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Original Article

Telomere length measurement in tumor and non-tumor cells as a valuable prognostic for tumor progression

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ABSTRACT

Telomere shortening has been supposed to be implicated in both aging and various human diseases especially carcinogenesis process. This phenomenon can lead to a chromosomal instability, contributing to a cell immortalization and tumor induction. In our study, we analyzed the role of telomere shortening in cancer progression, in Tunisian patients with digestive cancer. We measured the absolute telomere length in tumoral *vs* healthy adjacent tissues of each patient by using a q-RT PCR method and we investigated the relationship between telomere length and various sociodemographic and clinical parameters such as age, sex, tumor stage. In this pathological situation, we observed that, starting from 60 years of age, the telomere length increases in healthy mucosa and that in both healthy and cancer tissues, patients under 60 years have shorter telomeres, suggesting the telomere lengthening becomes more active with age. Finally, a positive correlation between normal and cancer tissues in both non-metastatic and metastatic stages, indicates telomere length in cancer tissue depends essentially on tumor stages. Our data allow us to suggest that telomere length depends on sex and age in healthy tissue while shortening and lengthening fluctuates considerably according to the tumor stage.

Introduction

Telomeres are composed by a repetitive DNA sequence (in humans: TTAGGG repeated up to 1500 times) which constitutes the end of each chromosome arm and associates with various proteins constituting the Shelterin complex [1]. This complex allows telomere folding to form the D-loop and the T-loop, protecting chromosomes from fusion or degradation and hence preventing chromosomal instability [2].

Since DNA polymerase cannot fully replicate the 5′ end of the newly synthesized strands, the telomeres shorten at each cell replication. After a critical number of divisions, the telomeres reach the Hayflick's limit and cannot longer be protected by the Shelterin complex, being thus recognized as double-strand breaks and triggering the DNA damage response. This eventually leads the cell to enter senescence or undergo apoptosis [3]. Otherwise, in case of defective cell cycle checkpoints, cells can escape senescence and continue to divide giving daughter cells with very short telomeres which may cause a genomic instability that characterizes the phenomenon of cell crisis. Under the pressure of such crisis, a potential reaction of the cell leading to telomere lengthening is necessary for further cell division. Two molecular mechanisms ensure telomere elongation and the maintaining of their function: the reactivation of telomerase, the reverse transcriptase enzyme necessary for complete telomere replication, or the "Alternative lengthening of telomeres" ALT, both giving rise to immortal cells [4,5]. From the above considerations the paradoxical role of telomere shortening becomes apparent: on one hand, telomere erosion triggers cell senescence or apoptosis leading to tumor suppression in normal cells, on the other hand it causes genetic instability and promotes tumorigenesis [6,7]. Several studies investigated the dynamic of telomere length

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Table 1
Clinicopathological parameters in digestive cancer.

Variables		Number of patients	Percentage
Age	<60	23	47
	≥60	26	53
Sex	Male	32	57
	Female	24	43
Tumor site	Colon	24	46
	Rectum	14	27
	Stomach	11	21
	Esophagus	3	6
Stages	I	7	17
	II	12	29
	III	6	14
	IV	17	40
Grades	Well	26	81
	Moderate	4	13
	Poor	2	6

during tumor occurrence. While the majority of studies found that telomeres undergo shortening during tumor transformation [8], some other studies proved an unchanged length of telomeres [9]. Other studies proved that telomere length is influenced not only from tumorigenesis but also from other factors [10].

The dynamic of telomere length during the tumorigenic process is obviously relevant to assess if telomere length can be considered as biomarker for aging and for risk, onset, progression and prognosis of some diseases such as cancer.

In the present study, we measured the telomere length (TL) in cancer tissues from digestive system and in the corresponding normal cells from the adjacent healthy mucosa in patients from La Rabta Hospital of Tunis. We compared telomere length of normal and cancer cells in each patient and then we studied the correlations between telomere length and clinicopathological parameters such as sex, age, tumor tissue, cancer stage and presence or absence of metastases, taking into account the individual variability.

Materials and methods

Patients and samples

We collected 54 pairs of biopsies extracted from both cancerous and adjacent healthy tissues of each tumor site during colonoscopy/fibroscopy. Our cohort consists of Tunisian patients suffering from digestive cancer enrolled in La Rabta Hospital of Tunis and before any chemotherapy treatment. Collected samples were immediately frozen in −80 °C. Our population is composed of 57% males and 43% females. The average age was 62.36 ±13.10 years (youngest = 33 years, oldest = 88 years). The distribution of patients according to the affected tissue was as following: 46% colon, 27% rectum, 21% stomach, 6% esophagus. Tumors were staged according to TNM classification method: 17% were classified as stage 1 (T1 or T2, N0, M0), 29% stage 2 (T3 or T4, N0, M0) 14% stage 3 (T, N1 or N2, M0) and 40% as stage 4 (T, N, M1) (Table 1) [11].

This study was conducted according to the principles of the declaration of Helsinki and all participants provided their informed consent to their participation in this study.

DNA isolation

Genomic DNA was extracted from tissues using the commercial kit ISOLATE II GENOMIC DNA (Bioline) according to the manufacturer's protocol. DNA was then diluted on 100 µl of DNase/RNase free water and stored in −20 °C until use. For fluorometric determination of DNA concentration, we used the Qubit® 2.0 fluorometer following manufacturer's instructions (Qubit® dsDNA_BR_Assay Kit, ThermoFisher, USA).

Quantitative real time PCR for absolute telomere length (aTL-qPCR)

The absolute telomere length (aTL) was determined by using a modified version of Cawthon's quantitative real-time PCR (q-PCR)-based method which uses the ratio of telomere-repeat copy number to single-copy-gene (SGC) copy number (T/S ratio) [12,13].

Telomere standard curve

Telomere standard curve was established by performing the aTL-qPCR assay on serial dilutions of known quantities of a synthesized oligomer standard containing 14 TTAGGG repeats (84 bp), with a molecular weight (MW) of 26,667.2 (Table 2). The weight of one molecule is calculated by dividing the MW by Avogadro's number (6.02×10^{23}). Therefore, weight of telomere standard is $26{,}667.2/6.02 \times 10^{23} = 0.44 \times 10^{-19}$ g. The highest standard concentration (TEL STD A) has 240 pg (60 pg × 4) of telomere oligomer, the lowest (TEL STD H) has 2,.4 $\times 10^{-5}$ pg of telomere oligomer. Plasmid DNA (*pBR322*) was added to each standard to maintain a constant amount of 20 ng of total DNA per reaction tube. To establish the standard curve, we used only the last 5 standard concentrations (TEL STD D-H). Cycles threshold (Ct) for each dilution were reported on the Y-axis of the graph, whereas X-axis represents the amount of telomere sequence in kb per reaction (Fig. 1A). The amount of telomere sequence in kb for each standard concentration is calculated as DNA concentration (g)/weight of telomere standard (0.44×10^{-19}), multiplied by number of base pairs (84 bp). DNA amount was then optimized by transforming telomere length values into log in order to detect experimental samples within the linear range. Correlation coefficient for telomere standard curve was 0.99.

