Differential Gene Expression Profiles Between Neuroblastomas With High Telomerase Activity and Low Telomerase Activity

By Eiso Hiyama, Keiko Hiyama, Masahiko Nishiyama, C. Partrik Reynolds, Jerry W. Shay, and Takashi Yokoyama

Hiroshima, Japan; Los Angeles, California; and Dallas, Texas

Purpose: Neuroblastoma shows remarkable heterogeneity, resulting in favorable or unfavorable outcomes. The authors previously reported that high levels of telomerase activity correlated with unfavorable tumors, and telomere shortening without telomerase activity correlated with tumor regression. To identify the genes responsible for the biological characteristics of neuroblastoma, the authors applied microarray techniques.

Methods: Mixtures of total RNAs extracted from 10 neuroblastoma tissues with high and 10 with low telomerase activity were labeled with Cy3 or Cy5 by reverse transcriptase reaction, respectively, and hybridized with our original microarrays prepared with a cDNA library of human fetal brain.

Results: Expression of 63 genes including *MYCN, hTERT, HSPCA,* and cell cycle–related proteins was found to be increased in neuroblastomas with high telomerase activity, whereas another 46 genes, including neural differentiating genes, were detected as highly expressed in tumors with low telomerase activity.

Conclusions: The expression profiling data indicated clusters of upregulated and downregulated genes tumors with high or low telomerase activity. The genes involved in differentiation/growth arrest of tumor cells were closely related to low telomerase activity in neuroblastoma. The genes overexpressed in tumors with high telomerase activity, including cell-cycle–related genes and transcriptional factors, would be candidates for novel prognosis-predicting factors as well as new therapeutic targets in aggressive neuroblastomas.
J Pediatr Surg 38:1730-1734. *© 2003 Elsevier Inc. All rights reserved.*

INDEX WORDS: Neuroblastoma, telomerase, gene expression, microarray, prognosis.

NEUROBLASTOMA, which arises from the embryonal neural crest, is one of the most common solid tumors in children and affects approximately 1 in 7,000 individuals.[1] Neuroblastoma shows remarkable biological heterogeneity, resulting in favorable outcome in some instances and aggressive growth despite multimodal therapy in other instances. To predict the biological behavior of the individual tumor, several prognosis-predicting markers such as *MYCN* gene amplification, loss of heterozygosity (LOH) in the short arm of chromosome 1, trk A expression, Ha-ras p21 expression, and DNA ploidy have been proposed.[2] However, each of these parameters alone is insufficient to predict the prognosis. Thus, massive efforts have been spent to elucidate the genes controlling neuroblastoma cells. Recently, microarray techniques have been developed and extensively applied in cancer research.[3,4] These technologies have emerged as indispensable research tools for gene expression profiling and detection of genetic aberrations.[5]

Telomeres are unique structures involved in the protection and replication of chromosomes in eukaryotes. Because of the end replication problem, serial cell division results in a gradual reduction of telomeres. The gradual shortening of telomeres has been proposed to be the clock by which cells count the number of mitoses and may contribute to cellular senescence. Telomerase, an RNA-dependent DNA polymerase, which compensates for the end replication problem, is expressed in germ-line cells but not in most somatic cells. The reactivation of telomerase and the stabilization of telomeres correlates with "immortality" in tumor cells.[6] Previously, we found that aggressive neuroblastomas expressed high levels of telomerase activity, whereas favorable tumors had no or low telomerase expression.[7,8] Diminishing of telomerease activity was concomitant with regression or matu-

From the Department of General Medicine, Graduate School of Biomedical Sciences, and Department of Translational Cancer Research, Research Institute for Radiation Biology and Medicine; Division of Hematology-Oncology, Childrens Hospital Los Angeles, Los Angeles, CA; and Department of Cell Biology, The University of Texas, Southwestern Medical Center at Dallas, Dallas, TX.

Presented at the 36th Annual Meeting of the Pacific Association of Pediatric Surgeons, Sydney, Australia, May 12-16, 2003.

Supported by Grant-in-Aid for Scientific Research (A) (No. 13307050) from the Ministry of Education, Culture, Sports, Science, and Technology and for Cancer Research (13-20) from the Ministry of Health, Labour, and Welfare of the Government of Japan.

