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Original Paper

Telomerase Activity in Neuroblastoma: is it a Prognostic Indicator of Clinical Behaviour?

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Neuroblastomas show remarkable biological heterogeneity, resulting in favourable prognosis or unfavourable prognosis due to aggressive growth despite multimodal therapy. Recently, we proposed that aggressive tumours express telomerase at a high level while the favourable tumours lack or have low telomerase expression. To evaluate the correlation between telomerase activity and other biological characteristics reported as prognostic markers (*MYCN* gene amplification, loss of heterogeneity (LOH) in the short arm of chromosome 1, *trk*-A expression, Ha-*ras* p21 expression, and DNA ploidy), we investigated these biological features in 105 untreated neuroblastomas. In these cases, 23 showed high telomerase activity, 78 showed low activity, and telomerase activity was undetectable in 4 cases. Most tumours with genetic alterations (*MYCN* amplification or 1p32 LOH) showed high telomerase activity. Most tumours with low or undetectable activity were aneuploid, and showed *trk*-A and Ha-*ras* expression. Three of the four tumours with undetectable telomerase activity regressed. In 2 of the tumours with low telomerase activity, the residual tumours maturated and showed repression of telomerase activity. Thus, the level of telomerase activity correlated with other genetic alterations and/or gene expression and may be a useful prognostic indicator in neuroblastoma. © 1997 Elsevier Science Ltd.

Key words: neuroblastoma, telomerase, telomere, biology, prognosis, regression, maturation, *MYCN*, *trk*-A, ploidy
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INTRODUCTION

NEUROBLASTOMA ARISES from the embryonal neural crest and is the most common solid tumour in children affecting approximately 1 in 7000 individuals. Clinically, neuroblastoma tumours show remarkable biological heterogeneity, resulting in both favourable and unfavourable prognosis, the latter due to aggressive growth despite multimodal therapy [1]. In order to predict the biological behaviour of an individual tumour more precisely, there have been several parameters proposed to predict prognosis of neuroblastoma patients. These parameters, which have been reported to have significant correlation with neuroblastoma's biological behaviour, include *MYCN* amplification [2], *trk*-A expression [3], Ha-*ras* p21 expression [4] and cellular DNA content [5]. However, each of these parameters appears to be insufficient to predict the prognosis of patients completely.

Telomeres are specialised structures at the ends of eukaryotic chromosomes which are thought to be important in the protection and replication of chromosomes [6]. Due to the end replication problem [7], cell division results in a gradual reduction of telomeres. Telomerase, an RNA-dependent DNA polymerase, which compensates for the end replication problem, is expressed in germ-line cells, but not in most of somatic cells, except haematopoietic cells, lymphocytes [8], skin keratinocytes [9] and cells in the crypt of the intestine [10],

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due to the repression of telomerase during development. This shortening of telomeres, without sufficient compensation by telomerase, has been proposed as the mitotic clock by which cells 'count' their divisions [11], and the progressive shortening of telomeric repeats may contribute to cellular senescence. The reactivation of telomerase and the stabilisation of telomeres appear to be concomitant with the attainment of immortality in tumour cells [12]. In our previous study [13], telomerase activity was detected in 94% of 100 neuroblastoma specimens, which suggests this enzyme may play an important role in neuroblastoma development. From the analysis of telomere length and semiquantitative analysis of telomerase activity in neuroblastoma [13, 14], it was discovered that although both aggressive and regressing neuroblastoma tumours contain short telomeres, the aggressive tumours expressed high telomerase activity while the regressing tumours showed no detectable or insufficient telomerase activity. Thus, telomerase activity may be a useful indicator of outcome in neuroblastoma. In the present study, to investigate the relationship between telomerase activity levels and other proposed prognosis-predicting factors, we compared these indicators in 105 clinical neuroblastoma cases; 79 of these cases were also included in a previous study [13].

PATIENTS AND METHODS

Patients

The patients with neuroblastoma in the present study were diagnosed at the Hiroshima University Hospital or referred for molecular analysis from other hospitals in Japan between 1983 and 1995. Patients were staged according to the standard clinical and pathological criteria of Evans [15].

Tissue samples

Tumours were obtained from 105 consecutive neuroblastoma patients untreated before surgery. In these patients, 21 adjacent normal adrenal glands were obtained. All tissues were stored at −80°C until use. Written informed consent was obtained from patients or parents before surgery.

