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The evaluation of telomere length and telomerase activity measurement in Neurofibromatosis type 1 NF1 tumors

Nörofibromatozis tip 1 (NF1) tümörlerinde telomer uzunluğu ve telomeraz aktivitesinin ölçülmesi ve değerlendirilmesi

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ABSTRACT

Objective: Neurofibromatosis type 1 (NF1) is an autosomal dominant disease that affects 1 in 2,500 people worldwide. The disease is developed due to the occurrence of mutations in NF1 gene. NF1 gene is coding cytoplasmic protein which is negative regulator of RAS proteins. The loss of neurofibromin results in activation of RAS cascade and cell proliferation. For this reason, NF1 gene is categorized as tumor suppressor gene. It is clinically characterized by cafe-au-lait spots, Lisch nodules, axillary and inguinal freckling, multiple peripheral nerve tumors, bone lesions, and a predisposition to malignancy. Variations in NF1 mutations may not correlate with the variations in clinical phenotype. This unclear genotype-phenotype correlations is assumed to be due to modifier genes. One of these modifier candidates is telomere length and telomerase activity. Telomeres are repetitive nucleotide sequences located at the ends of chromosomes and protect them from fraying and sticking to each other. The length of telomeres is shortening in each cell division. Nevertheless, this shortening can be prohibited

ÖZET

Amaç: Nörofibromatozis tip 1 (NF1), dünyada her 2.500 kişiden birini etkileyen otozomal dominant bir hastalıktır. Hastalık, NF1 genindeki mutasyonlar nedeniyle gelişir. NF1 geni, RAS proteinlerinin negatif regülatörü olan sitoplazmik bir proteini kodlamaktadır. Nörofibromin kaybı, RAS kaskadının aktivasyonuna ve hücre çoğalmasına neden olur. Bu nedenle NF1 geni, tümör baskılayıcı gen olarak kategorize edilir. Klinik olarak cafe-au-lait lekeleri, Lisch nodülleri, koltuk altı ve kasık çilleri, çoklu periferik sinir tümörleri, kemik lezyonları ve maliniteye yatkınlık ile karakterizedir. NF1 mutasyonlarındaki varyasyonlar, klinik fenotipteki varyasyonlarla ilişkili olmayabilir. Bu genotip-fenotip korelasyon yokluğunun modifiye edici genlerden kaynaklandığı varsayılmaktadır. Modifiye edici gen adaylarından biri de telomer uzunluğu ve telomeraz enzim aktivitesidir. Telomerler, kromozomların uçlarında yer alan ve onları yıpranmaya ve birbirine yapışmaya karşı koruyan tekrarlayan nükleotid dizileridir. Her hücre bölünmesinde telomerlerin uzunluğu kısalır ve bu kısalma, telomerlerin 3' ucuna

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by the enzyme telomerase which adds a species-dependent telomere repeat sequence to the 3' end of telomeres. However, telomerase activity usually diminished after birth in somatic cells. Researches done in last years have shown the importance of telomere and telomerase activity and they are causally connected to human disease. However, the number of research on this concept for NF1 patients is very few.

Methods: The DNA and proteins isolated from tumors of nine NF1 patients was analyzed by quantitative PCR based technics. Telomere length measurement were done using the DNA samples. The pathological status of tumor tissues was confirmed by routine pathological examination. Telomerase activity were evaluated from proteins isolated from acquired tumor samples.

Results: Considering the preliminary results, higher telomerase activity is measured in some NF1 tumors and also variations in telomere size were detected.

Conclusion: These primary data indicate that telomere length may play an important role in NF1-associated tumor's progression and could provide information about the telomere-targeted therapeutic approaches for treatment of telomere dysfunction in the clinic.

Key Words: Neurofibromatosis type 1, modifier genes, telomere length, telomerase activity

telomer tekrar dizisi ekleyen telomeraz enzimi tarafından engellenebilir. Bununla birlikte, somatik hücrelerde telomeraz aktivitesi genellikle doğumdan sonra azalır. Son yıllarda yapılan araştırmalar, telomer ve telomeraz aktivitesinin önemini ve hastalıklarla ilişkili olduğunu göstermiştir. Ancak NF1 hastaları için bu kavramla ilgili araştırmalar son yıllarda başlamış olup sayısı çok azdır.

Yöntem: Dokuz NF1 hastasının tümör dokularından elde edilen DNA ve protein örnekleri kantitatif PCR tabanlı tekniklerle analiz edildi. Tümör dokularının patolojik durumu, rutin patolojik inceleme ile doğrulandı. DNA örnekleri kullanılarak telomer uzunlukları ölçüldü. Telomeraz aktivitesi, elde edilen tümör örneklerinden izole edilen proteinlerden değerlendirildi.

