparative analysis, Controls$_3$ controls that presented two samples, separated in time

a From a total of 45 “During treatment-BRCA1/2/X” patients, 15 were BRCA1/2 and 30 BRCAX
amplified in a total reaction volume of 10 μl containing 1×
Gotaq quantitative PCR Master Mix (Promega, Madison,
Wisconsin), 500 nM of primer Tel1, 1,000 nM of primer
Tel2, 10–30 ng of DNA and a common thermal cycling
profile for both “t” and “s” determination. For 36B4 reac-
tions, the concentrations of primers were 500 nM of 36B4u
and 500 nM of 36B4d. All samples were analyzed in trip-
llicate using an ABI 7900HT thermal cycler, in 384-well
format. TL was calculated as previously described [17].

Measurement of telomere length and short telomere
content by High Throughput Q-FISH

For TL by High Throughput Q-FISH measurement,
peripheral blood mononuclear cells were hybridized with a
PNA-tel Cy3-labeled probe. TL was determined as previ-
ously described [26].

DAPI and Cy3 signals were acquired simultaneously in
separate channels using a Leica TCS-SP5 confocal
microscope (Wechsler, Germany) and maximum intensity
projections from image stacks were generated for image
quantification. In all cases, background noise was sub-
tracted from the image prior to quantification. The “aver-
age telomere fluorescence” values represent the average
Cy3 pixel intensity for the total nuclear area. Percentage of
cells with short telomeres was calculated as those with
intensity values within the first quartile.

Telomerase assay

Protein extracts were obtained from peripheral blood
mononuclear cells cultured in RPMI supplemented with
20 % fetal bovine serum and phytohemagglutinin during
4–5 days, according to the recommendations of the man-
ufacturer of the TRAPEze telomerase detection kit (Merck
Millipore, Darmstadt, Germany). The average telomerase
activity was determined in each sample using 0.5, 0.25 and
0.125 μg of protein extract and normalized with the
internal control included in the assay.

Telomere shortening rate

When possible, we calculated the difference in t/s values
between blood samples extracted in two different time
points (average of 6 years). The result was divided by the
number of years that separated both samples. A positive
result indicated shortening, while a negative value indi-
cated elongation of the telomere.

Statistical analysis

TL measurements were adjusted for age using the line of best
fit for controls. Thus, the difference between the actual and
the predicted value was calculated for each sample. Follow-
ing this method we adjusted t/s values obtained by quantita-
tive PCR (\( y = -0.0174 * X + 1.96 \)), the kb obtained by
High Throughput Q-FISH (\(-0.0587 * X + 12.007\)) and the
percentage of short telomeres (0.1501 * X + 13.331).

The Kolmogorov–Smirnov test was used to evaluate
whether TL measurements obtained from healthy controls,
familial breast cancer and sporadic cancer cases followed a
normal distribution. As a normal distribution could not be
assumed for healthy controls, a Mann–Whitney \(U\) test was
applied (Table S2; Fig. 3a, b) Unpaired Student \(t\) test for
normal distributions was applied in the comparative ana-
lysis of telomerase activity, telomere shortening rate
(Tables S3, S4) and percentage of short telomeres among
the familial breast cancer groups (Fig. 4).

Spearman correlation test was used to establish whether
correlation between variables were statistically signifi-
cant (Fig. 1).

Statistical calculations were performed using SPSS
version 18 (SPSS, Inc., Chicago, Illinois) and Graph pad
Prism 5.03(San Diego, California). Nominal two-sided \(p\)
values less than 0.05 were considered statistically signifi-
cant. Graphics were performed using Graph pad Prism 5.03
and Microsoft Office Excel 2007.

Results

Controls

We evaluated the TL distribution in 330 healthy women as
a function of age in order to obtain a regression line to
adjust the t/s values from both the familial breast cancer
and the sporadic series. As expected, we found a decrease
in TL with age (Fig. S1).

