Cukurova Medical Journal
ÇUKUROVA ÜNİVERSİTESİ TIP FAKÜLTESİ

Cukurova Med J 2018;43(Suppl 1):167-172
DOI: 10.17826/cumj.431395

ARAŞTIRMA / RESEARCH

Telomerase activity in patients with neurofibromatosis type-1

Nörofibromatozis tip1 hastalarında telomeraz aktivitesi

Serhan Küpeli[1], Ayçin Arkan[1], Faruk İncecik[2], Şakir Altunbaşak[2]

[1]Çukurova University Faculty of Medicine, Department of Pediatric Oncology and Pediatric Bone Marrow Transplantation Unit, [2]Department of Pediatric Neurology, Adana, Turkey.

Cukurova Medical Journal 2018;43(Suppl 1):167-172

Abstract

Purpose: In various studies low telomerase activity have been shown to have a protective role in some types of cancer. Patients with neurofibromatosis type-1 (NF1) are at risk of developing certain types of cancer. Prognosis is generally better in patients with NF1 than those without NF1. In the present study, we aimed to measure telomerase activity in patients with NF1 and in controls.

Materials and Methods: Telomerase activity was investigated in peripheral blood samples with human telomerase reverse transcriptase (hTERT) mRNA by using real time reverse transcriptase polymerase chain reaction (RT-PCR) method and Light Cycler 480 system.

Results: hTERT expression was investigated in blood samples of 48 patients with a diagnosis of NF1 and in 37 controls. Telomerase activity was positive in 36 of 48 (75%) patients with NF1 and 23 of 37 patients (73%) without NF1. Telomerase activity was positive in 31 of 36 patients (86%) with NF1 having a benign or malignant tumor, whereas it was positive in 5 of 12 NF1 patients (41.6%) without a tumor.

Conclusion: Detection of hTERT expression in patients with NF1 can be used as a useful marker for tumorigenesis. Additional studies need to be done to know whether detection of hTERT expression in low-grade tumors would help predict progression to high-grade tumors.

Key words: Neurofibromatosis Type-1, tumorigenesis, telomerase activity, hTERT expression.

Öz

Amaç: Çeşitli kanser türlerinde yapılan çalışmalarda düşük telomeraz aktivitesinin bazı kanser tiplerinde koruyucu etkisi olduğundan bahsedilmektedir. Nörofibromatozis Tip-1 (NF1) hastalarının bazı kanser türleri için yüksek risk altında olduğu bilinmektedir. İlginç olarak NF1 hastalarında ortaya çıkan kanserlerde prognoz genel olarak aynı tanılı NF1 olmayan hastalara göre daha iyi olmakta ve sağkalım daha uzun olmaktadır. Bu çalışmada NF1 hastalarında telomeraz aktivitesinin ölçülerek kronik hastalığı olmayan bireylerdeki değerlerle karşılaştırılması amaçlanmıştır.

Gereç ve Yöntem: Telomeraz aktivitesinin belirlenmesi amacıyla hTERT mRNA çalışması periferik kan örneklerinden "real-time reverse transcriptase polymerase chain reaction" (RT-PCR) yöntemiyle ve "Light Cycler 480" sistemi kullanılarak yapıldı.

Bulgular: Çocukluk çağında NF1 tanısı almış 48 hasta ve kontrol grubu olarak 37 hastanın kan örneklerinde hTERT ekpresyonu çalışıldı. NF1 hastaları arasında telomeraz aktivitesi 36 hastada (%75) saptanırken telomeraz aktivitesi kontrol grubunda 27 çocukta (%73) pozitif bulundu. Malign veya benign tümörü olan 36 NF1 hastasının 31'inde (% 86) telomeraz pozitifliği mevcut iken herhangi bir tümör saptanmayan 12 NF1 hastasının 5'inde (%41.6) telomeraz aktivitesi pozitif bulundu. Aradaki fark istatistiksel olarak anlamlı idi.