Bulgular: İlk sonuçlara bakıldığında, bazı NF1 tümörlerinde daha yüksek telomeraz aktivitesi ölçülmüş ve ayrıca telomer boyunda da varyasyonlar tespit edilmiştir.

Sonuç: Bu öncül veriler, telomer uzunluğunun NF1 ile ilişkili tümörlerin ilerleme sürecinde önemli rol oynayabileceğini göstermiştir. Bu durum klinikte telomer işlev bozukluğunun tedavisi için telomer hedefli terapötik yaklaşımlar için de önemlidir.

Anahtar Kelimeler: Nörofibromatozis tip 1, modifiye edici genler, telomer uzunluğu, telomeraz aktivitesi

INTRODUCTION

Neurofibromatosis type 1 (NF1), also known as von Recklinghausen's disease, is manifested by an autosomal dominant clusters, numerous café au lait spots on the skin, freckles, and large-small, tumors called neurofibromas. Sometimes, tumors can develop in bone, such as scoliosis, in the brain, cranial nerves and spinal cord (1).

The disease occurs due to the mutations in the NF1 gene in the 17q11.2 region of chromosome 17. The NF1 gene is one of the largest genes in the human genome with a length of 350 kilobases and encodes mRNA containing 60 exons of 11-13 kb. Some of its exons (such as 9a, 23a, 48a) are formed by alternative splicing. The NF1 gene has been very conserved throughout evolution. There is 98% similarity between human and mouse and 68% between human and *Drosophila*, indicating that it encodes an important protein for living organism. Intron 27b contains three functional genes, named EVI2A, EVI2B and OMGP, which are transcribed in the opposite direction of the NF1 gene. Intron 39 also contains a pseudogene called AK3 (2).

Although the expression of the NF1 gene is common

in the body, its expression level is especially high in the nervous system. NF1 encodes the cytoplasmic protein neurofibromin. Neurofibromin is a protein with a molecular weight of 327 kD, consisting of 2818 amino acids. Neurofibromin isoforms are formed by alternative splicing in the NF1 gene. It is thought that these isoforms may play a role in the diversity of the clinical picture of NF1 (3).

The region of neurofibromin spanning exons 21-27a shows homology with the catalytic domain of guanosine triphosphatase activating proteins (GAP). The region of exon 21-27a is called the GAP-related region (GRD) (4,5).

NF1 disease clinically affects various organs and systems of the body, primarily the peripheral nervous system. Its main characteristic findings are neurofibroma formation, café au lait spots, axillary and inguinal freckles, lisch nodules, bone deformities, learning disabilities and a predisposition to tumor development. Neurofibromas, which cause clinical and aesthetic distress in patients, are benign tumors of the peripheral nerve sheath. These tumors are classified as cutaneous, subcutaneous and plexiform. Unlike the skin and subcutaneous neurofibromas, plexiform neurofibromas often enlarge and have the potential to become malignant. About 15% of NF1 patients develop malignant tumors. While the majority of these tumors involve the nervous system, some develop leukemia. There is no relationship between the mutations detected in NF1 patients and the phenotype of the disease. The clinic varies a lot; Even within the same family, individuals with the same mutation show a different clinical picture. An individual in our study group had a mild phenotype despite two different pathological mutations (5). Modifier genes are thought to be responsible for this clinical variability. The modifying effect of various candidate genes for clinical variations is being studied (6). Although it has been emphasized in recent years that telomere length may also be effective among these changes, these studies are few in number and need to be supported by new studies.

Telomeres are repetitive nucleotide sequences at the ends of linear chromosomes and play a critical role in the termination of chromosome replication. The structure of telomeres was first described by Hermann Müller (7) and Barbara McClintock (8) in studies with *Drosophila* and Zea mays. Muller emphasized that these structures in telomeres of chromosomes exist to maintain integrity, he used the term telomere for the first time, and then McClintock emphasized that these structures are important for chromosome stability. Elizabeth Blackburn, Carol Greider, and Jack Szostak received the Nobel Prize in 2006 for their work on the protection of chromosomes by telomeres and telomerases (9,10). Thanks to the intensive studies that took place after that, a lot of information has been gained about telomeres. Telomeres are non-coding "TTAGGG" repeats located at the ends of chromosomes (11). At the 3' ends, the G-rich single strand DNA folds over itself to form a loop structure, and this loop is called the "T-loop" (12,13). Due to the nature of DNA replication, telomeres shorten with each cell division and when it reaches a critical length, cells stop dividing (14).