Address reprint requests to Eiso Hiyama, MD, Department of General Medicine, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3, Kasumi, Minami-ku, Hiroshima, 734-8551, Japan.

© 2003 Elsevier Inc. All rights reserved.

0022-3468/03/3812-0008$30.00/0

doi:10.1016/j.jpedsurg.2003.08.042

ration. Although there is no evidence that telomerase directly affects cell growth of aggressive neuroblastoma, we proposed that telomerase activity might be a useful indicator of outcome. In the current study, we classified neuroblastomas into 2 groups according to the levels of telomerase activity and then analyzed the gene expression profiles between these 2 groups of tumors using microarray techniques.

MATERIALS AND METHODS

A total of 212 patients with neuroblastoma, whose tumors were obtained before any treatments, were enrolled in this study. They were diagnosed at the Hiroshima University Medical Hospital or consulted for molecular analysis from other hospitals in Japan between 1983 and 2000. Patients were staged according to the standard clinical and pathological criteria of INSS.[9] These patients were treated postoperatively with various antitumor agents except for the cases in stage 1 tumors or detected by mass screening. All tumor samples were obtained at surgery and stored at −80°C until use. Written informed consent was obtained from all patients or parents before surgery.

Telomerase activity was measured by the telomeric repeat amplification protocol (TRAP) assay as described earlier.[10] Tumor tissues were homogenized in CHAPS lysis buffer and an aliquot of the extract containing 0.5 μg of protein was used for each assay. The assay was done using a commercial kit, TRAPeze XL kit (Serological Co, Gaithersbrug, MD) that is a semiquantitative fluorescent-labeled polymerase chain reaction (PCR) system for estimation of relative telomerase activity with the use of a PCR internal control. The levels of telomerase activity were quantified by the ratio of the fluorescence intensity of the entire TRAP ladder to the sulforhodamine-intensity of internal control and expressed as the number of oligonucleotides (TS primer) extended by telomerase (TPG, total product generated unit).

We prepared target cDNA clones from a cDNA library of human fetal brain. A set of 6,272 human fetal brain cDNA clones were amplified by PCR and spotted onto poly L-lysine–coated glass slides using a custom robot arrayer (Genex 2000, Kaken Genetics, Chiba, Japan). Total cellular RNA was extracted from tumor tissues by the acid-guanidium-phenol chloroform method. From each group of high or low telomerase activity, we selected 10 representative tumors from which sufficient good-quality RNA was obtained. Ten micrograms of total RNA mixture derived from the 10 representative tumors in each group were labeled with Cy3 or Cy5, respectively, and hybridized with the slide. After hybridization, slides were washed, dried, and scanned by laser confocal scanner (Scan Array 5000, GSI Luminics, Ottawa, ON, Canada). Hybridization intensity of each spot was normalized to the mean intensity of all noncontrol spots for each channel. This microarray analysis was done in triplicate, and only the clones with consistent results were analyzed further.

To validate the microarray results, expression levels of the representative genes identified as differentially expressed in each group were estimated by fluorescent-based quantitative real-time reverse transcriptase (RT) PCR using TaqMan probe. Amplification reaction was carried out in a 96-well reaction plate (Applied Biosystems, Foster City, CA) in a spectrofluorometric thermal cycler (ABI PRISM 7700 Sequence Detecter; Applied Biosystems).

RESULTS

Among the 212 tumor specimens, telomerase activity was detected in 195 (92%). The levels of telomerase activity ranged between 0 and 754.8 TPG. High telomerase activity (≥100 TPG) was detected in 32 tumors, whereas low telomerase activity (<10 TPG) was detected in 153 tumors, and the remaining 10 tumors showed moderate levels (10 to 99 TPG). Among the 10 tumors with high telomerase activity, 2 were stage 3, and 8 were stage 4. All 10 patients died of tumor without multimodal therapy. The other 10 cases of low telomerase activity consisted of 2 stage 1, 1 stage 2A, 3 stage 2B, 2 stage 3, and 2 stage 4S cases. All patients are alive and disease free, and the 4S tumors regressed spontaneously.