Sonuç: NF1 hastalarında benign veya malign tümör gelişimi açısından telomeraz aktivitesi ölçümü tümör belirleyici işlevi görebilir. Düşük dereceli tümörlerde hTERT ekspresyonunun yüksek dereceli tümörlere progresyonu tahmin etmede yardımcı olup olamayacağı konusunda daha fazla çalışmaya ihtiyaç vardır.

Anahtar kelimeler: Nörofibromatozis Tip1, tümörigenez, telomeraz aktivitesi, hTERT ekspresyonu

Yazışma Adresi/Address for Correspondence: Dr. Serhan Küpeli, Çukurova University Faculty of Medicine, Department of Pediatric Oncology and Pediatric Bone Marrow Transplantation Unit, Adana, Turkey.
E-mail: serhankupeli@cu.edu.tr
Geliş tarihi/Received: 7.6.2018 Kabul tarihi/Accepted: 17.7.2018 Published online: 15.9.2018

INTRODUCTION

The end of the human chromosomes are made up of telomeric repeats which elongates up to 15 kilobases[1]. Telomeric bases help stabilization of DNA and protect it against new combinations (end to end fusion) and enzymatic degredation[2]. DNA polymerases need an RNA primer for initiation of DNA synthesis in 5'→3' direction. Telomeric shortening occurs after each cell division and telomer shortening is proposed to be the main mechanism in cellular aging[1]. Telomerase activity is among the cellular mechanisms preventing telomere shortening. Telomerase, a complex of ribonucleoproteins, helps stabilization of telomere length by adding hexamere repeats $(TTAGGG)_n$ to the end of the chromosomes. It contains an RNA of 159 nucleotides to facilitate replication of telomeric repeats in its structure. The other two components of telomerase are reverse transcriptase and protein I[3-5].

Recently, in various types of cancer, a strong relationship between telomerase activity and malignant diseases was revealed. For this reason, telomerase enzyme has been proposed to be a potential tumor marker for widespread use[6-9]. In humans telomerase activity exists exclusively in germ cells and stem cells and telomerase expression has not been shown in differentiated somatic cells. Furthermore, a low-level telomerase activity could be detected in lymphocytes[10,11]. For stem cells and almost all types of cancer, telomerase activity is an obligation for unlimited proliferation[12]. Regaining of telomerase activity and stabilization of telomere length have a role in obtaining immortality for cancer cells. Short telomere length is thought to be an indicator for cumulative cellular aging and increased risk of death after cancer[13].

Neurofibromatosis Type-1 (NF1) is a cancer predisposition syndrome and is characterized by cafe au lait spots, axillary and/or inguinal freckling, Lisch nodules and dermal and/or plexiform neurofibromas. These patients are under risk of certain types of cancer. Interestingly, prognosis and survival are superior in some malignant disorders in patients with NF1 compared with patients without NF1[14]. Telomerase activity might be helpful in earlier detection of tumorigenesis in a non-invasive way. In the present study, we aimed to compare telomerase activity in patients with NF1 and without NF1 and in patients with tumor (benign or malignant) and without tumor.

MATERIALS AND METHODS

The study was conducted in Department of Pediatric Oncology and Pediatric Neurology, Çukurova University Faculty of Medicine between March 2014 and September 2017. The study was approved by Ethical Committee of Çukurova University Faculty of Medicine.

Inclusion criteria were; having diagnosed with NF1 before the age of 18 years, taking no chemotherapy and/or radiotherapy in another center and giving an informed consent. Control group included children without a chronic disease who visited Outpatient Clinic of Pediatrics. After gathering informed consent from patients and controls, data forms were filled which include information about age, sex, NF1 criteria, diagnosis, presence of benign or malignant tumor, telomerase activity, treatment details and outcome. Hamartomas and optic gliomas were diagnosed radiologically with magnetic resonance imaging. Telomerase activity was investigated in peripheral blood samples with hTERT mRNA by using real time RT-PCR method and Light Cycler 480 system.