SCG standard curve

A similar procedure was followed to establish a SCG standard curve, in order to check the amplification for every sample performed and to determine genome copies per sample. The SCG standard curve was generated by performing serial dilutions of the *36B4* oligomer standard (75 bp) with a MW of 23,268.1 (Table 2). As done for telomere standard, the weight of one molecule is calculated by dividing MW by Avogadro's number (6.02×10^{23}). Therefore, weight of 36B4 standard is $23{,}268.1/6.02 \times 10^{23} = 0.38 \times 10^{-19}$ g. The highest concentration standard (SCG STD A) has 800 pg (200 pg × 4) of 36B4 oligomer, the lowest (SCG STD M) has 8×10^{-8} pg of SCG oligomer. To establish the standard curve, we used only the last 6 standard concentrations (SCG STD F-M). Plasmid DNA (*pBR322*) was added to each standard to maintain a constant amount of 20 ng of total DNA per reaction tube. Cycles threshold (Ct) for each dilution were reported on the Y-axis of the graph, whereas X-axis represents *36B4* copy number per reaction. The number of SCG copies for each standard concentration is calculated as DNA concentration (g)/weight of *36B4* standard (0.38×10^{-19}), divided by 2 since there are two copies of *36B4* per diploid genome. DNA amount was then optimized by transforming SCG copy number values into LOG in order to detect experimental samples within the linear range. Correlation coefficient for SCG standard curve was 0.99 (Fig. 1B).

qRT-PCR assay

All samples were diluted to obtain a final concentration of 5 ng/µl and run on Mx3000P System (Stratagene). Three different

Table 2
Oligomers used for qRT-PCR (designed by O'Callaghan and Fenech [13]).

	Oligomer name	Oligomer sequence 5′–3′	Amplicon size
Standards	Telomere standard	(TTAGGG)14	84 bp
	36B4 standard	CAGCAAGTGGGAAGGTGTAAT CCGTCTCCACACACAAGGCCA GGACTCGTTTGTACCCGTTGAT GATAGAATGGG	75 bp
qPCR primers	Telo_F	CGGTTTGTTTGGGTTTGGGTTTG GGTTTGGGTTTGGGTT	>76 bp
	Telo_R	GGCTTGCCTTACCCTTACCCTTAC CCTTACCCTTACCCT	
	*36B4*_F	CAGCAAGTGGGAAGGTGTAATCC	75 bp
	*36B4*_R	CCCATTCTATCATCAACGGGTACAA	
	*β-globin*_F	GCTTCTGACACAACTGTGTTCACTAGC	82 bp
	*β-globin*_R	CACCAACTTCATCCACGTTCACC	

master mixes were prepared for each sample. The first one contained primers for telomere amplification, the second contained *36B4* primers for SCG amplification and the third contained *β-globin* primers. Since in many cancers there is a strong genomic instability that can lead to aneuploidy, the number of genetic copies of *36B4* could undergo variations; therefore, we used *β-globin* as a second single copy- gene to check this possibility. In the 96-wells plate samples were loaded as follows: each column corresponds to an individual sample, normal and tumor samples from the same patient were loaded in replicates into adjacent columns; in the first two rows of the plate the telomeric sequences, in the second two rows the SCG and in the following two *β-globin* amplicons were amplified. Finally, a No Template Control (NTC) was boosted for each master mix. Individual samples are analyzed in duplicate. Cycling conditions were: 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min, followed by a dissociation curve. Only Ct values with a standard deviation ≤5% were accepted. In each standard curve the *y* values of the equation were substituted with Ct values from q-RT-PCR to calculate total telomere length in kb per human diploid genome. Ct values for both telomere and *36B4* gene (Single Copy Gene, SCG) amplification in each sample were determined after performing qRT-PCR. The logarithm (log 10) of telomere length was obtained by replacing the *y* with Ct values from qRT-PCR in the straight-line equation ($y = 4.306x + 47.5$) of the telomere standard curve, for each sample. The same was done for the SCG. By raising these values to the tenth power, we obtained the absolute telomere length and the number of copies of the *36B4* gene, respectively. By computing $10^{\log(L)}$ and $10^{\log(SGC)}$, we obtained the T/S ratio by dividing telomere length by the number of SCG copies. This ratio gives a total telomere length in base pair (bp) per human diploid genome. It can be also used to give a length per telomere by dividing by 92 (the total number of telomeres in one cell).

Statistical analysis

The comparison between mean TL in tumor tissues and in corresponding normal mucosa, were determined by Wilcoxon signed rank (paired) test.

In order to see TL timing according to age, we analyzed the TL in two distinct groups within cancer and normal tissues, according the median age (60.5): patient with age<median and >median, that is age ≤60 and age >60. We tested significance by Mann–Whitney test.

Moreover, to test an association between normal and tumor with age changes, we carried out Spearman's correlation.

A perfect Spearman correlation of +1 or −1 occurs when each variable is a perfect linear function of the other. To look for a possible correlation between TL and tissue type, we divided samples into different groups according to the tissue in which the tumor developed and we used the Wilcoxon signed-rank test to evaluate the significance of results. The same was done regarding the tumor stage. Samples were first divided into four groups according to the stage (I, II, III, IV) and were compared to each other; then stages I, II and III were grouped together and compared with stage IV. The association between TL in tumor samples and the stage, for each group, was calculated with the Spearman's correlation. To compare telomere lengths in men and women and to compare TL in cancer and normal within the same sex we used the Mann–Whitney test. Finally, we divided tumor samples into two groups: those exhibiting shorter telomeres than in control normal tissue and those with equal or longer telomeres than normal mucosa and we compared telomere length in the healthy mucosa tissues by using Mann–Whitney test because the two groups are not paired.

Using Shapiro–Wilk normality test, we could affirm the normal distribution of our data ($p < 0.05$ means that the distribution is not normal/Gaussian)

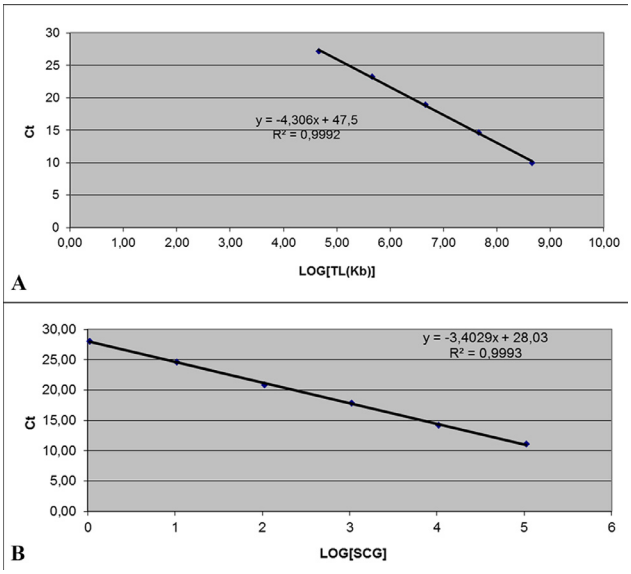

Fig. 1. Standard curves used to calculate (A) telomere length per reaction per tube and (B) genome copies using *36B4* copy number.

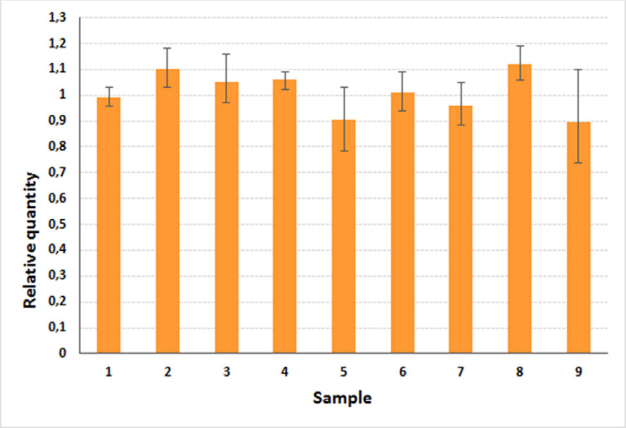

Fig. 2. *36B4/β-globin* relative ratio in some representative samples.