Telomerase assay

Extracts of tissue specimens and assays of telomerase activity were performed as described previously [12, 16]. Briefly, frozen samples were homogenised in CHAPS lysis buffer. After 25 min incubation on ice, the lysate was centrifuged. An aliquot of extract containing 6 μg of protein was used for each TRAP (telomeric repeat amplification protocol) assay unless otherwise indicated. For RNase treatment, 5 μl of extract was incubated with 1 μg RNase (Boehhringer Mannheim Corp., Indianapolis, Indiana, U.S.A.) for 20 min at 37°C. Assay tubes were prepared by sequestering of 0.1 μg of CX primer (5′-CCCTTACCCTTACCCTTACCCTAA -3′) under wax barrier (Ampliwax™, Perkin-Elmer, Foster City, California, U.S.A.). Each extract was assayed in 50 μl of reaction mixture containing TS oligonucleotide (5′-AATCCGTCGAGCAGAGTT -3′) as described previously [12, 13, 16]. Each reaction mixture also contained an Internal Telomerase Assay Standard (ITAS) for the identification of false-negative tumour samples that contain telomerase assay inhibitors [17]. After 30 min incubation at room temperature for telomerase-mediated extension of the TS primer, the reaction mixture was subjected to 31 PCR cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 45 s. The PCR product was electrophoresed on a 10% polyacrylamide gel. For estimation of telomerase activity, serial dilutions of positive extracts were re-examined: 10-fold dilution contained 0.6 μg of protein and 100-fold dilution contained 0.06 μg. The levels of telomerase activity were classified into three groups: undetectable, low (telomerase mediated signals positive but negative using 100-fold diluted extracts), high (positive using 100-fold diluted extracts).

Telomere length analysis by Southern blot analysis

Genomic DNA was isolated from frozen tissues as previously described [14]. For TRF (terminal restriction fragment) analysis, 2 μg of DNA were digested to completion with 10 units of *Hin*f I, electrophoresed on 0.8% agarose gels and then blotted on to nitrocellulose filters. The filters were hybridised to a ^{32}P-labelled $(TTAGGG)_4$ probe and washed as previously reported [14], and then autoradiographed. We estimated the mean length of TRFs at the peak position of hybridisation signal.

Analysis of MYC*N gene amplification by Southern blot analysis*

Two μg of DNA were digested to completion with 10 U of *Eco*RI, electrophoresed on 1.0% agarose gels and then blotted on to nitrocellulose filters. The filters were hybridised to an *MYCN* probe (PN-*myc*·1, Oncor Inc. Gaithersburg, Maryland, U.S.A.) and autoradiographed as previously described [14].

Loss of heterozygosity (LOH) analysis of 1*p*32

Restriction fragment length polymorphisms (RFLP) at an *Eco*RI site in the *MYCL* gene was analysed as previously described [13]. In addition, the tetranucleotide polymorphism 16 kb upstream from the *MYCL* gene was analysed using the reported primers [18]. One primer set was end-labelled with [γ-^{32}P]ATP, and the PCR (polymerase chain reaction) was carried out using the reported conditions. The amplified DNA products were separated by 5% denaturing polyacrylamide gel electrophoresis and then subjected to autoradiography using X-ray film and a BAS2000 bioimage analyser (Fuji, Tokyo, Japan).

Northern blot analysis

For each sample, 10 μg of total RNA were electrophoresed on 1.5% agarose gels and blotted on to nitrocellulose filters. These filters were separately hybridised with ^{32}P-labelled *trk*-A probe and β-actin probe, washed with 0.1 × SSC (standard saline citrate) and 0.1% SDS (sodium dodecyl sulfate) at 50°C and then autoradiographed. The *trk*-A probe was a gift from Luis Parada [19]. The *trk*-A RNA hybridisation signal for each tumour was standardised to the β-actin signals. The intensity of the *trk*-A signal was evaluated by BAS2000 bioimage analyser (Fuji) and the individual tumours were classified into one of three categories: negative, low (< half of β-actin signal) and high (≥ half of β-actin signal).