Leukocyte extraction

Leukocytes were separated by lysis of erythrocytes in peripheral blood samples taken from patients with NF1 and controls. Initial volume was calculated with 10000/WBC=X µl formula. Calculated volume was placed on shaker for 10 minutes in eppendorf tubes after adding erythrocytes whic is two times larger than the calculated volume. After 10 minutes eppendorfs were centrifuged for 30 seconds in 13000 rpm. After supernatant was expelled 900 µl erythrocyte was added on pellet. After placing for 5 minutes on shaker, eppendorfs were again centrifuged for 30 seconds in 13000 rpm. Supernatant was thrown completely and 500 µl 0.9 % NaCl was put on pellet. Eppendorfs were again centrifuged for 30 seconds in 13000 rpm and NaCl was thrown completely from eppendorfs.

RNA isolation

400 µl lysis buffer from High Pure RNA isolation kit was put on pellet. For isolation of RNA from leukocytes, filter and collection tubes were prepared and numbered. Prepared mixture was put into filter tubes and caps were closed. After centrifuge for 15

seconds in 8000 rpm, collection tubes were emptied. Contents of filter tubes were transferred into collection tubes. After adding 90 µl DNase incubation buffer and 10 µl DNase I each sample was incubated for 15 minutes. Filter tubes were centrifuged for 15 seconds in 8000 rpm after filling with 0.5 ml wash buffer I. Collection tubes were emptied and contents of filter tubes were again transferred into collection tubes. Filter tubes were centrifuged for 15 seconds in 8000 rpm after adding 0.5 ml wash buffer II. Collection tubes were emptied and contents of filter tubes were again transferred into collection tubes. After adding 0.2 ml wash buffer II filter tubes were spinned for 2 minutes in 13000 rpm and collection tubes were thrown. Contents of filter tubes were transferred into eppendorf tubes with caps. The RNAs obtained were deposited in -80 °C.

cDNA synthesis

For complementary DNA (cDNA) synthesis the RNAs were placed in room temperature. cDNA synthesis was started by using Transcriptor First Strand cDNA Synthesis kit. For each sample, 9 µl pure total RNA, 2 µl random hexamer primer and 0.5 µl PCR grade water were put to a total volume of 13 µl. The mixture was placed on thermal cycler for 10 minutes. After adding 4 µl Transcriptor Reverse Transcriptase Reaction Buffer, 0.5 µl protector RNase inhibitor, 2 µl deoxynucleotide mix and 0.5 µl Transcriptor Reverse Transcriptase a total volume of 20 µl was formed. The mixture was then placed on thermal cycler for 10 minutes in 25 °C, 60 minutes in 50 °C and 5 minutes in 85 °C, and cDNAs were obtained.

Real time PCR step

For each sample, 0.5 µl primer (hTERT gene), 0.5 µl probe (Real Time Ready), 10 µl probe master, 4 µl grade water were put to a total volume of 15 µl. 5 µl cDNA was added to the mixture and the study was conducted on plates of Light Cycler 480 system with Real Time PCR Heat Protocol. In denaturation step, the mixture was placed for 10 minutes in 95 °C for 1 cycle. In amplification step (annealing) 45 cycles were spent for 10 seconds in 90 °C, 30 seconds in 60 °C, 1 second in 72 °C and reading was obtained in 72 °C. In cooling step the mixture was placed in 40 °C for 1 minute. To ensure accurate interpretation and comparison of the results beta-actin primer was used. At the end of the study, the results were evaluated buy using the $2^{-\Delta\Delta CT}$ values of the samples.

Statistical analysis

The data was evaluated by using SPSS 16 version and for descriptive variables percent, frequency, median, mean were used. For comparison between groups chi-square test was used. P values <0.05 were considered statistically significant.

RESULTS

hTERT expression was studied in blood samples taken from 48 NF1 patients diagnosed in childhood and 37 patients without a chronic disease as control group. NF1 group was comprised of 33 males (68.8%) and 15 females (31.2%) with a median age of 12 years and control group consisted of 21 males (56.7%) and 16 females (43.3%) with a median age of 12 years.

Table 1. Diagnostic criteria in patients with NF1.