Results

Genomic DNA normalization

The absolute telomere length (aTL) was determined by using a quantitative real-time PCR (q-PCR)-based method which uses the ratio of telomere-repeat copy number to single-copy-gene (SGC) copy number (T/S ratio). For this study we used the single-copy-gene *36B4* which encodes for the acidic ribosomal phosphoprotein P0. In order to assess the consistency of our results, we first determined by quantitative PCR the relative ratio of *36B4* gene copies to *β-globin* gene copies, for each different sample. Relative ratios could be considered as reflecting relative length differences in telomeric DNA and taken into account only if the number of *36B4* gene copies per cell that are effectively PCR-amplified, is the same in all individuals. The relative ratio *36B4/β-globin* versus the reference DNA, was about 1, indicating that equal copy numbers of the *36B4* gene per cell were amplified in all DNA samples. By one sample *T*-test we assessed that the difference from 1.0 was never significant with $p > 0.20$ (Fig. 2).

Correlation of telomere length in normal and tumoral tissues

Average telomere length in all tumor samples (280,091 kb) was slightly shorter than in healthy control tissue (301,176 kb). This difference was not significant (Signed-rank test, 2-tailed $p = 0.80258$) (Fig. 3).

Patient-by-patient comparison of telomere length in tumor tissue and in adjacent healthy mucosa showed that cancer tissues had shorter telomeres in 29 patients (53.7%), longer telomeres in 23 patients (42.6%) and unchanged telomeres in 2 patients (3.7%). T/S ratio for each sample was reported in a bar chart to compare the total telomere length in normal and cancer tissue in each patient (Fig. 4).

Then, we divided tumor samples into two groups: group I, those exhibiting shorter telomeres than in control normal tissues (29 patients, 53.7%, $p = 3.7 \times 10^{-9}$), henceforth C < N and group II, those with longer or equal telomeres than in normal tissues (23 + 2 patients; 46.2%, $p = 5.9 \times 10^{-8}$), henceforth C ≥ N, and we

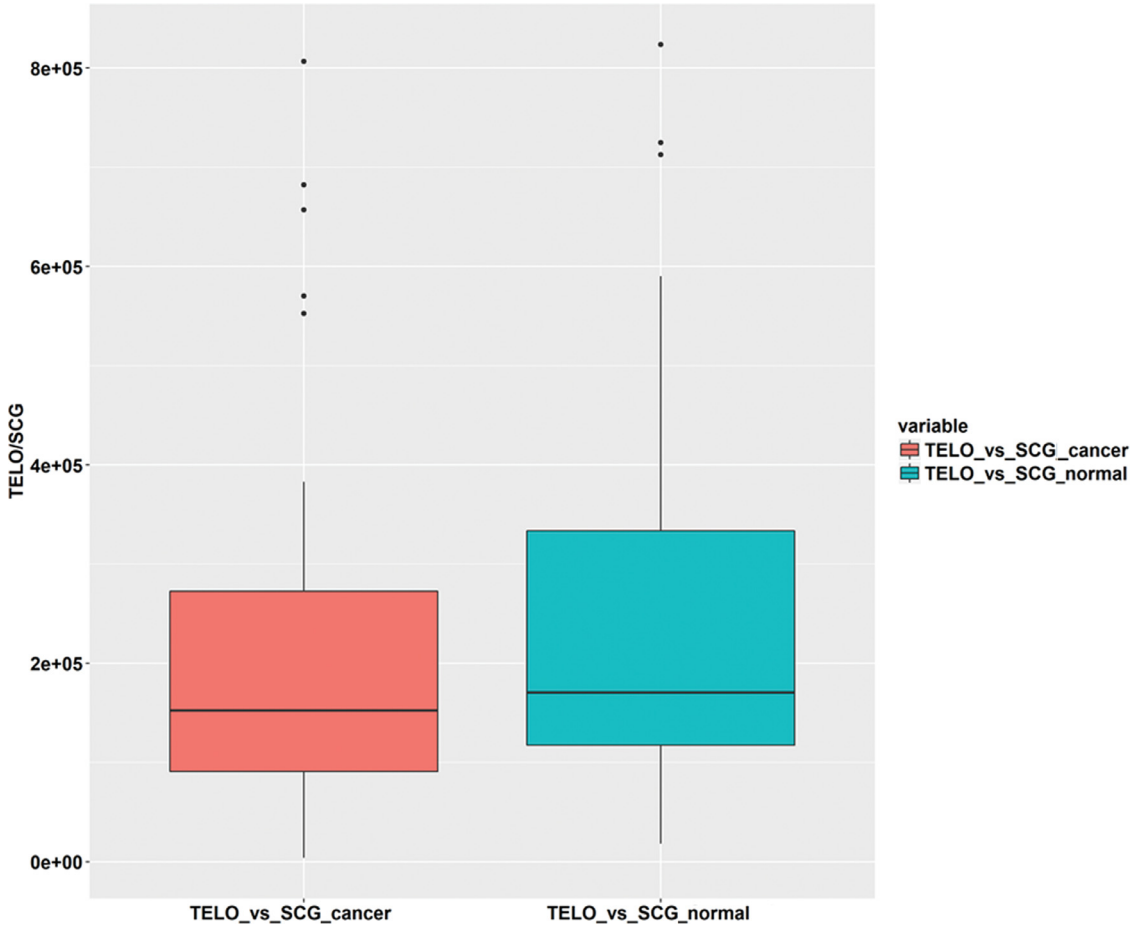

Fig. 3. The boxplot shows the mean of TL in cancer and in normal tissue.

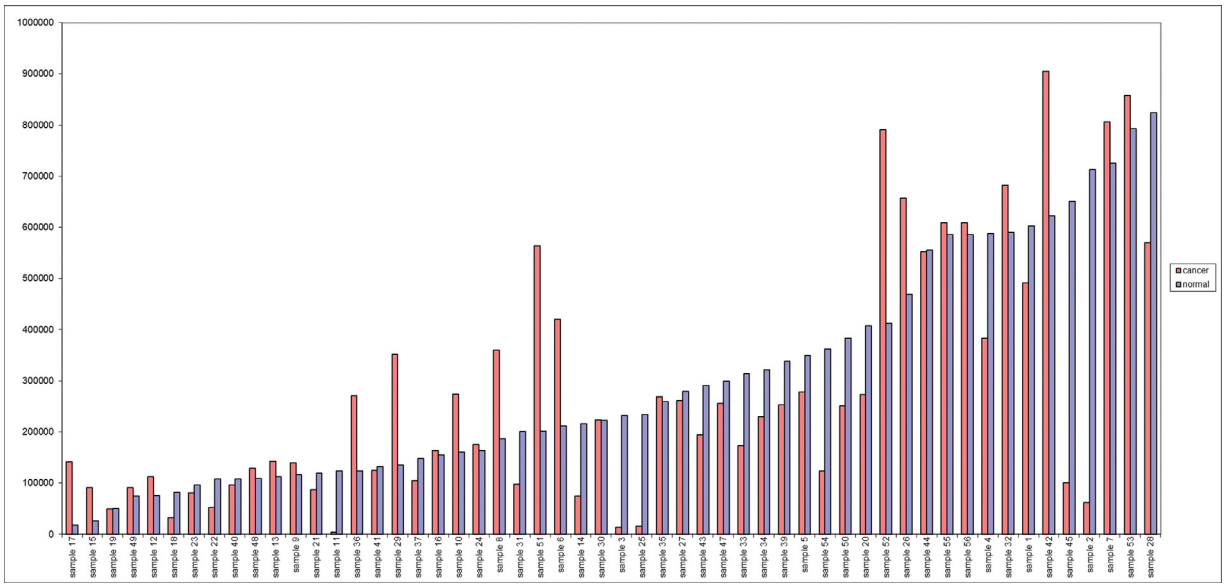

Fig. 4. In the graph the T/S ratio in both normal and cancer tissue for each patient was reported.