In recent years, it has been emphasized that telomere length can be a powerful new cellular marker for the prognosis of various tumors, including leukemia and breast cancer. Jones et al. (15), showed that telomere length is important for MPNSTs (Malignant peripheral nerve sheath tumors) in NF1, but there is no information about how the situation is in plexiform and cutaneous neurofibroma. Moreover, this study is the only one showing the relationship between NF1 and telomeres and needs to be supported by further studies. The same group of researchers previously studied the status of telomerase enzyme in MPNST and raised the possibility that telomerase enzyme could be used as a biomarker (16). For these reasons, in this study we planned to search the status of telomere length in NF1-related tumors. We aimed to determine the amount of telomere lengths and telomerase activity, which may be important for NF1 clinic, to reveal the differences and its use as a possible tumor type-specific cellular marker (17).

MATERIAL and METHOD

Patients

In this study, the plexiform and cutaneous tumor specimens were surgically taken from nine patients under appropriate conditions and fresh frozen and stored under appropriate conditions. The healthy skin sample tissue of one patient was used as the control. The pathological status of tumor tissues was confirmed by routine pathological examination.

DNA Isolation

In this study, different tumor samples were taken surgically from NF1 patients; two dermal neurofibroma (dNF), four plexiform neurofibroma (pNF), two rhabdomyosarcoma samples (RMS) and one malignant peripheral nerve sheath tumor (MPNST). These samples were fresh frozen after surgery and kept in -80°C for later use. These fresh frozen tissues were first crushed in a ceramic mortar with the aid of liquid nitrogen. Then the sample pieces were collected in a 2 ml tube and homogenized by the help of TissueRupture II Homogenizer (Qiagen) device. The DNA isolation from these samples were performed using GeneJET Genomic DNA Purification Kit (Thermo Fisher, Cat no: K0721).

Telomere Length Measurement

Telomere length measurement were done by using the DNA samples. For the RT-PCR reaction of telomeres, primers of the ß-Globin gene (single gene copy; SCG) was used as the "housekeeping" gene, as described by O'Callaghan and his group, together with standard primers (17).

The name and the sequence of the primers (10 pmoles/µl) used for this purpose are given below:

Telo 1	5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3'
Telo 2	5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'
SCG 1	5'-GCTTCTGACACAACTGTGTTCACTAGC-3'
SCG 2	5'-CACCAACTTCATCCACGTTCACC-3'

The RT-PCR reaction for telomeres were prepared as follows: 0.2 µl *Telo* 1 primer, 1,8 µl *Telo* 2 primer, 10 µl Qiagen PCR mastermix, 2 µl DNA sample and 6 µl nuclease free water. The RT-PCR reaction for *B-globin* gene as housekeeping gene, were prepared as follows: 0.6 µl SCG 1 primer, 1,4 µl SCG 2 primer, 10 µl Qiagen PCR mastermix, 2 µl DNA sample and 6 µl nuclease free water. The reaction condition for both genes were as follows: 95°C for 10 minutes for denaturation, 95°C for 10 seconds, 60°C for 5 seconds and 72°C for 11 seconds. This was repeated for 45 cycles. The reaction was performed in Rotor Gene-Q Thermocycler (Qiagen, Maryland, USA). The comparison of gene expression results was analyzed by calculating $2^{-\Delta\Delta Ct}$ values.

Quantitative Telomerase Screening

In this study, protein isolation was performed from frozen tumor tissues. The samples were prepared as explained before using liquid nitrogen and homogenizer device (TissueRuptur II, Qiagen). Then, each sample was suspended in 200 µl of 1X lysis buffer and incubated on ice for 30 min. It was then centrifuged at 12,000 xg for 30 min at 4°C. The supernatant was transferred to a new tube. For protein concentration measurement, 160 µl is poured into 1.5 ml tube. The isolated proteins were stored at -80°C for later use. Before measuring telomerase activity, a small amount of each sample was taken in another 1.5 ml tube and inactivated by keeping at 85°C for 10 minutes and used as negative control group. As a positive control group the cell pellet included in the kit was used.

The samples for telomerase activity analysis was prepared according to datasheet of TRAPeze® Telomerase Detection Kit (Merck, Catalogue No: S7700) which is based on the TRAP assay (Telomeric Repeat Amplification Protocol). The reaction was performed in Rotor Gene-Q Thermocycler (Qiagen, Maryland, USA). Data analysis is performed by comparing the obtained cycle threshold values (Ct) with the standard curve and comparing the telomerase activity of the samples.

