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The comparison of telomere length in cancer patients: Plasma, whole blood and tumor tissue

Mine Urfali[1], Fatma Silan[2], Furkan Erturk Urfali[3], Atila Gurgen[4], Ozturk Ozdemir[2]

[1]Kutahya Health Sciences University, Faculty of Medicine, Department of Medical Genetic , Kutahya, Turkey
[2]Canakkale 18 March University, Faculty of Medicine, Department of Medical Genetic,Canakkale, Turkey
[3]Kutahya Health Sciences University, Faculty of Medicine, Department of Interventinal Radiology, Kutahya, Turkey
[4]Ordu State Hospital, Department of Psychiatry, Kutahya, Turkey

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Abstract

Telomer dysfunction triggers numerical and structural chromosomal instability and initiates tumorigenesis. Classical biopsies provide information about parts of tumor tissue, but cancer is divided into subgroups according to its mutational and behavioral characteristics. In this study, we aimed to investigate whether the results of cellfree-DNA were compatible with those obtained from tissues and to investigate whether cellfree-DNA telomere length is an alternative non-invasive method for the diagnosis of cancer. This study included the Q-PCR telomere measurement of tumor tissue, peripheral blood and plasma samples in patients with various cancers and peripheral blood and plasma samples of a control group. The telomeric DNA length and T/S ratios were calculated using the T/S ratio ($2^{-\Delta\Delta Ct)}$ formula. The median value for the plasma relative T/S ratio of the cancer group was statistically significantly higher than control group. In the cancer group, the lowest relative T/S ratio was found in plasma samples. The mean T/S ratio of whole blood was higher than tumor tissue, and similarly the relative T/S ratio of tumor tissue was higher than plasma T/S ratio(whole blood>tumor tissue>cfDNA). In cancer patients, the longer telomere length suggests that plasma cellfree-DNA telomere length could be a new molecular marker in cancer diagnosis and follow-up.

Keywords: Cell-free DNA, cancer, qPCR, telomere length

Introduction

Telomeres are nucleic complex structures responsible for genomic stability and replicative potential, consisting of guanine-rich DNA sequences located at the ends of eukaryotic cell chromosomes. The functions of telomeres are basically to prevent cancer development and replicative senesence [1]. Telomer dysfunction results in malignant cell transformation due to aging and genomic instability [2]. As a result of telomeric DNA erosion or dysfunction of telomeric proteins, DNA damage response (DDR) pathways are activated and telomeres are repaired [3]. Persistent DDR stimulates replicative senescence as a tumor-suppressing feature. If cells with various genetic defects (e.g., p53 and pRb defects) continue to divide despite aging, telomere dysfunction leads to chromosome fusion and fractures occur. This is the period in which telomere dysfunction determines procancer genotype formation [4]. Telomer dysfunction results in numerical and structural chromosomal instability, which is common in cancers. There is an increasing number of studies on the use of telomere length as a biomarker for cellular damage and aging. The relationship between telomere length with cancer risk, development and prognosis was determined in previous studies [5-7].

Cell-free DNA (cfDNA) refers to small nucleic acid particles in circulation resulting from apoptosis, necrosis, and active secretion [8]. Plasma concentration significantly increases in pregnancy, cancer, and some other diseases [9]. The results of many studies support the idea that cfDNA can be used as an alternative biomarker for early diagnosis, prediction of prognosis, treatment selection, and follow-up [10]. Specific mutations, methylation and microsatellite differences can be investigated based on cfDNA determined using blood samples [9].

Circulating tumor DNA(ctDNA) and circulating tumor cells (CTC) have been reported to be homogeneous molecular markers of tumor tissue and metastasis [11]. Cancer with many different somatic mutations can show clonal evolution during treatment

***Corresponding Author:** Mine Urfali, Kutahya Health Sciences University, Faculty of Medicine, Department of Medical Genetic , Kutahya, Turkey
E-mail: mine.urfali@ksbu.edu.tr

[12]. Murtaza et al. stated that cfDNA reflected this clonal evolution better than tumor tissue [13]. Thus, it has been reported that ctDNA can be used to monitor the clonal evolution of tumor tissue real time and to demonstrate the mechanism by which resistance is formed to treatment.