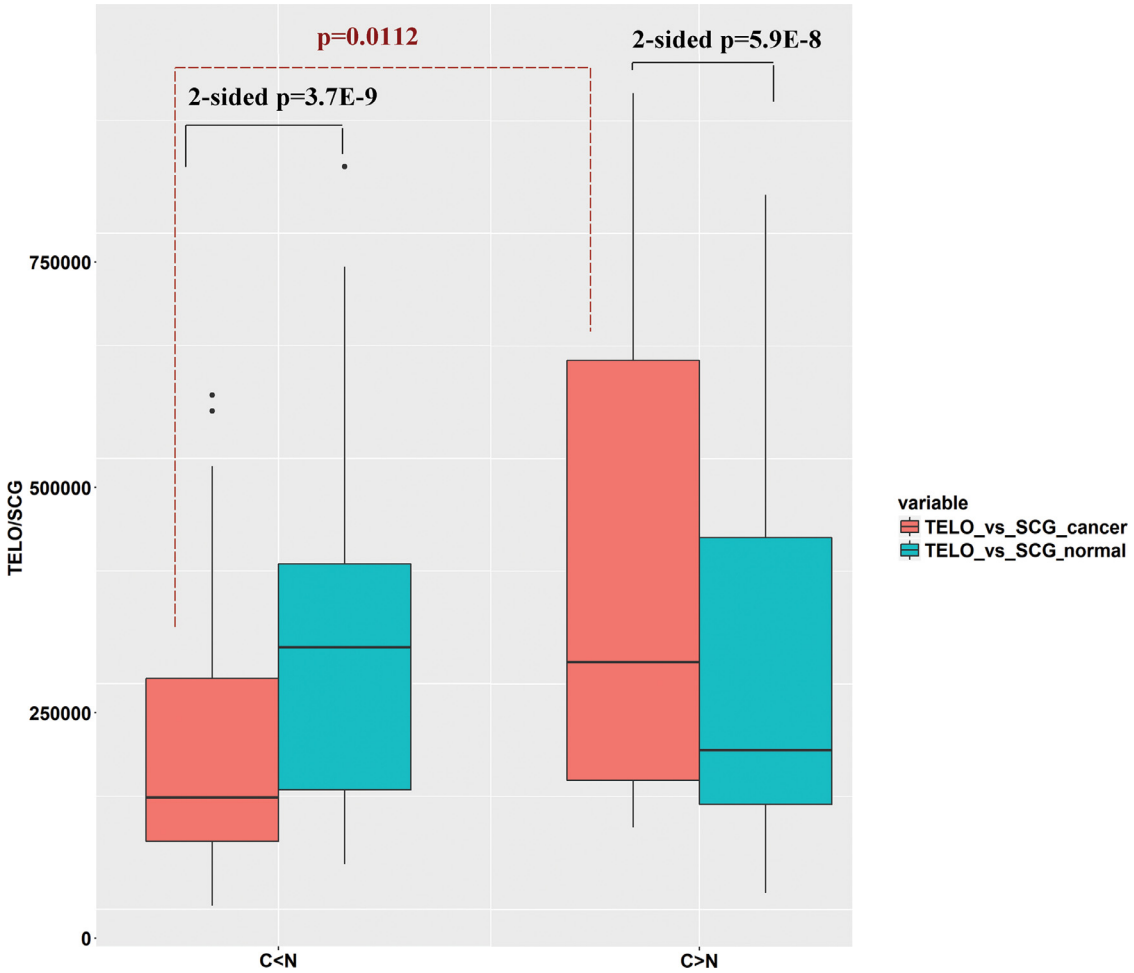

Fig. 5. The boxplot shows the mean of TL in cancer and in normal tissue, in group I (shorter telomeres in cancer than in control normal tissues C < N) and in group II (equal or longer telomeres in cancer than normal tissues C > N).

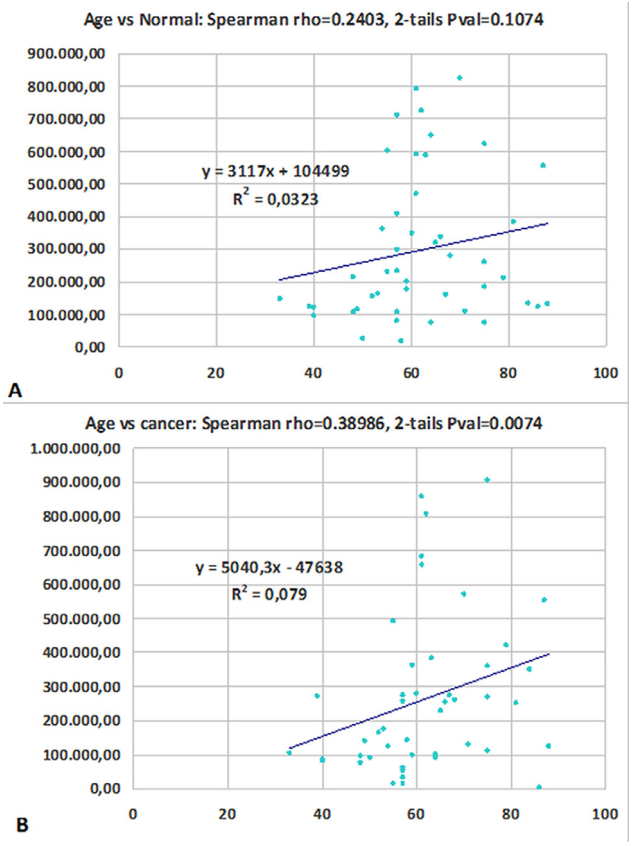

Fig. 6. Scatter plot shows a Spearman's correlation between TL in normal (A) and cancer (B) tissues and age. As regards normal tissues this correlation is not significant.

compared telomere length in the healthy and tumor mucosa tissues among the two sample groups. Interestingly, we found that telomeres in the tumor mucosa of patients of group C < N, were significantly ($p = 0.0112$) shorter (mean TL = 182,333 kb) than tumor telomeres of group $C>N$ (mean TL = 383,659 kb). Moreover, telomeres in the healthy mucosa of patients of group C > N were shorter, even though not significantly, (mean TL = 270,044 kb) than telomeres of C < N patient healthy mucosa (mean TL = 314,690 kb) (Fig. 5).