The study was approved by the Institutional Ethics Committee of TOBB University of Economy and Technology (Date: 29.02.2019 and Number: KAEK-118/032).

RESULTS

Telomere Length Measurement

For measuring telomere length, DNA isolation were performed from fresh frozen tumor samples of 9 different patients including 4 plexiform (pNF) samples, 2 dermal (dNF), 2 Rhabdomyosarcoma (RMS) and 1 MPNST tumor samples. The length of telomeres was measured by using *Telo* primers and compared to *B-globin* (*SCG*) gene primers as housekeeping gene. The result was compared to control group which is the healthy skin sample from one of the patients. The results of individual samples are given in Table1. The average mean of samples was shown in Figure 1.

Considering our preliminary results, plexiform neurofibroma have the lowest telomere length and MPST sample have the highest telomere length compare to control sample. The length of dermal plexiform and rhabdomyosarcoma tumors has been very close to each other. Moreover, variations in telomere size were detected. Comparing to the healthy DNA sample, although the telomere length of dermal neurofibromas are shortened, plexiform neurofibromas show smaller telomere length. However, the telomere length of MPNST is higher than healthy DNA sample. The list of samples and the mean values of telomere lengths calibrated to healthy DNA sample has been given in Table 1.

Table 1. Telomere length calibrated to control (accepted as 1)

Type of Tumor	Patient Code	Telomere Length
Dermal Neurofibroma	dNF-1	0,69
	dNF-2	0,57
Plexiform Neurofibroma	pNF-1	0,05
	pNF-2	0,04
	pNF-3	0,0001
	pNF-4	$6*10^{-8}$
Rhabdomyosarcoma	RMS-1	0,49
	RMS-2	0,11
MPNST	MPNST-1	1,72

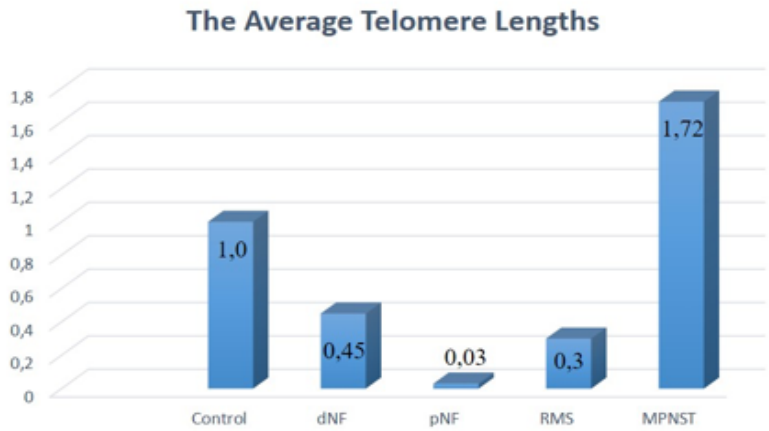

Figure 1. The overall results of average telomere lengths of dermal neurofibroma, plexiform neurofibroma, rhabdomyosarcoma and MPNST regarding control sample

Telomerase Activity Analysis

To measure telomerase activity of the tumor samples, proteins were isolated according to datasheet of TRAPeze® RT Telomerase Detection Kit Assay. The result was shown in Figure 2.