NF1 criteria	n	%
"cafe-au-lait" spots, axillary/inguinal freckling	18	37.5
"cafe-au-lait" spots, optic glioma	9	18.7
"cafe-au-lait" spots, family history	8	16.6
"cafe-au-lait" spots, neurofibroma	3	6.2
"cafe-au-lait" spots, freckling, Lisch nodule, optic glioma	2	4.2
"cafe-au-lait" spots, Lisch nodule	2	4.2
"cafe-au-lait" spots, freckling, Lisch nodule, neurofibroma, family history	2	4.2
"cafe-au-lait" spots, freckling, Lisch nodule	1	2.1
"cafe-au-lait" spots, freckling, optic glioma	1	2.1
"cafe-au-lait" spots, Lisch nodule, optic glioma	1	2.1
"cafe-au-lait" spots, dysplasia of tibia	1	2.1

There was no statistically significant difference between patients in NF1 and control groups in regard of gender or age (p> 0.05). Cafe au lait spots were present in all patients with NF1. Diagnostic criteria of NF1 in our patients are summarized in Table 1.

In 36 patients (75%) in NF1 group benign or malignant tumor development was detected. Most of them were benign tumors and there were more than one tumor in 5 patients. Hamartomas were the mostly diagnosed tumors detected in 20 patients (41.7%). The second most frequent tumor was optic glioma found in 13 patients (36.2%). At the end of

the study we did not detect any tumoral development in 12 patients (25%). Tumor status of NF1 patients was shown in Table 2.

Table 2. Tumor status of NF1 patients

Tumor	n	%
Hamartoma	16	33.3
Optic glioma	9	18.7
Optic glioma and hamartoma	3	6.2
Acute lymphoblastic leukemia	2	4.2
Astrocytoma	1	2.1
Hodgkin lymphoma	1	2.1
Malignant peripheral nerve sheath tumor	1	2.1
Neuroblastoma and optic glioma	1	2.1
Neurofibroma and hamartoma	1	2.1
Neurofibroma	1	2.1
None	12	25.0

Chemotherapy and/or radiotherapy was administered to 9 patients (18.7%) in NF1 group due to acute lymphoblastic leukemia (ALL) in 2 patients, optic glioma in 3, Hodgkin lymphoma in 1, neuroblastoma with optic glioma in 1, astrocytoma in 1 and malignant peripheric nerve sheath tumor (MPNST) in 1. The other 27 patients with tumor did not receive chemotherapy and/or radiotherapy because they had asymptomatic hamartoma, neurofibroma and/or optic glioma without impairment in visual acuity or in visual field. Patients and treatment details who were given chemotherapy and/or radiotherapy were summarized in Table 3. All patients responded well to treatment and are in remission for 6 months to 3 years.

hTERT expression was detected in 36 patients (75%) in NF1 group and in 27 patients (73%) in control group. There was no statistically significant difference according to hTERT expression between patients in NF1 and control groups (p=0.83). Among 36 patients having benign or malignant tumors in NF1 group 31 patients (86%) had hTERT expression. Of 12 patients without a tumor in NF1group 5 (41.6%) had telomerase activity. The difference was statistically significant between the groups (p=0.002). hTERT expression in NF1 patients and controls were shown in Table 4.

Table 3. Patients and treatment details who were given chemotherapy and/or radiotherapy

Age, gender	Tumor	hTERT expression	Treatment	Outcome
16 y, M	Hodgkin lymphoma	Negative	Chemotherapy, radiotherapy	NED, 3 years
4 y, F	Neuroblastoma, optic glioma	Positive	Chemotherapy, surgery	NED, 3 years
4 y, F	ALL	Positive	Chemotherapy	NED, 2 years
17 y, F	Astrocytoma	Negative	Surgery, radiotherapy	NED, 3 years
11 y, M	Optic glioma	Positive	Chemotherapy	NED, 2 years
1 y, M	Optic glioma	Positive	Chemotherapy	NED, 6 mo.s
15 y, F	MPNST	Positive	Chemotherapy, radiotherapy	NED, 2 years
14 y, F	Optic glioma	Positive	Chemotherapy, radiotherapy	NED, 3 years
4 y, F	ALL	Positive	Chemotherapy	NED, 3 years