The aim of the current study was to compare the telomere length of cfDNA with the length determined in peripheral blood and solid tumor tissues and to investigate whether circulating cfDNA could be an alternative non-invasive method for the genetic studies of tumor tissue in the early diagnosis and treatment follow-up of cancer.

Materials and Methods

The study included patients that presented to Çanakkale State Hospital for the diagnosis or treatment of cancer between January 2016 and November 2016. Forty patients diagnosed with pathological cancer, who had tumor samples available and agreed to participate in the study, were included in the sample. Twenty people without cancer were evaluated as the control group. All participants were informed about the study and signed a consent form. Ethical approval was obtained from the Clinical Ethics Committee of Çanakkale 18 Mart University (number: EK-2014-102).

Telomeric repeat sequences were compared between three types of biological samples: whole blood, plasma, and tumor tissue. Peripheral blood samples were taken into tubes containing ethylenediaminetetraacetic acid(EDTA) and centrifuged at 3,800 rpm for 10 minutes immediately after sampling and divided into plasma and blood products. The plasma and blood samples were transferred to separate cryo tubes and stored at -20 °C for later use. Tissue samples were obtained from the remaining material of macroscopic tumor tissue after samples were taken for a pathological analysis.

CfDNA and genomic DNA were extracted from 200 µL serum and whole blood sample, respectively using the High Pure PCR template preparation (Roche) DNA kit according to the manufacturer's protocol and eluted in 25 µL of elution buffer. Tumor was extracted from the tissue samples using the Qiagen tissue kit according to the manufacturer's protocol. All DNA samples were diluted to the 5-30ng/µl concentration and stored at -80 °C until use. The telomere length of each DNA sample was measured as the ratio of telomere repeat copy number to single copy gene relative to a standard pooled DNA using quantitative PCR according to a protocol described by Cawthon [14,15].

We used primer sets previously designed by Richard Cawthon in our study. Primers were synthesized by standard HPLC method and delivered dry. (ThermoFisher-scientific). For primers diluated 10 pmol / µl for PCR reaction. The primers used in telomere PCR were as follows: forward telomere primer; Tel1; 5'-GGTTTT TGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT-3' reverse telomere primer; Tel2; 5'-TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA -3' forward human Beta globulin primer; HBG1;5'-GCTTCTGACACAACTGTGTTCACTAGC-3' reverse human Beta globulin primer; and HBG2;5'-CACCAACTTCATCCACGTTCACC-3'. Real-time PCR was performed using LightCycler2.0 (Roche, Manheim, Germany). PCR reaction (10µL) for the telomere or betaglobulin amplification consisted of LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany) (ref12239264001), $MgCl_2$ (final concentration 2mM), 200 nmol/L telomere or 300 nmol/L betaglobulin specific primers, and 30 ng DNA sample. The thermal cycling conditions for telomere amplification and followed melting curve analysis were 95 °C for 10 min followed by 25 cycles of 95 °C for 10 s, 58 °C for 60 s, 72 °C for 10 s, and one cycle of 95 °C for 5 s, 40 °C for 60 s, and 97 °C for the continue mode. The thermal cycling conditions for betaglobulin amplification and followed melting curve analysis were 95 °C for 10 min followed by 35 cycles of 95 °C for 10 s, 56 °C for 15 s, 72 °C for 10 s, and one cycle of 95 °C for 5 s, 40 °C for 60 s, and 97 °C for the continue mode. For quality control and calibration of PCR efficiency, each sample was run in duplicate and the same calibrator sample (human genomic DNA, Roche, USA) (lot10423225) was used on each plate. The melting curve analysis was performed after every reaction to verify the specificity and identity of the PCR products. All samples had a standard deviation of less than 0.5 for the threshold cycle (Ct) values. The Ct values and melting curve analysis were automatically generated by the LightCycler analysis software. The coefficients of variation (CVs) for the Ct values of the whole telomere, betaglobulin and T/S ratio were <4% for each group. The Ct values were used the calculate the T/S ratio for each sample using the following formulas: T/S ratio = $[2^{Ct(telomere)} / 2^{Ct(beta\ globin)}]^{-1} = [2^{[Ct(telomere)-Ct(beta\ globin)]}]^{-1} = 2^{-\Delta Ct}$, $\Delta Ct = [Ct(telomere)-Ct(beta\ globin)]$; relative T/S ratio = $2^{-(\Delta Ct1-\Delta Ct2)} = 2^{-\Delta\Delta Ct}$; and $\Delta\Delta Ct = \Delta CtT - \Delta Ct_{calibrator}$