Sporadic cases

We tested whether chemotherapy itself has an effect on
telomere shortening. To address this hypothesis we evalu-
ated the TL distribution of patients included in the “During
treatment” category. We observed a strong correlation
between telomere shortening and treatment duration
\(( r = -0.43; \ p = 3.71E-05; \  \text{Fig. 1a})\). We did the same
evaluation in the “Post-treatment” category, and we
observed that the distribution had the opposite shape, with
a significant positive correlation between TL and time after
treatment \(( r = 0.17; \ p = 0.02; \  \text{Fig. 1b})\).

We next did a comparative analysis of TL during dif-
f erent periods of the treatment time line (Fig. 2). We
compared the median TL of each group, with the median
TL of the control group. The TL \((t/s \text{ values adjusted by}
age)\) in patients before treatment did not differ compared to
controls (Fig. 2, black line). From the beginning of the treatment, we observed a shortening effect that became statistically significant in patients treated for more than 90 days and remained significantly shortened until 360 days after the treatment. Patients whose samples were extracted later than 360 days after the end of the treatment tended to recover normal TL values, and did not show significant differences when compared to controls. The results of this analysis are summarized in Table S2.

In patients treated with therapy based in taxane derivatives (Table 1), we observed a more significant telomere shortening effect in those treated with FEC–T/T–FEC (red line) compared to those treated with AC–T/T–AC (green line) however, the latter seemed to recover their TL faster (Fig. 2).

Familial breast cancer cases

Cross-sectional study

We performed this analysis to explore both, the role of BRCA1/2 mutations as TL modifiers and the possible correlation between telomere shortening and cancer treatment. Healthy BRCA1/2 mutation carriers (n = 54) did not present shorter telomeres when compared to controls (n = 330), while significantly shorter telomeres were found in affected BRCA1/2 mutation carriers (n = 50; p = 0.044) and BRCAX patients (n = 63; p = 0.048; Fig. 3a).

In view of the behavior of telomeres in sporadic breast cancer cases according to the treatment status, we decided to reanalyze the data from the familial breast cancer series. We established two categories consisting of cancer patients from whom samples were extracted within 2 years from the beginning of the treatment, called “During treatment BRCA1/2/X”, and a second group composed of patients whose samples were taken more than 2 years after the beginning of treatment, called “Post-treatment BRCA1/2/X”. We used 2 years as a cut-off, because in the sporadic series we observed that the recovery phase starts approximately 2 years since diagnosis.

No significant differences were found between “Post-treatment BRCA1/2/X” patients and controls (Table 2-controls1). However, the “During treatment BRCA1/2/X” group presented significant shortened telomere t/s values compared with the controls (p = 0.0005) and the “Post-treatment BRCA1/2/X” groups (p = 0.007; Fig. 3b).

We also evaluated the leukocyte telomerase activity in this cohort of patients (Fig. 3c). We found that healthy BRCA1/2 mutation carriers presented similar telomerase activities to the healthy controls (Table 2-controls2). Interestingly, all cancer patients, independently of the mutation type and the treatment time, presented significantly lower levels of telomerase activity, being patients “During treatment” the ones showing the lowest values (p = 0.0009; Table S3).

In parallel, we used High Throughput Q-FISH technology in order to validate our results using an alternative technology. We found the same results regarding TL for the “During treatment BRCA1/2/X” group (Fig. 4a), confirming the presence of a significantly higher percentage of short telomeres (<3 kb) for this group of patients (p = 0.01) compared to the control population (Table 2-controls2; Fig. 4b).

Longitudinal study

We performed a longitudinal retrospective study in order to analyze telomere shortening rate in a set of 31 BRCA1/2 mutation carriers with two independent blood samples taken separately by an average period of 6 years: 15 were healthy carriers, 9 affected carriers with the two samples obtained once the chemotherapy had finished, and 7 affected carriers in which the first sample was obtained “During treatment” and the second extraction point in the “Post-treatment”. Additionally we analyzed the telomere shortening rate in a set of 25 controls from which we also had samples taken at two time point (Table 2-controls2).
Fig. 2 Telomere length evolution during treatment and after treatment. Mann–Whitney test was used to determine significant differences between controls and the different subgroups. The discontinuous lines represent significantly shortened telomeres compared to controls for all the patients together (black line) and for the two major drug schedules (red and green lines).