Association between telomere length and clinicopathological parameters

We next examined several variables (such as age, sex, tissue localization) for potential associations with telomere-length changes in both healthy and tumoral tissues. Age was the first parameter we took into consideration. Our results showed an increase in telomere length with age in tumor tissues (Spearman correlation rho = 0.38 $p = 0.0074$) (Fig. 6B) and a similar trend in normal tissue too, although in this case it is not significant (Spearman correlation rho = 0.24 $p = 0.1074$) (Fig. 6A).

To analyze in depth how telomere length varies according to age, we plotted, for each age x, the TL averaged across either all age $<x$ or all age $>x$ values, both in normal and in cancer tissue (Fig. 7A).

We can observe that between about 55 and 63 years of age, there is a sharp transition in all the average lengths. The critical age value corresponds approximately to the median age of patients (60.5), thus we divided the control and cancer samples into two groups: above and below median age. Both in cancer and normal tissues there a significant difference in TL between the two ranges of age (Fig. 7B)

We then investigated TL distribution in cancer and normal tissues as a function of the sex. Since male samples did not show a Gaussian distribution (not shown), statistical analysis was performed applying the Mann–Whitney test. Females showed statistically significant shorter telomeres than males in healthy tissues (177,108 kb versus 283,522 kb; $p = 0.047$), but not in cancer ones. Instead, the TL difference between normal and cancer was not significant into each sex class (Fig. 8).

As for tumor localization, telomere length in different cancer tissues did not show any statistically significant difference, even if the mean TL in tumor tissues was shorter in colon (201,685 kb) than in rectal (252,498 kb), stomach (268,980 kb) and esophageal cancer (281,034 kb) (Fig. 9). Moreover, in normal samples we did not find any significant difference in telomere length among tissues. In normal mucosa, the mean telomere length was as follows: 224,563 kb in the colon, 325,321 kb in the rectum, 265,597 kb in the stomach and 290,997 kb in the esophagus. Rectum seemed to be the tissue where there was the biggest difference in TL between normal and cancer, but this difference was not significant.

Next, we investigated if there existed any significant correlation between TL in normal and cancer, in the different tumor stages, and we did not find any evidence for such a correlation (Fig. 10).

Instead, Spearman's correlation showed that telomere length was correlated between normal and cancer tissue in stage II and IV (Fig. 11A, B), where an increase of TL in cancer tissues corresponds to an increase in normal ones. The same analysis could not be done for stage I and III because of the scarcity of patients at these stages. However, we grouped stage I, II and III, since these stages did not show any metastases, contrary to stage IV. Taken together, stages I, II and III, showed correlation ($p = 0.029$) of TL between normal and cancer tissues since when TL increases in healthy tissue, it increases also in tumor (Fig. 11C). Moreover, in tumor tissues, at the transition from one tumor stage to another, significant differences were observed in TL, which increased from stage I to II, then decreased from stage II to III and again increased from stage III to IV. These differences were significant according to the Student's *t*test (Table 3), whereas, according to Mann–Whitney test, the difference between stage III and IV was not significant.

Discussion

The role of telomere shortening in carcinogenesis is a paradox; telomere erosion is considered a "mitotic clock" as it limits the replicative potential of cells and it acts as a barrier against carcinogenesis. However, telomere shortening is also an early event of carcinogenesis and it contributes to tumor transformation via increasing chromosomal instability, which plays a key role in tumor initiation [14]. In the stage of cellular crisis, characterized by chromosomal instability, it is possible that telomerase, the enzyme responsible for telomere lengthening, is reactivated, inducing cell immortalization and leading to tumor progression [15]. Telomere length analysis has emerged as a molecular feature to understand the implication of telomere shortening in cancer progression [16], particularly in patients with colorectal tumors [8,17].

Based on the above considerations, to add new and original contribution to this issue, we studied telomere length in cancer tissues and in the normal adjacent mucosa from different tracts of the digestive system, in order to investigate if and how it impacts carcinogenesis process. Moreover, we analyzed the correlation between telomere length and some clinicopathological characteristics. Unlike the majority of similar studies that used qRT-PCR [12] to calculate telomere length in tumor tissue against a control, healthy tissue [18], we developed an adaptation of the basic qPCR-based technique developed by O'Callaghan and Fenech [13]. This

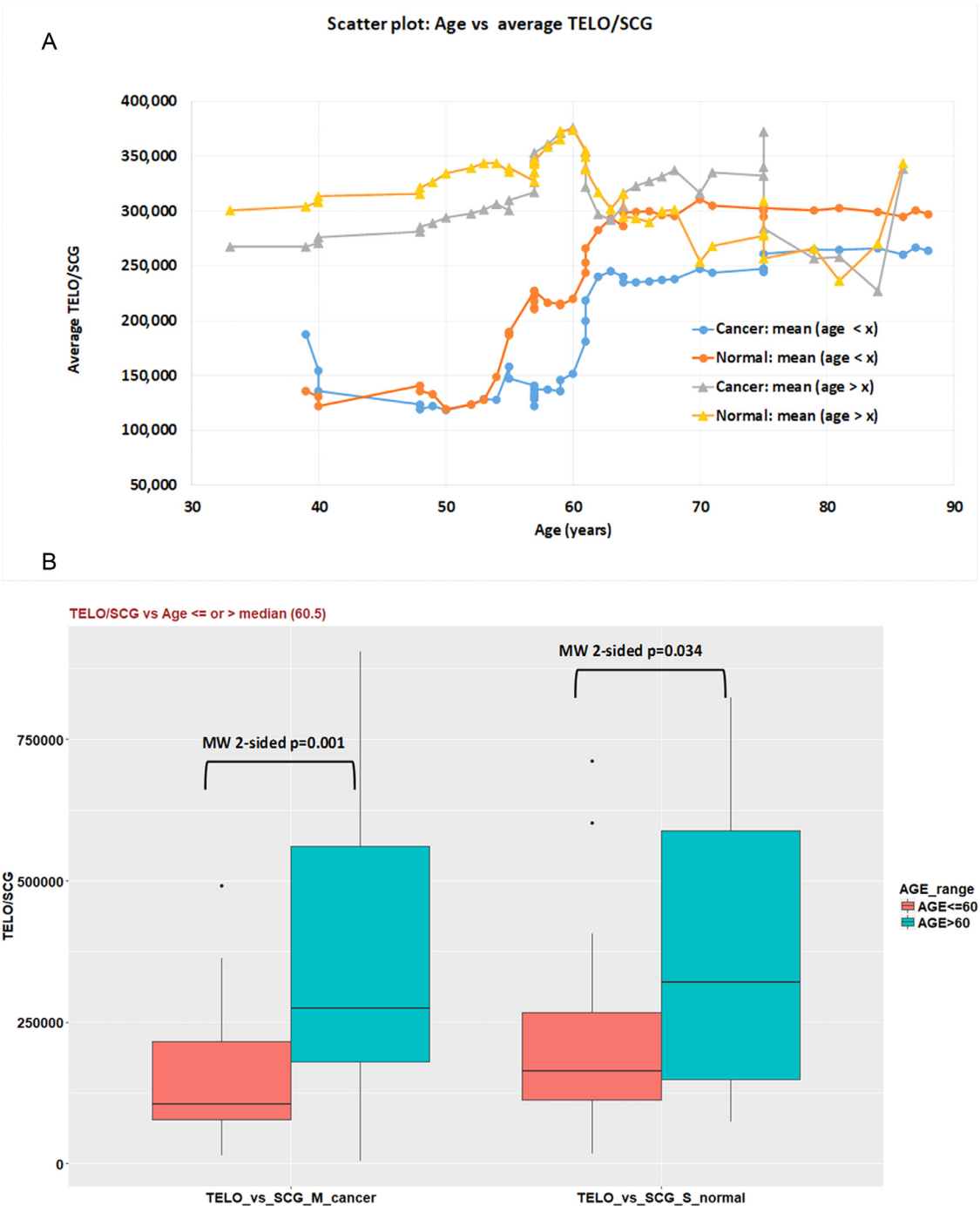

Fig. 7. (A) The scatter plot shows average TL trend according to age. (B) Difference in TL according to age.