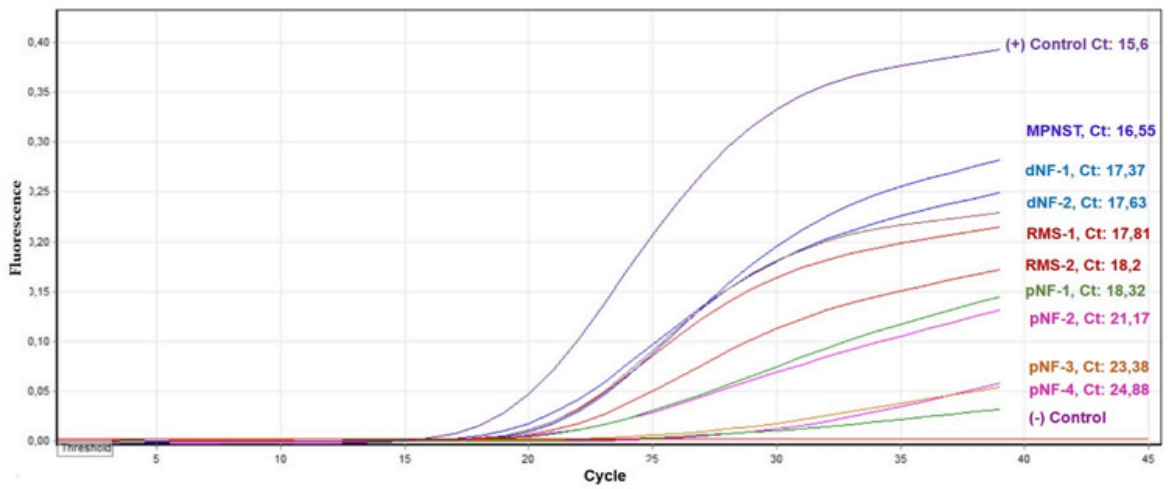

Figure 2. Measurement of telomerase activity from patients' tumor tissue [(+) Control: Cell with high telomerase activity; dNF: dermal neurofibroma, pNF: Plexiform neurofibroma, RMS: Rhabdomyosarcoma, MPNST: Malignant Peripheral Nerve Sheath Tumor, (-) Control: Heat inactivated sample]

DISCUSSION

NF1 is an autosomal dominant disease that affects 1 in 2,500 people worldwide. The disease is developed due to the occurrence of mutations in NF1 gene which codes for neurofibromin. The loss of neurofibromin results in activation of RAS cascade and cell proliferation. For this reason, NF1 gene is categorized as tumor suppressor gene. Variations in NF1 mutations may not correlate with the variations in clinical phenotype. This unclear genotype-phenotype correlations is assumed to be due to the modifier genes.

One of these candidate modifiers is telomere length and telomerase enzyme activity. Telomeres are repetitive nucleotide sequences located at the ends of chromosomes and protect them from fraying and sticking to each other, so telomere length has crucial role in maintaining of genomic integrity in normal cells. The length of telomeres shortens in each cell division. Excessive shortening of telomere length during cell division can be end up with loss of genes located near to telomere region and finally cause chromosomal instability.

Telomere length can be used as cellular marker to study the mechanism of aging, stress, obesity, cancer and many other diseases (18). As an example, we can talk about ischemic heart disease (19), Alzheimer's Disease (20), osteocarcoma in females (21), lung cancer among smokers (22), familial thyroid cancer (23), diabetes (24) and dementia (25) that have shown the correlation with telomere length. On the other hand, studies related to age-related macular degeneration (19), colorectal cancer (26), and death from infectious disease, cancer, cardiac or cerebrovascular disease (27) they have observed no significant relationship with telomere length.

Telomere length is maintained by telomerase enzyme which adds a species-dependent telomere repeat sequences to the 3' end of telomeres. However, telomerase activity usually diminished after birth in somatic cells (28). Thus, to explain the differences between telomere length and telomerase activity should be studied and analyzed together. There are

many methods and protocols to study telomere length and telomerase activity, such as STELA method (Single TElomere Length Analysis), which is expensive and requires more technological lab facilities (29). However, qRT-PCR, although it does not give information on chromosome basis as in STELA method, is still a powerful tool to measure telomere length and telomerase activity in basic level and give us enough information (18).

Research in recent years has shown the importance of telomere and telomerase activity and their cause-and-effect association with human disease. However, the number of research on this concept for NF1 patients is very limited. According to literature, any changes in telomere length and telomerase activity can affects the disease progression. This preliminary study shows that both telomere length and telomerase activity of plexiform neurofibroma has the most decrease comparing to other tumor samples. As we know plexiform neurofibroma is the early stage of malignancy considered as premalignant and telomerase activity is low in premalignant tumors (30). Even though we had only one MPNST sample, we got the highest telomere length for MPNST (Table 1, Figure 1) in the contrary to Jones et al (15). However, we should point out that Jones et al. have analysed results for 19 MPNST samples by STELA (16, 19). Moreover, we monitored telomerase activity for all samples however, we could only compare the results relative to positive and negative control (Figure 2). Comparing the Ct values for telomerase activity we can see the highest decrease for plexiform tumors and the lowest decrease for MPNST, comparing to positive control which is offered by the kit (whose have the highest telomerase activity). Telomerase activity of dermal neurofibromas and Rhabdomyosarcomas are almost the same. If we evaluate the results from both telomere length and telomerase activity, we can clearly observe that the results are compatible. Our results showed similarities with Mantripragada et al. (16) studies.

In conclusion, according to results; statistical analyzes were not found to be significant due to the small number of samples. However, the preliminary obtained data indicate that telomere length may play an important role in NF1-associated tumor progression and could provide information about the telomere-targeted therapeutic approaches in future.

ETHICS COMMITTEE APPROVAL

* The study was approved by the Institutional Ethics Committee of TOBB University of Economy and Technology (Date:29.02.2019 and Number: KAEK-118/032).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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