Fig. 3 a Distribution of telomere length (t/ts) values adjusted for age for the familial breast cancer groups according to mutational status. The Mann–Whitney test was used to test for significant differences in telomere length between controls and the different subgroups. b Distribution of telomere length (t/ts) values adjusted for age for the familial breast cancer groups according to treatment status (Mann–Whitney test). c Comparative analysis of telomerase activity levels in the familial breast cancer series; Student’s Unpaired t test was used to determine significant differences among groups.
Healthy BRCA1/2 carriers presented a faster telomere shortening rate compared to the control group, although the differences were not significant; while “Post-treatment BRCA1/2” patients presented a telomere shortening rate very similar to the control group. By contrast, “During treatment—BRCA1/2” patients presented a different pattern, going from short telomeres at the first measurement, to normal telomeres 7 years later (Fig. 5). In this case the elongation speed was very pronounced, and statistically different from the normal shortening of the control population ($p = 0.011$). The results of this analysis are summarized in Table S4.

**Discussion**

We previously reported that BRCA1/2 might be TL modifiers, because mutation carrier patients presented shorter telomeres than the control population and sporadic breast cancer cases [17]. However, two recent reports did not find any relationship between the presence of BRCA1/2 mutations and telomere shortening, nor between TL and an increased cancer risk [18, 19]. The three studies were done in a retrospective manner, and in none of them treatment status, which might constitute a confounding factor, was evaluated independently as a possible modifier of TL. We tried to overcome this possible bias by working with two independent cohorts of women with breast cancer (sporadic and familial) and two different approaches (cross-sectional and longitudinal).

According to the results obtained in the sporadic set of patients treated with different chemotherapy schedules and followed for a maximum period of 1,855 days, telomere shortening starts at 91–180 days after the beginning of treatment and reaches its maximum effect between 91 and 180 days after finishing treatment. Then a recovery phase starts, and it lasts for a maximum of 1,855 days after which the original TL is recovered (Table S2). Similar results were observed in the familial breast cancer series in both, cross-sectional and longitudinal studies.

There are some reports suggesting a possible deleterious effect of chemotherapy on TL and/or telomerase activity, although some of these seem to be contradictory.

The first authors, who addressed this question, reported significant telomere shortening accompanied by a significant reduction in telomerase activity in the blood cells of pediatric leukemia patients after the administration of cancer treatment [27]. Another early longitudinal study in breast cancer patients undergoing chemotherapy, reported a clear negative effect on different hematological parameters, including telomere shortening, after high doses of FEC + T [28]. On the other hand, in a recent longitudinal study performed in a set of 33 breast cancer patients undergoing AC + T/T + AC chemotherapy no changes were detected in TL, at 3 and 12 months after the end treatment [29].

It is probable that in this case patients were already at the “recovery phase” proposed in this study. Surprisingly, increased expression of the senescence markers p16 and ARF was detected, which have been correlated with telomere damage in primary cells [30].

Engelhardt et al. were the first to raise the question of whether telomere shortening is permanent or telomeres regain their initial length after discontinuation of chemotherapy [27].

In this regard, our results confirm there is a telomere recovery after treatment, although we have observed that the shortening effect and the time to reach the “recovery
phase” varies depending on the treatment type, with a shortening effect more severe at the initial phase of the treatment for patients under the AC + T/T + AC regimen, while those treated with FEC + T/T + FEC present a more delayed “recovery phase”. It seems that telomere stability is affected by conventional chemotherapies in different manner depending on the specific drug mechanism of action (alkylating agents, anti-replicative molecules, spindle poisons, anti-topoisomerase I and II) being the effects on the telomere heterogeneous.