Table 3
Correlation between telomere length and tumor stages.

Stages	*p*-valueStages		
	Shapiro normality test	*T* test	Mann–Whitney
T1	*p*-value = 0.3121	T1 < T2, *p*-value = 0.005892	T1 < T2, *p*-value = 0.007272
T2	*p*-value = 0.02699	T2 > T3, *p*-value = 0.01203	T2 > T3, *p*-value = 0.02587
T3	*p*-value = 0.106	T3 < T4, *p*-value = 0.03796	T3 < T4, *p*-value = 0.104
T4	*p*-value = 0.03032		

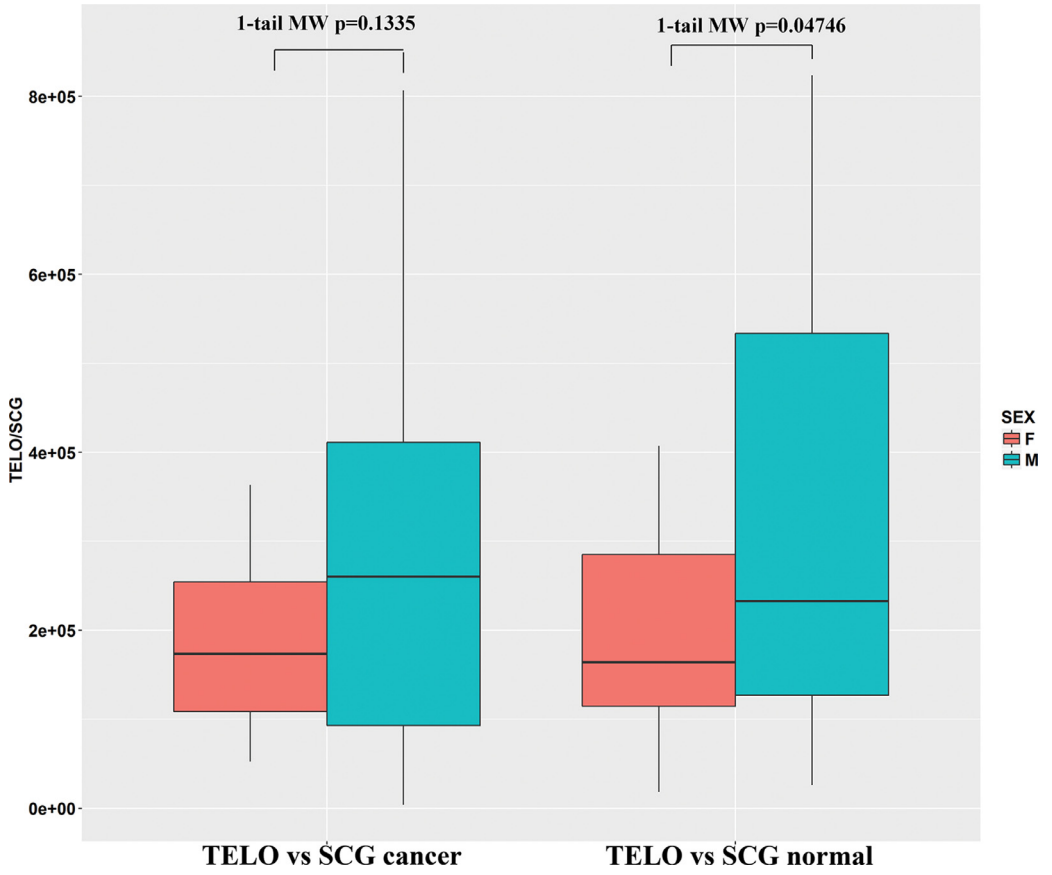

Fig. 8. The boxplot shows the mean telomere length in cancer and in normal tissues, in females and males.

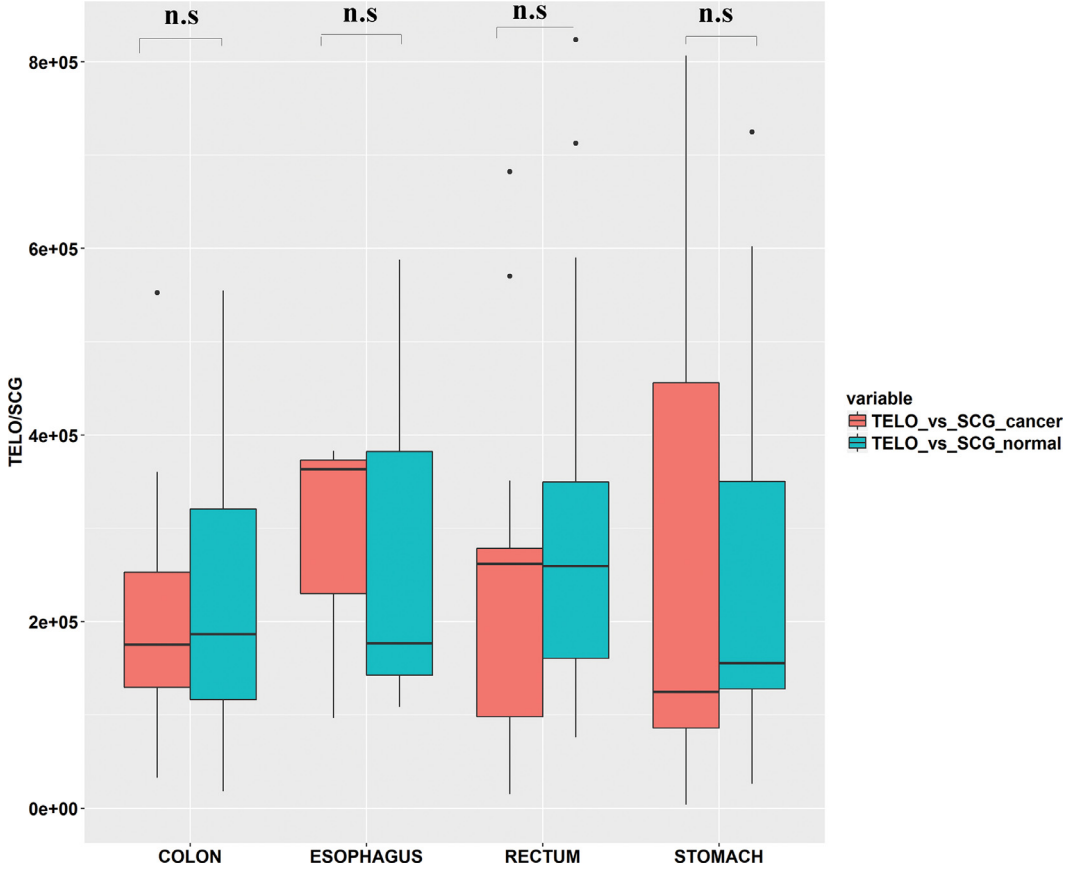

Fig. 9. The boxplot shows mean TL in cancer *vs* normal mucosa, in different tissues.