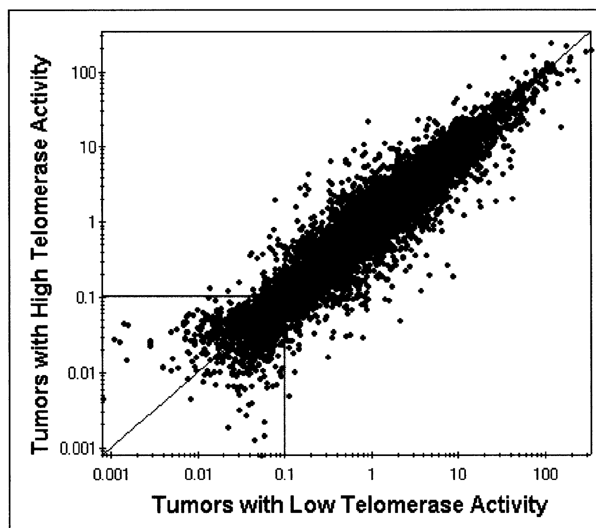

Fig 1. Scatter plots of the cDNA microarray analysis. Cy5: neuroblastoma tissues with high telomerase activity and Cy3: those with low telomerase activity. We excluded genes from further analysis when both Cy3 and Cy5 intensities were below 0.1 and defined "overexpression" when the intensity ratio of Cy3:Cy5 or Cy5:Cy3 was greater than 2.0.

The microarray with 6,272 spots derived from a fetal brain cDNA library was hybridized with Cy3-labeled cDNAs derived from low telomerase tumors and Cy5-labeled cDNAs with high telomerase tumors. Scatter plot analysis is shown in Fig 1. We excluded the spots with both Cy3 and Cy5 intensities below 0.1 and defined the significant "overexpression" when the intensity ratio of Cy3:Cy5 or Cy5:Cy3 was consistently greater than 2.0 in triplicates. Among the 6,272 clones, 201 and 181 clones consistently upregulated in tumors with high or low telomerase activity, respectively. All these clones were sequenced, compared with databases, and annotated. After the exclusion of unknown genes and redundancy, 63 (Table 1) and 46 (Table 2) genes were identified as specifically overexpressed in tumors with high and low telomerase activity, respectively. Some of these differentially expressed genes detected by microarray analysis were examined by quantitative real-time RT-PCR analysis for confirmation. The overall expression levels of these genes estimated by TaqMan assay well correlated with the microarray expression profiles (data not shown).