For primary dimerization and non-specific binding, melting curve analysis was performed. Telomere and beta globilin PCR products were analyzed in channel 530 at 84 °C and 86 °C Tm respectively.

Statistical analysis

Statistical analysis of the data was performed using SPSS (version 21.0; IBM Corporation, Armonk, NY). The conformity of the data to normal distribution was evaluated with the Shapiro-Wilk test. The Mann Whitney-U test was used for the analysis of non-normally distributed numerical data, respectively. The chi-square test was used for categorical data, and the relationship between numerical variables was evaluated by calculating Spearman's correlation coefficient. Then, the Wilcoxon test was conducted to determine the T/S ratio differences between the blood-plasma, blood-serum and plasma-serum samples. When P was <0.05, it was considered statistically significant. The results were given as mean ± standard deviation, minimum and maximum values.

Results

Quantitative measurement of telomere length

There was no statistically significant difference in age between the cancer and control groups (mean age; 59 ± 15 (33-88) and 52.8 ± 9.5 (36-67) years, respectively). The mean plasma relative T/S ratio was 0.45(0.02-3.17) ± 0.58 in the cancer group and 0.04(0.02-0.13) ± 0.03 in the control group, and it was observed to be statistically significantly higher in the patients with cancer ($p < 0.001$) (Table 1). The mean relative T/S ratio of the whole blood samples was 1.94(0.13-11.50) ± 1.96 for the cancer group and 1.92(0.30-4.21) ± 1.09 for the control group, indicating no

statistically significant difference (p: 0.380) (Table 1).

Features of cancer patients

Among the patients with cancer, the origin of the primary tumor was the breast in 40% (n: 16), colon in 40% (n: 16), stomach in 7.5% (n: 3), lung in 7.5% (n: 3), and rectum in 5% (n: 2). There was no statistically significant difference in the relative T/S ratios of the plasma (p:0.155), whole blood (p:0.119) and tissue (p:0.940) samples according to the cancer type (Figure 1).

Comparison of whole blood, tissue and plasma telomere lengths in the cancer group

In patients with cancer, the relative T/S ratio values of whole blood, tissue and plasma samples significantly differed ($p < 0.001$) (Table 2). There was a significant difference in the relative T/S ratios between the whole blood (1.94(0.13-11.50) ±1.96) and plasma samples (0.45 (0.02-3.17) ±0.58), with the mean value of the whole blood samples approximately 4.3 times higher than that of the plasma samples ($p < 0.001$). In addition, the mean relative T/S ratio of the whole blood samples (1.94(0.13-11.50) ±1.96) was 2.2 times greater than that of the tissue samples (0.85 (0.03-2.20)±0.48), and this was statistically significant (p: 0.001). In tissue samples (0.85 (0.03-2.20)±0.48), the mean relative T/S ratio was 1.8 times higher than that of plasma samples (0.45 (0.02-3.17)±0.58), and this was at a statistically significant level (p: 0.002) (Table 3).