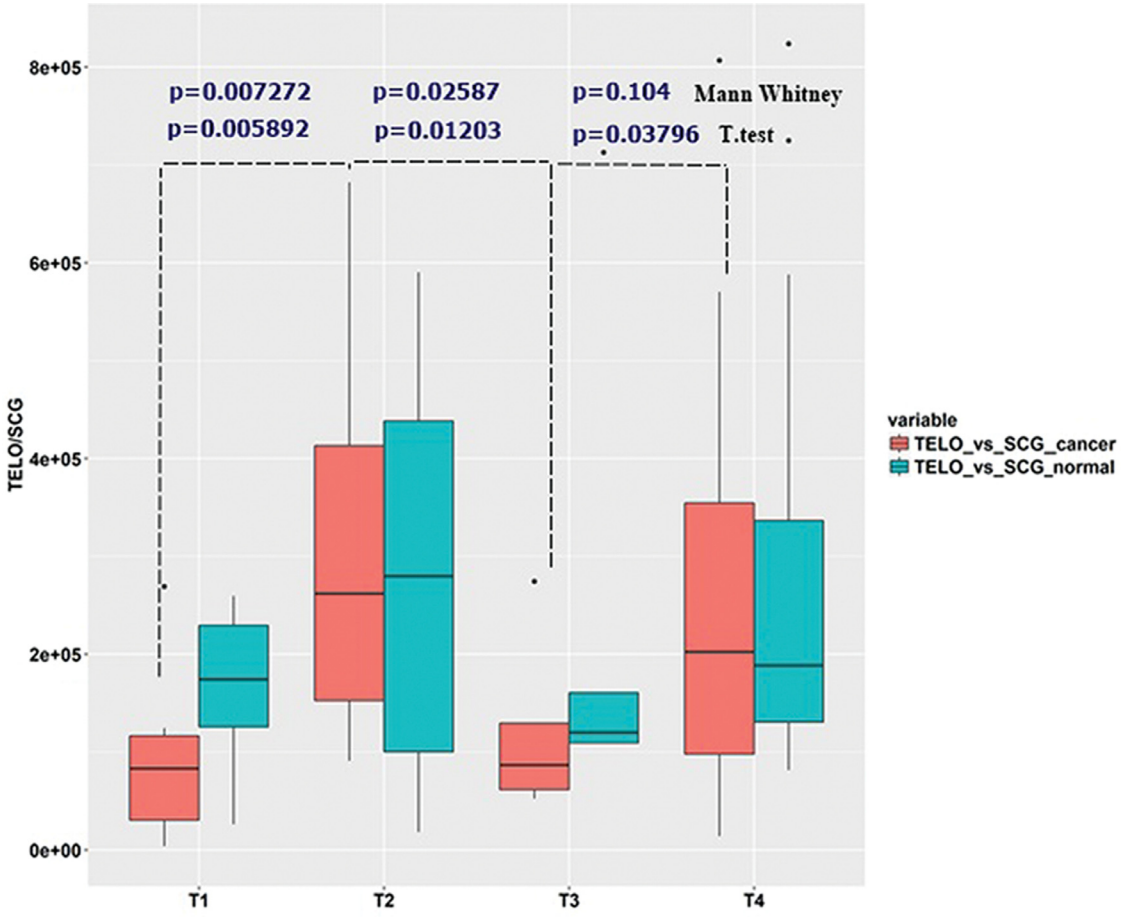

Fig. 10. The boxplot shows the mean of TL in cancer *vs* in normal, in different stages.

method allowed us to calculate the aTL according to the number of copies of a housekeeping gene.

Notably, we used tumor-adjacent healthy tissue as control, instead of peripheral blood leukocytes (PBL) [18]. Usually, telomere length studies are performed on PBL because blood samples are more readily available than biopsies. However, using PBL as control may present objective limits because some studies proved that telomerase is expressed in hematopoietic cells to ensure immortality [19]. Moreover, the number of cell divisions changes accordingly to tissue type [20]; therefore, even in the same individual, the telomere length is variable in different tissues [21,22]. Thus, the appropriate control for solid tumors should be collected from the adjacent healthy tissue, close to the tumor site, which is more difficult to obtain than blood samples. Additionally, as it will be discussed later, in PBL, telomere length decreases with age [23–25], while in normal colon from healthy people, telomere length declines with age until 60–70 and increases in the oldest individuals [26]. In each individual subject, leukocyte telomere length would eventually be an important indicator of aging status [23,24,27–29] without any correlation with cancer onset wherever. Comparing paired tumor tissues with the adjacent healthy ones, we avoid confusing information on TL due to aging and/or to the tissue-specificity; therefore, differences in Telomere Length will concern just the tumoral process. Many studies suggest that patient-matched tumors and adjacent normal tissue are in general more informative, even if, the main difficulty is to collect clearly identified normal samples due to proximity to tumor tissues [30,31].

Despite the number of researches dealing with this topic, a well-defined theory regarding the role of telomere length in cancer was not yet postulated. Several studies showed that telomere length decreased in cancer [4,8] even if this result is controversial [26,32]. Individuals with short telomeres can be at increased risk for cancer, since short telomeres lead to genomic instability – a hallmark of cancer. However, individuals with long telomeres also display an increased risk for major cancers, thus creating a cancer-telomere length (TL) paradox [7].

In the present research, our data showed that telomere length did not significantly differ in tumor *vs* normal tissues, even though, on average, telomeres are slightly shorter in the tumors.

We could divide our patients into two groups: those with shorter telomeres in tumor than in the adjacent healthy tissue ($C < N$) and those exhibiting equal or longer telomeres in tumor tissue than in control ($C \geq N$). Interestingly, it can be noted that shorter are telomeres in normal tissue of a patient, greater is the increase in telomere length in cancer. These findings are in agreement with what reported by Le Balc'h et al. [33], who measured telomere length in colorectal cancers using TRF assay and found that telomeres in the healthy control tissue of patients with longer telomeres in tumor were significantly shorter than their control counterparts. Our and Le Balc'h's results can be explained assuming that when telomeres are under a given threshold in healthy tissue then telomere expansion starts earlier, or more efficiently, in tumor cells. In many different studies it was reported that TL decreases with aging [8,34], nevertheless this is not true for all tissues. In PBL, telomere length decreases with age [23–25].