MPNST: Malignant peripheral nerve sheath tumor, NED: No evidence of disease, ALL: Acute lymphoblastic leukemia

Table 4. hTERT positivity in NF1 patients and controls

hTERT	NF1patients	Controls	p	NF1patients having tumor	NF1 patients with no tumor	p
Positive	36	27	0.83	31	5	0.002
Negative	12	9		5	7	

hTERT: human telomerase reverse transcriptase

DISCUSSION

Telomerase activity was shown to be present in various malignant tumors and it is proposed to be a valuable diagnostic marker. It was reported that, telomerase activity is correlated with telomerase reverse transcriptase (TERT) expression[15,16]. NF1 is a cancer predisposition syndrome which is characterized by cafe au lait spots, axillary and inguinal freckling, Lisch nodules, dermal and/or plexiform neurofibromas. It is known that NF1 patients are at risk of certain types of cancer. Brain tumors, malignant peripheral nerve sheath tumors, optic gliomas, soft tissue sarcomas and some benign tumors like hamartomas and neurofibromas are among the most frequent tumors diagnosed in patients with NF1. Interestingly, prognosis in NF1 patients with a malignant tumor is better than the

patients with the same diagnosis and without NF1.

In the present study, we aimed to detect telomerase activity by means of hTERT expression in patients with NF1 and to compare the results with other patients without a chronic disease. Detecting hTERT expression might be helpful in earlier detection of tumorigenesis in a non-invasive way. NF1 patients with and without a tumoral development were compared according to hTERT expression. hTERT expression was detected in 36 patients in NF1 group and in 27 controls (p=0.83). Among 36 patients having benign or malignant tumors in NF1 group 86% had hTERT expression. 12 patients without a tumor 5 (41.6%) had hTERT expression in the same group (p=0.002).

Mantripragada et al. investigated telomerase activity in MPNST samples of patients with NF1 and they employed NF1 patients with benign tumors as control group. They found telomerase activity positivity in 61% of the patients with MPNST but none in NF1 patients with benign tumors[6]. Similarly, Levy et al. detected more telomerase activity in NF1 patients with MPNST compared with NF1 patients with benign tumors[17]. In our study, NF1 patients with a tumor showed hTERT expression in a rate of 86%, whereas it was 41.6% in NF1 patients without a tumor (p=0.002). Similarly, hTERT expression was detected in most of the patients (77.7%) with a tumor who were given chemotherapy and/or radiotherapy. We could not divide the patients in NF1 group into patients having malignant or benign tumors as pathological grading of most of the tumors was unknown in patients with NF1. Most of the tumors in NF1 group were diagnosed radiologically not with pathological examination. Instead, the patients in NF1 group were divided as having a tumor and not having a tumor.

In the present study, peripheral blood samples were employed for determining hTERT expression. Mononuclear cells in peripheral blood can be activated by antigenic stimulation of soluble factors or cancer cells in metastatic lymph nodes. Lee et al. have reported that telomerase activity can be detected in mononuclear cells in peripheral blood. They have studied telomerase activity in patients with head and neck cancers and found statistically significant difference in regard of telomerase expression in cancer patients compared with control group[18].

Small number of malignant and benign tumors in patients in NF1 group and studying hTERT expression in only peripheral blood samples are among the limitations of the present study. It would be exciting to know the hTERT expression results in tumor specimens of NF1 patients having a malignant or benign tumor. Due to the high cost of studying hTERT in tissue samples, it is planned to be done in another research.

In conclusion, hTERT expression acts as an indicator of tumor presence in NF1 patients. It can be a promising marker in detecting tumor progression in patients with NF1. Additional studies need to be done to know whether detection of hTERT expression in low-grade tumors would help predict progression to high-grade tumors.