Table 1. Genes That are Overexpressed in Tumors With High Telomerase Activity

Ratio*	Description	ACCN#
25.30	SPTF-associated factor 65 gamma (STAF65)	NM_014860
22.20	DNA-binding transcriptional activator (MYCN)	NM_006316
21.83	Gamma-aminobutyric acid A receptor, alpha 5 (GABRA5)	NM_000810
14.38	MAGE-4a antigen (MAGEA4)	U10687
13.96	cDNA FLJ10674 FIS, Clone NT2RP2006436	AK001536
11.52	Serine (or cysteine) proteinase inhibitor (SERPINA3)	NM_001085
7.23	Cellular retinoic acid binding protein 1 (CRABP1)	NM_004378
5.81	Neuromedin U (NMU)	NM_006681
5.58	Protease, serine, 12 (neurotrypsin, motopsin) (PRSS12)	NM_003619
5.22	Nuclear autoantigenic sperm protein (histone-binding) (NASP)	NM_002482
4.59	Vasoactive intestinal peptide (VIP)	NM_003381
4.44	Neuroblastoma-amplified protein (LOC51594)	NM_015909
4.32	Interleukin 8 (IL8)	NM_000584
4.14	Kinesin-like 1 (KNSL1)	NM_004523
3.95	DEAD/H (ASP-GLU-ALA-ASP/HIS) box polypeptide 1 (DDX1)	NM_004939
3.77	MAD2 mitotic arrest deficient-like 1 (YEAST) (MAD2L1)	NM_002358
3.59	Enhancer of invasion 10 (HEI10),	NM_021178
3.34	KIAA0008 gene product (KIAA0008)	NM_014750
3.31	Ribonucleotide reductase M2 polypeptide (RRM2)	NM_001034
3.23	RAB6 interacting, kinesin-like (Rabkinesin6) (RAB6KIFL),	NM_005733
3.18	Protein disulfide isomerase-related protein (P5)	NM_005742
3.11	CHK1 checkpoint homolog (S. Pombe) (CHEK1)	NM_001274
3.04	Cyclin-dependent kinase inhibitor 3 (CDKN3)	NM_005192
2.88	Chromosome condensation-related SMC-associated protein 1 (CNAP1)	NM_014865
2.83	Pro-galanin	M77140
2.80	Centromere protein F (350/400KD, Mitosin) (CENPF)	NM_016343
2.74	Steroidogenic acute regulatory protein (STAR)	NM_000349
2.71	Hypothetical protein FLJ10970 (FLJ10970)	NM_018286
2.59	Telomerase reverse transcriptase (TERT)	AB086950
2.54	Cyclin B1 (CCNB1)	NM_031966
2.52	Transmembrane 4 superfamily member 4 (TM4SF4)	NM_004617
2.47	Hypothetical protein FLJ20288 (FLJ20288)	NM_024668
2.45	Clone 25028 mRNA sequence	AF131846
2.45	cDNA FLJ10952 FIS, clone PLACE1000374	AK001814
2.43	TRAIL receptor 2 (TNFRSF10E)	AF016266
2.38	Topoisomerase (DNA) II alpha (170KD) (TOP2A)	NM_001067
2.36	Cell division cycle 2, G1 to S and G2 to M (CDC2)	NM_001786
2.36	Nuclear factor I/B (NFIB)	NM_005596
2.34	CDC28 protein kinase 2 (CKS2)	NM_001827
2.34	CDC6 cell division cycle 6 homolog (S. cerevisiae) (CDC6)	NM_001254
2.33	Hypothetical protein MGC5306 (MGC5306)	NM_024116
2.33	Phosphoribosyl pyrophosphate synthetase 1 (PRPS1)	NM_002764
2.25	Catenin (cadherin-associated protein), alpha-like 1 (CTNNAL1)	NM_003798
2.25	Capillary morphogenesis protein 2 (CMG2)	NM_058172
2.25	Adrenomedullin (ADM)	NM_001124
2.24	Chloride channel, nucleotide-sensitive, 1A (CLNS1A)	NM_001293
2.22	Heat shock 90kD protein 1, alpha (HSPCA)	NM_005348
2.20	Bloom syndrome (BLM)	NM_000057
2.20	Deleted in lymphocytic leukemia, 1 (DLEU1)	NM_005887
2.19	Serine/threonine kinase 15 (STK15)	NM_003600
2.16	Aldehyde dehydrogenase 1, soluble (ALDH1)	NM_000689
2.14	Human Ig J chain gene (IGJ)	M12759
2.14	Flap structure-specific endonuclease 1 (FEN1)	NM_004111
2.13	Solute carrier family 1 (neutral amino acid transporter), member 5 (SLC1A5)	NM_005628
2.13	Multifunctional polypeptide similar to SAICAR synthetase and air carboxylase (ADE2H1),	NM_006452
2.11	Centromere protein E (312KD) (CENPE)	NM_001813
2.11	Cyclin A2 (CCNA2)	NM_001237
2.09	Cleavage and polyadenylation specific factor 3, 73kd subunit (CPSF3)	NM_016207
2.07	Prostaglandin I2 (prostacyclin) synthase (PTGIS)	NM_000961
2.07	PRO2000 protein (PRO2000)	NM_014109
2.02	Galactokinase 2 (GALK2)	NM_002044
2.00	Nucleolar protein ANKT (ANKT)	AB18454

*Ratio represents the fold of overexpression in the tumors with high telomerase activity (Cy5) to those with low telomerase activity (Cy3).