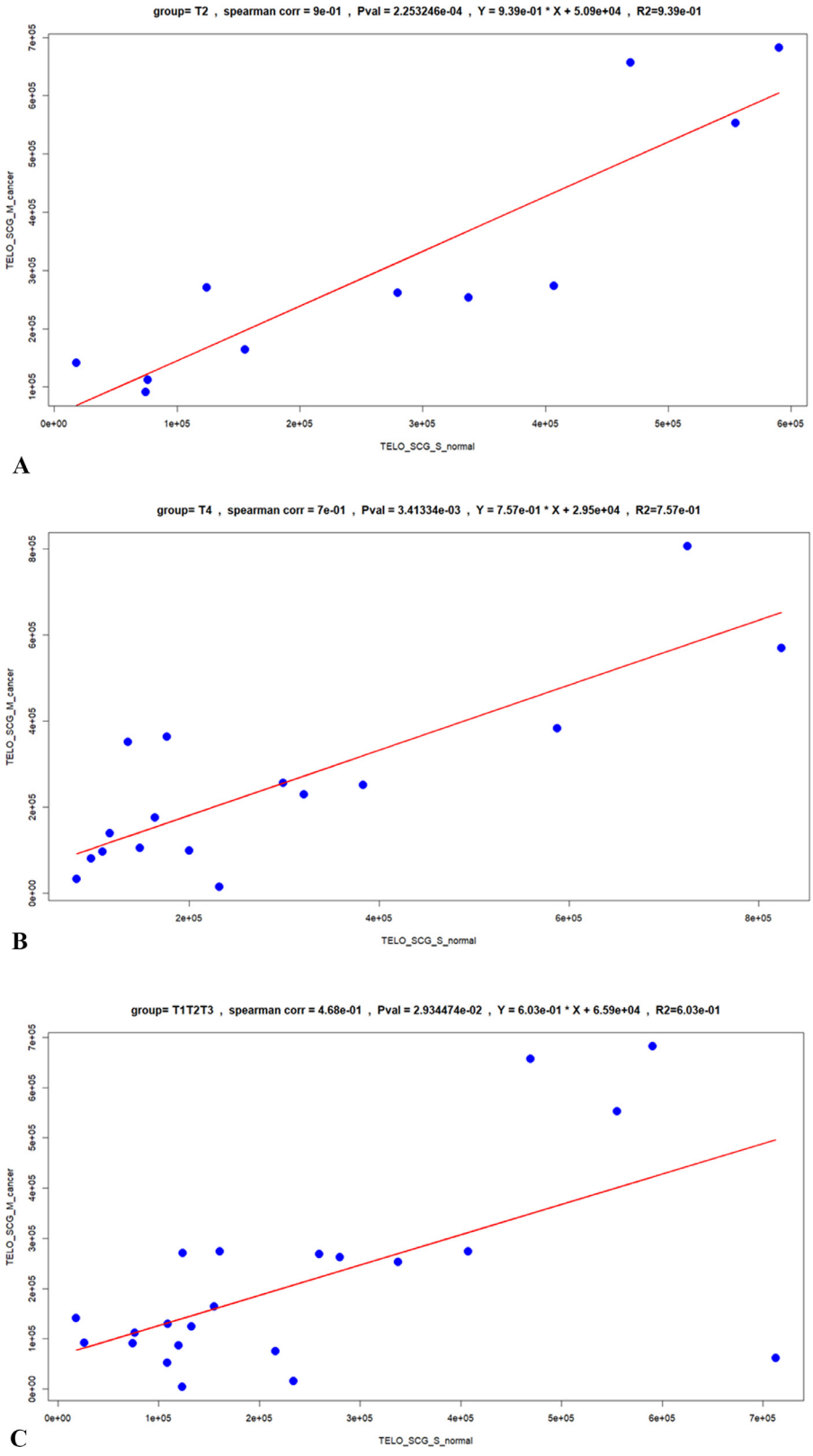

Fig. 11. Scatter plot shows Spearman's correlation between TL in cancer and in normal tissues at stage II (A), at stage IV (B) and at stages I, II, III grouped together (C).

However, O'Sullivan et al. [26] showed that, in normal colon from people with no history of colorectal malignancy, telomere length was inversely correlated with age until 60–70 years of age; but, surprisingly, in oldest people, telomere length was positively associated with age. In our study, we observed that around 60 years of age, in healthy tissue, there is a sharp increase in telomere length, and that in both normal and tumoral tissues, patients aged under 60 years have shorter telomeres than patients aged over 60 years. The positive correlation between telomere length and age in the oldest patients is possibly a consequence of selective

survival of elderly patients with long telomeres in colonocytes. Another explanation, not necessarily alternative, of this phenomenon could be that the telomere expansion mechanisms become more efficient with time. Several studies showed a link between short telomere length and various type of cancer such as bladder cancer [35–37], renal cell carcinoma [36,38], non-Hodgkin lymphome [39], lung cancer [40], and head and neck tumors [36], but a significant correlations between short telomere length and colorectal and breast cancer was not observed [41,42]. Since the risk of getting colorectal cancer progressively increases from the age of 50 [43], and since we found the increase of telomere length in healthy tissues, in people aged from 60 years, we can speculate that telomere elongation in normal tissue may be a risk factor for cancer development. Moreover, colorectal cancer risk is greater in males than in females[43] and we found a significant difference between the two sexes, with males having longer telomeres than females, which may support the hypothesis that telomere elongation in the healthy mucosa of digestive organs is a risk factor for gastrointestinal cancer development.

Even if it is widely believed that females have longer telomeres than males [44,45], results from different studies were contradictory [46,47]. O'Sullivan et al. [26] observed that, in the 80–90 age group, males show longer telomeres than females, and this is consistent with our data, although we had few samples within this age range. Moreover, while our results showed a statistically significant difference between males and females, with shorter telomeres in females, the difference in telomere length between normal and cancer tissues is not significant into each sex class.

To assess if the telomere length depends on tumor localization, we analyzed four different tissues from the gastrointestinal system: oesophagus, stomach, colon and rectum. In none of these four tumor tissues we found a correlation with telomere length. In healthy samples we did not find any significant difference in telomere lengths among tissues

To evaluate the timing of telomere shortening/elongation, we studied the correlation of telomere length among different stages of cancer. Some studies found that telomere length does not significantly differ with tumor stage [4,48]. Others, instead, reported a strong correlation between telomere length and cancer stage, being telomeres in early-stage cancers significantly shorter than those of advanced tumors [49,50]. In our study, we observed a significant homogeneity in the distribution of telomere length among patients in stages 1 and 3 (as shown by Shapiro–Wilk normality test) which include the patients with the shortest telomeres. Instead, we observed that stages 2 and 4, those which exhibit the longest telomeres, were heterogenous not showing a normal distribution, (Shapiro test: $p = 0.026$ and $p = 0.03$). For T2, the telomere elongation was significant as compared to T1 and T3. Concerning T4, the great heterogeneity in telomere length of this group of patients, could be explained by the great variability of metastasis process. Further enlarged studies, based on T4 stratification, will be necessary to understand the telomere length heterogeneity causes in metastasis group. Interestingly, we found a positive correlation between normal and cancer tissues in both non-metastatic stages I, II, III and in the metastatic stage IV. This correlation suggests the need of further studies to assess the molecular rationale of the coordinated stage-related telomere length variability in tumors and contiguous healthy tissues.

Summarizing, telomeres shorten at stage 1 reaching a critical value (telomere lengths are homogeneous and all patients have the same critical value); as cells enter crisis, telomeres start elongation (stage 2). Then cells quickly divide until they reach the Hayflick limit again, therefore telomeres become short once more (homogeneous telomere at stage 3); finally, telomeres lengthen another time at stage 4.

In conclusion, among different stages of cancer, from stage 1 to 4, we could observe an alternation between shortening and lengthening which may lead us to hypothesize the action of different biological processes. Further studies are necessary in order to conclude if a telomerase is acting at stages 1 and 3 and/or the ALT is active at stages 2 and 4. Even though based on a limited number of cases and deserving further studies, our results suggest that the understanding of telomere length behavior during tumor progression might be a future perspective for a valuable prognostic biomarker.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cancergen.2019.07.007.

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