Some authors have evaluated the role of several chemotherapy drugs in vitro and in cancer patients at a more mechanistic level [21, 31, 32]. For instance, the alkylating agent cyclophosphamide, was shown to induce in spermatogonial cells a significant increase in DNA damage, which was localized preferentially at telomeres. This telomere DNA damage was accompanied by telomere shortening and a significant reduction in both telomerase mRNA levels and significantly diminished telomerase activity [21]. These negative effects may be explained due to the high guanine content of the telomere sequence and the TERC gene, which may increase the risk of DNA–DNA crosslink damage by cyclophosphamide [21].

In another longitudinal study using 6 cycles of cyclophosphamide, doxorubicin, vincristine, and prednisone in a cohort of 14 non-Hodgkin lymphoma patients, significant telomere shortening was maintained up to 2 years, after the treatment ends. The same study evaluated the anti-metabolite drug 5-fluorouracil alone in a set of 10 colon cancer cases. TL was measured 2 and 12 months after the end of the treatment showing an initial telomere attrition that was recovered after 1 year [31]. The authors propose a possible mechanism based on whether these drugs are able to target hematopoietic stem cells, or only mature progenitors, to explain a long- or a short-term effect in telomere shortening [31]. In addition, it is also known that mitotic inhibitors such as taxol, which are normally included in conventional breast cancer chemotherapy schedules, can causes telomere uncapping, triggering telomere dysfunction in cancer cells [32].

Taking all this evidences together we can assume some variation in the telomere dynamics during and after the treatment, depending which type of anticancer chemotherapy drugs are included in the patients treatment.

In the familial breast cancer model we followed both cross-sectional and longitudinal approaches in order to test whether the BRCA genes and chemotherapy are modifiers of TL and/or telomerase activity. In the cross-sectional approach we found similar results as in our previous study [17], familial cases presented shorter telomeres than controls; however, a substantial proportion of the samples had been taken during or after a short time of chemotherapy administration. When we corrected for treatment status we were not able to detect any effect of the BRCA1/2 mutations on telomere shortening, neither in healthy carriers nor in post-treated BRCA1/2 patients, suggesting that the treatment is the real TL modifier (Fig. 3b). These results could explain partially, the discrepancies reported in the literature regarding the role of the BRCA1/2 genes as modifiers of TL [17]. In a recent report by Pooley et al., BRCA1/2 mutation carriers showed longer telomeres than controls, and the authors suggests that an increase in telomerase levels could be associated with this event [18]. However, in the present study we have not found higher levels of telomerase activity in healthy BRCA1/2 patients, while treated patients...
(independently of their mutational status), presented both shorter telomeres and significant lower telomerase activity levels.

Regarding the longitudinal study, BRCA1/2 affected carriers with two TL measurements covering the “Post treatment” period, did not present significant differences in the shortening rate compared to healthy BRCA1/2 carriers and/or controls. However, BRCA1/2 patients, with the first sample taken during treatment and the second sample taken 7 years later (during the post-treatment period) presented a recovery of TL after chemotherapy, confirming our observations in the sporadic series (Fig. 5).

To summarize, our study has focused on the effect of treatment on TL in both familial and sporadic breast cancer cases. We cannot rule out that BRCA1 and BRCA2 might be minor modifiers of TL, but it appears that treatment is the true cause of telomere shortening. The rates of telomere shortening and recovery may vary depending on the treatment. These results stress the need to perform prospective and retrospective studies, considering the variability found as a consequence of the treatment status (untreated, during treatment, and post-treatment) of the patients, to obtain conclusive results about the relationship between TL and disease.

Acknowledgments We thank Alicia Barroso and Victoria Fernandez for their technical support. This work was partially funded by project FIS PI12/00070 from the Carlos III Health Institute and the Spanish Network on Rare Diseases (CIBERER) and by project SAF2012-35779 (Spanish Ministry of Economy and Competitiveness). M.A.B.’s Laboratory is funded with the Spanish Ministry of Science and Innovation, Projects SAF2008-05384 and 2007-A-200950 (TELOMARKER), European Research Council Advanced Grant GA#232854, the Körber Foundation, Botín Foundation, and Lilly Foundation.

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

Ethical Standards The authors declare that this work complies with current Spanish laws.

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