Acknowledgement

We thank to Yusuf Gozet, M.Sc., Onur Ucar, M.Sc., and Mert Kuez for technical and laboratory support.

The study was funded by Çukurova University BAP Coordination Unit with the project number: TSA-2017-5843.

REFERENCES

1. Dahse R, Fiedler W, Ernst G. Telomeres and telomerase: biological and clinical importance. Clin Chem. 1997;43:708-14.
2. Rhyu M. Telomeres, telomerase, and immortality. J Natl Cancer Inst. 1995;87:884-94.
3. Feng J, Funk WD, Wang S, Weinrich S, Avilion A, Chiu C et al. The RNA component of human telomerase. Science. 1995;269:1236-41.
4. Harrington L, McPhail T, Mar V, Zhou W, Oulton R, Program AE et al. A mammalian telomerase-associated protein. Science. 1997;275:973-7.
5. Nakamura TM, Morin GB, Chapman KB, Weinrich SL, Andrews WH; Lıngner J, Harley CB, Cech TR. Telomerase catalytic subunit homolog from fission yeast and human. Science. 1997;277:955-9.
6. Mantripragada KK, Caley M, Stephens P, Jones CJ, Kluwe L, Guha A et al. Telomerase activity is a biomarker for high grade malignant peripheral nerve sheath tumors in neurofibromatosis type 1 individuals. Genes Chromosomes Cancer. 2008;47:238-46.
7. Shay JW, Bacchetti S. A survey of telomerase activity in human cancer. Eur J Cancer. 1997;33:787–91.
8. Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL et al. Specific association of human telomerase activity with immortal cells and cancer. Science. 1994;266:2011–5.
9. Greider CW. Telomerase activity, cell proliferation,

and cancer. Proc Natl Acad Sci USA. 1998;95:90–2.
10. Hiyama K, Hirai Y, Kyoizumi S, Akiyama M, Hiyama E, Piatyszek MA et al. Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. J Immunol. 1995;155:3711–5.
11. Ge P, Zhang B, Zhang J, Gao Z, He W. Telomerase activity of peripheral blood mononuclear cells from patients with laryngeal carcinoma. Am J Clin Oncol. 2004;27:191-4.
12. Meeker AK, Coffey DS. Telomerase: a promising marker of biological immortality of germ, stem, and cancer cells. A review. Biochemistry (Mosc). 1997;62:1323-31.
13. Weischer M, nordestgaard BG, Cawthon RM, Freiberg JJ, Tybjærg-Hansen A, Bojesen SE. Short telomere length, cancer sur-vival, and cancer risk in 47102 individuals. J Natl Cancer Inst. 2013;105:459–68.
14. Ater JL, Xia C, Mazewski CM, Booth TN, Freyer DR, Packer RJ et al. Nonrandomized comparison of neurofibromatosis type 1 and non-neurofibromatosis type 1 children who received carboplatin and vincristine for progressive low-grade glioma: A report from the Children's Oncology Group. Cancer. 2016;122:1928-36.
15. Liu Y, Snow BE, Hande MP, Yeung D, Erdmann NJ, Wakeham A et al. The telomerase reverse transcriptase is limiting and necessary for telomerase function in vivo. Curr Biol. 2000;10:1459–62.
16. Tomoda R, Seto M, Tsumuki H, Iida K, Yamazaki T, Sonoda J et al. Telomerase activity and human telomerase reverse transcriptase mRNA expression are correlated with clinical aggressiveness in soft tissue tumors. Cancer. 2002;95:1127–33.
17. Levy P, Vidaud D, Leroy K, Laurendeau I, Wechsler J, Bolasco G et al. Molecular profiling of malignant peripheral nerve sheath tumors associated with neurofibromatosis type 1, based on large-scale real-time RTPCR. Mol Cancer. 2004;3:20.
18. Lee BJ, Wang SG, Choi JS, Lee JC, Goh EK, Kim MG. The prognostic value of telomerase expression in peripheral blood mononuclear cells of head and neck cancer patients. Am J Clin Oncol. 2006;29:163-7.