Table 2. Genes That Are Overexpressed in the Tumors With Low Telomerase Activity

Ratio*	Description	ACCN#
13.28	Glucagon receptor (GCGR)	NM_000160
12.46	Neurotrophic tyrosine kinase, receptor, type 1 (NTRK1)	NM_002529
12.17	C-type (calcium dependent, carbohydrate-recognition domain) lectin, superfamily member 9 (CLECSF9)	NM_014358
9.79	Small inducible cytokine subfamily A (cys-cys), member 21 (SCYA21)	NM_002989
9.66	Small inducible cytokine subfamily A (cys-cys), member 19 (SCYA19)	NM_006274
9.20	Protein phosphatase 1, regulatory (inhibitor) subunit 1A (PPP1R1A)	NM_006741
7.69	Lymphotoxin beta (TNF superfamily, member 3) (LTB)	NM_009588
7.32	Neuropeptide FF-amide peptide precursor (NPFF)	NM_003717
6.94	Ras homolog gene family, member 1 (ARHI)	NM_004675
6.74	Amine oxidase, copper containing 2 (retina-specific) (AOC2),	NM_009590
6.56	Endothelin converting enzyme-like 1 (ECEL1)	NM_004826
6.16	Amiloride-sensitive cation channel 1, neuronal (degenerin) (ACCN1)	NM_001094
5.55	Phospholipase A2, group IID (PLA2G2D)	NM_012400
5.46	T-cell specific protein	X00437
5.09	Calcium/calmodulin-dependent protein kinase (CAM kinase) II β (CAMK2B)	NM_001220
4.94	Synaptic vesicle protein 2B homolog (SV2B)	NM_014848
4.08	CDW52 antigen (campath-1 antigen) (CDW52),	NM_001803
3.75	Fucosyltransferase 1 (FUT1)	NM_000148
3.49	Candidate mediator of the p53-dependent G2 arrest (REPRIMO),	NM_019845
3.39	Phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma) (PLA2G7)	NM_005084
3.30	CD48 antigen (B-cell membrane protein) (CD48)	NM_001778
3.23	Retinol binding protein 5, cellular (RBP5)	NM_031491
3.23	Actinin, alpha 2 (ACTN2)	NM_001103
3.15	Interleukin 7 (IL7)	NM_000880
3.14	Protein kinase (cAMP-dependent, catalytic) inhibitor beta (PKIB)	NM_032471
3.09	Capping protein (actin filament), gelsolin-like (CAPG)	NM_001747
2.89	cDNA FLJ32724 FIS, clone TEST12000951	AK057286
2.84	Secretogranin II (chromogranin C) (SCG2)	NM_003469
2.72	Natural killer cell transcript 4 (NK4)	NM_004221
2.71	5-hydroxytryptamine (serotonin) receptor 3A (HTR3A)	NM_000869
2.65	Cathepsin H (CTSH)	NM_004390
2.64	Bcl-2-related ovarian killer protein-like (BOKL)	NM_032515
2.63	Melanocortin 4 receptor (MC4R)	NM_005912
2.62	Deoxyribonuclease I-like 3 (DNASE1L3)	NM_004944
2.59	Interleukin 18 (interferon-gamma-inducing factor) (IL18)	NM_001562
2.58	Synaptotagmin 5 (SYT5)	NM_003180
2.56	Clone 23582	AF038190
2.45	MEMD protein (ALCAM)	Y10183
2.38	Granzyme a (granzyme 1, cytotoxic t-lymphocyte-associated serine esterase 3) (GZMA)	NM_006144
2.37	Protein kinase C (PKC) type beta II	X07109
2.37	Vanilloid receptor-like protein (VRL)	NM_016113
2.36	Peripherin (PFPH)	NM_006262
2.34	Aldolase C, fructose-bisphosphate (ALDOC)	NM_005165
2.30	Regulator of G-protein signalling 5 (RGS5)	NM_003617
2.28	Neuronatin (NNAT)	NM_005386
2.25	GM-CSF receptor beta chain (CSF2RB)	NM_000395

*Ratio represents the fold of overexpression in the tumors with low telomerase activity (Cy3) to those with high telomerase activity (Cy5).

DISCUSSION

The patients whose tumors had high telomerase activity showed significantly worse outcomes. Thus, high telomerase activity in neuroblastoma is a useful prognostic factor. In the current study, we identified 63 genes that were overexpressed in the tumors with high telomerase activity and 46 genes overexpressed in those with low telomerase activity. The feasibility of this approach was shown from the fact that several genes well known to be highly expressed in aggressive neuroblastomas such as *MYCN,* neuroblastoma-amplified protein (*LOC51594*), and *hTERT,* were overexpressed in the former group, whereas trk A was overexpressed in the latter group. It was proved previously that MYC transcription factor directly activates telomerase in primary human fibroblasts through the induction of *hTERT* expression.[11] Thus, N-*myc* might have a similar function for *hTERT* expression.

In general, the overexpressed genes in tumors with high telomerase activity included genes involved in cell cycle, apoptosis-escape, protein synthesis, and transcription (Table 1). In addition, *MAGEA4* gene, which had

been detected in melanoma, was also overexpressed as previously reported in aggressive neuroblastoma.[12] Overexpression of *GABRA5* (gamma-aminobutyric acid A receptor-alpha 5), a well-known imprinting gene in chromosome 15,[13] suggested the existence of loss of imprinting in aggressive neuroblastoma. *BLM* (Bloom syndrome) gene is a repair gene of double strand breaks,[14] and it might coact with telomerase, which repairs the single strand telomere end.