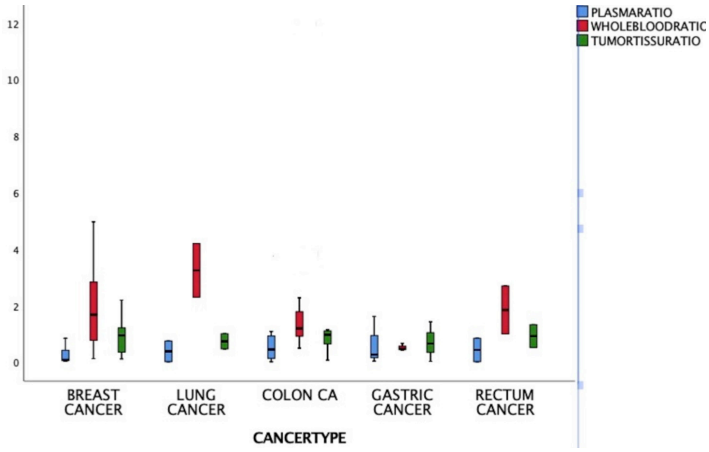

Figure 1: According to cancer type; comparison of telomere lengths of plasma, whole blood and tissue samples

Table 1. Comparison of whole blood and plasma telomere lengths between the cancer and control groups

	Cancer (n:40)	Control (n:20)	P
Plasma relative T/S ratio	0.45 (0.02-3.17) ± 0.58	0.04 (0.02-0.13) ± 0.03	<0.001
Whole blood relative T/S ratio	1.94 (0.13-11.50) ± 1.96	1.92 (0.30-4.21) ± 1.09	0.380
Mann-Whitney U test			

Table 2. Comparison of plasma, whole blood and tumor tissue telomere lengths in patients with cancer

	Mean (Min-Max) ±SD	Chi-square test
Whole blood T/S ratio	1.94(0.13-11.50) ±1.96 n:40	30.821 P < 0.001
Tumor tissue T/S ratio	0.85 (0.03-2.20)±0.48 n:39	
Plasma T/S ratio	0.45 (0.02-3.17)±0.58 n:40	

Table 3. Binary comparison of plasma-whole blood-tissue telomere lengths in patients with cancer

	Z	P
Whole blood-plasma	-4.731	<0.001
Whole blood-tissue	-3.475	0.001
Tissue–plasma	-3.070	0.002
Wilcoxon testi		

Discussion

CfDNA, which is a new and ideal marker in the diagnosis and follow-up of cancer, has been shown to be effective for clinical use in liquid biopsies and it can also allow for the follow-up of patients with cancer. Several studies have reported that the concentration of cfDNA, which can be obtained from plasma, serum and other body fluids, is increased in patients with cancer [11,16–18]. However, a clear cut-off value has not yet been determined. In addition, mutation, methylation and microsatellite changes in tumor tissues are not specific to cancer alone [19], and telomeres may be superior to other variables in this area.

The most important advantage of this study, which also makes it superior to previous research, is the comparison of telomere lengths in tumor tissue, whole blood and cfDNA samples in patients with cancer and the analysis of both cfDNA and whole blood in controls. In our study, only blood and plasma telomere lengths were evaluated in case-control comparisons based on relative T/S ratio values. The plasma telomere length of the cancer group was significantly longer than that of the control group (Table 1). Similarly, Fu et al. [17] and Wan et al.[20], who evaluated HBV-infected patients, reported that serum telomere was longer in patients with hepatocellular cancer (HCC) than those without HCC. In these studies, the median and interval values were similar to our study. In our study, as a contribution to the literature, we also evaluated other types of cancer and performed a tumor tissue analysis. Although the number of samples was low and there were

different types of cancer, the similarity of the range and median values can be interpreted as telomere length showing a similar increase regardless of cancer type. In contrast, Shi et al. found that shortened serum cfDNA telomere was significantly associated with a higher risk of gastric cancer progression [21]. Further studies that analyze a large number of patients with each type of cancer may indicate whether telomere length can be helpful in determining the type of cancer.

Plasma cfDNA was not in normal chromosomal structure and fragmented, Wu et al [22] calculated the telomeric cfDNA by using the qPCR method using the SINE and LINE nucleotide sequences instead of the single copy gene as the reference gene. They found that telomere cfDNA was shorter in patients with breast cancer than in healthy controls. The different results obtained from our study can be attributed to the variation of the SINE and LINE repeats from person to person. Cawthon [14] similarly suggested that the quantification of cfDNA telomere length should be performed using a single copy gene, and that Alu repeat numbers might provide erroneous results since they varied from one patient to another.