On the other hand, in the tumors with low telomerase activity, neuronal transmitters such as neuropeptide Y, synaptic vesicle protein, and neuronatin (NNAT) were overexpressed (Table 2). Moreover, these tumors showed overexpression of several receptors such as glucagon receptor, trk A, and melanocortin 4 receptor. These data suggest that signal transduction is running well in these tumors to induce regression or maturation of neuroblastoma. The overexpression of the candidate mediator of the p53-dependent arrest (*REPRIMO*) and BCL2-related ovarian killer proteinlike (*BOKL*) genes might indicate the existence of a functional apoptosis pathway in these tumors. Recently, it was shown that telomere shortening without sufficient telomerase activity induces p53-dependent cell apoptosis.[15] Thus, the existence of the p53-dependent apoptotic mechanism in neuroblastoma should be clarified.

Differentiation/growth arrest of tumor cells with low telomerase activity suggested that the signal transduction of differentiation/growth arrest is closely related to telomerase activity in neuroblastoma. Because high telomerase activity is related to poor prognosis, genes for cell cycle, apoptosis escape, protein synthesis, and transcription factors would be candidates for new prognostic factors. Moreover, these genes would be candidates for new therapeutic targets in aggressive neuroblastomas.

REFERENCES

1. Young JL, Ries LG, Silverberg E, et al: Cancer incidence, survival, and mortality for children under 15 years of age. Cancer 56:598-602, 1987
2. Brodeur GM: Neuroblastoma: Biological insights into a clinical enigma. Nat Rev Cancer 3:203-216, 2003
3. Schena M, Shalon D, Davis RW, et al: Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 270:467-470, 1995
4. Osin P, Shipley J, Lu YJ, et al: Experimental pathology and breast cancer genetics: New technologies. Recent Results Cancer Res 152: 35-48, 1998
5. Hiyama E, Hiyama K: Clinical Application of Microarrays as a Tool of Understanding Cancer Biology, in Pandalai SG (ed): Recent Research Developments in Cancer, Vol 4—2002 Part II. Kerala, India, Transworld Research Network, 2002, pp 675-694
6. Hiyama E, Hiyama K: Clinical utility of telomerase in cancer. Oncogene 21:643-649, 2002
7. Hiyama E, Hiyama K, Yokoyama T, et al: Correlating telomerase activity levels with human neuroblastoma outcomes. Nature Med 1:249-255, 1995
8. Hiyama E, Hiyama K, Ohtsu K, et al: Telomerase activity in neuroblastoma: Is it a prognostic indicator of clinical behavior? Eur J Cancer 33:1932-1936, 1997
9. Kushner BH, Cheung NK, LaQuaglia MP, et al: International neuroblastoma staging system stage 1 neuroblastoma: A prospective study and literature review. J Clin Oncol 14:2174-2180, 1996
10. Kim NW, Piatyszek MA, Prowse KR, et al: Specific association of human telomerase activity with immortal cells and cancer. Science 266:2011-2015, 1994
11. Greenberg RA, O'Hagan RC, Deng H, et al: Telomerase reverse transcriptase gene is a direct target of c-Myc but is not functionally equivalent in cellular transformation. Oncogene 18:1219-1226, 1999
12. Cheung IY, Cheung NK: Detection of microscopic disease: Comparing histology, immunocytology, and RT-PCR of tyrosine hydroxylase, GAGE, and MAGE. Med Pediatr Oncol 36:210-212, 2001
13. Song J, Koller DL, Foroud T, et al: Association of GABAA receptors and alcohol dependence and the effects of genetic imprinting. Am J Med Genet 117B:39-45, 2003
14. Mohaghegh P, Hickson ID: Premature aging in RecQ helicase-deficient human syndromes. Int J Biochem Cell Biol 34:1496-1501, 2002
15. Saretzki G, Sitte N, Merkel U, et al: Telomere shortening triggers a p53-dependent cell cycle arrest via accumulation of G-rich single stranded DNA fragments. Oncogene 18:5148-5158, 1999