In our study, there was no significant difference between the cancer and control groups in terms of the whole blood T/S ratio values (Table 1). The literature contains different views concerning the use of whole blood telomere length in the evaluation of cancer risk and prognosis. Some studies report that telomere shortening in blood may be a risk or prognostic factor for cancer [7,23] while others suggest that increased telomere length measured in peripheral blood may be a risk factor for cancer [24,25]. These conflicting views on telomere length in peripheral blood may be an indication that whole blood does not reflect cancer well. In our study, the selection of cancer and control groups from patients of similar age and gender was considered to be effective in the telomere length results.

In the current study, tissue telomere length was measured only in the cancer group, and it was not compared with the control group or healthy tissues of the patients with cancer. In our study, the longest telomere was observed in peripheral blood, followed by tumor tissue while the shortest telomere was in cfDNA obtained from serum (whole blood > tumor tissue >cfDNA) ($p < 0.001$). These values statistically significantly differed (Tables 2, 3). There was also no correlation or difference plasma/tissue/whole blood telomere length between the subtypes of cancer (Figure 1). In many studies, especially those evaluating colorectal cancer, it has been observed that telomere length in cancer tissues is shorter than normal tissues [5,26-28]. In a study comparing telomere length obtained from the tissue and blood samples of patients with colon cancer, Vals-Baınutista et al. determined that the tumor tissue samples indicated shorter telomere length compared to normal colon tissue and whole blood samples of the same patients. However, they determined that telomere length obtained from the normal colon tissue samples was shorter than that of the whole blood samples among the patients, which they attributed to inflammation. The authors also suggested that normal colon mucosa would not be suitable for use as a control [29].

We found plasma telomere length to be shorter than whole blood telomere length in both patients with cancer and the healthy controls. In a recent study, Zinkova et al. reported greater serum and plasma telomere lengths compared to whole blood in healthy volunteers [30]. In contrast to our study, the previous authors used the same amount of serum and plasma to determine the concentration of telomeric sequences due to different concentrations of DNA in these samples. In our study, we used the same amount of DNA; thus, we found relative telomere length in patients with cancer and controls.

In the current study, the shortest telomere length was observed in the plasma samples in patients with cancer. To the best of our knowledge, there is no previous study comparing tissue and cfDNA telomere length. Tumor tissue is not only composed of cancer cells but it also contains some blood vessels and connective tissue, and it only provides information about the biopsy area. However, in patients with cancer, cfDNA originates only from cancerous cells (including areas of possible metastasis) and necrotic-apoptotic cells throughout the body. This result shows that genetic analyses of cfDNA are more reliable and better reflect the cancer status of patients.

There is a need for further studies to analyze a large number of patients with different types of cancer in order to clarify whether cfDNA would be useful in determining cancer type, particularly among patients with tumors of unknown primary origin. The main limitation of our study was the limited number of participants, even though different samples of each participant were evaluated. In our study, although the tumor tissue telomere length of the patients with cancer was not compared with the control group, peripheral blood cells provided important data as healthy tissue since there was no bone marrow metastasis in our patients.

Conclusion

In our study, the plasma cfDNA telomere length of the cancer group was longer than that of the control group. Circulating telomere fragments in cell free DNA is significantly higher in cancer patients. Considering that these telomere products are originated from cancer cells in circulation, we anticipate that they can be used as a biomarker for diagnosis, evaluation of response to treatment and recurrence for cancer patients. Furthermore, in patients with cancer, plasma cfDNA telomere length was significantly shorter compared to the whole blood and tumor tissue samples, indicating that plasma cfDNA could be used as a cancer biomarker, and cfDNA analysis was more sensitive than tumor tissue analysis.

Conflict of interests
The authors declare that they have no competing interests.

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Ethical approval
Ethical approval was obtained from the Clinical Ethics Committee of Çanakkale 18 Mart University (number: EK-2014-102).

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