Immunohistochemical analysis

Immunostaining for Ha-*ras* p21 was performed using an antibody specific to Ha-*ras* p21 (Oncogene Science, Manhasset, New York, U.S.A.) according to the report described previously [4]. Briefly, immunohistochemical analysis was done in formalin-fixed and paraffin-embedded tissue sections by the avidin–biotin complex (ABC) immunoperoxidase staining technique. The sections were counterstained lightly with haematoxylin or methyl green. The intensity of the immunostaining was evaluated by light microscopy and the

individual tumours were classified into one of three categories: negative, low (weakly or partially positive) and high (definitely positive).

Flow cytometric analysis of cellular DNA content

Frozen samples were thawed at 20°C and cut into small pieces. Suspensions of single nuclei were prepared using the detergent–trypsin procedure of Vindeløv and associates [20], and stained with propidium iodide (Becton Dickinson, Mountain View, California, U.S.A.). Measurement of DNA cellular content was performed using the FACScan flow cytometer (Becton Dickinson). In the analysis of 2×10^4 nuclei per each sample, the DNA index (DI) was determined by calculating the ratio of the modal channel number for tumour G0/G1 phase cells to that for normal diploid cells. Tumours with a DI of 1.11–1.89 were defined as aneuploid. Tumours were considered tetraploid if a peak occurred with a DI of 1.90–2.10 in at least 20% of the analysed cells or if a peak corresponding to a G2-M tetraploid cell population was present. When more than two different distinct G0–G1 cell populations with DI = 1.0 were present, the tumour was classified as polyploid.

Statistical analysis

For statistical analysis, we divided untreated neuroblastomas into two groups: tumours with undetectable or low telomerase activity and tumours with high telomerase activity (positive using 100-fold dilution of extract). Between these two groups, age at diagnosis were compared using the Mann–Whitney test and other factors were compared using Fisher's exact test or the chi-square test, when appropriate.

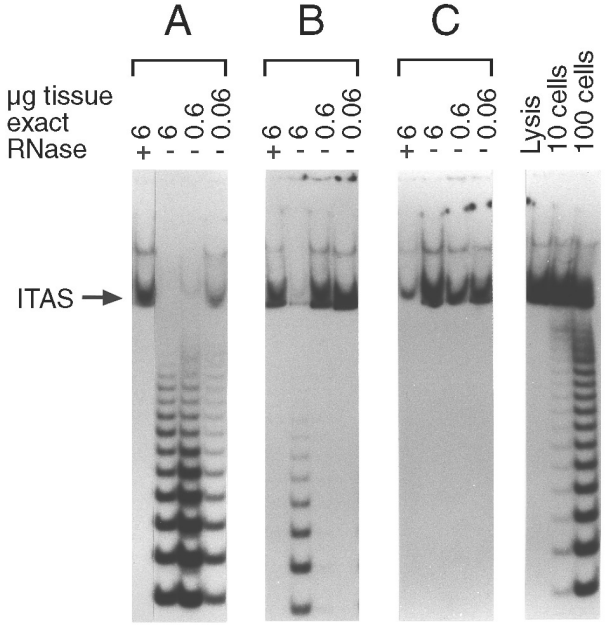

Figure 1. Telomerase activity in neuroblastoma tissues. Telomerase activity signals with (+) or without (−) RNase pretreatment of extract. Tumour samples were examined by serial dilution (extracts containing 6, 0.6 and 0.06 μg of protein). Case A showed high activity with strong 6-bp ladder signals using extracts containing 6 and 0.6 μg of protein and retained signals using extract containing 0.06 μg of protein (high telomerase activity). Case B showed low telomerase activity (telomerase-mediated signals positive but negative using 100-fold diluted extracts). Case C showed no detectable telomerase activity.

RESULTS

Telomerase activity in neuroblastomas

Telomerase activity was detected in 101 (96%) of 105 untreated neuroblastoma specimens using the TRAP assay. According to the serial dilution analysis of telomerase assay, the 101 tumours with positive telomerase signals were divided into two groups: 23 tumours (23%) had high telomerase activity (e.g., retained a TRAP signal after 100-fold dilution of the extract) and 78 tumours (77%) had low telomerase activity (Figure 1). No obvious differences in histology were observed between these two groups. However, telomerase activity was undetectable in 4 tumours (4%), of which 3 were stage IV-S tumours which regressed and 1 was a stage I tumour which was surgically removed.

The relationship between telomerase activity and clinical data are shown in Table 1. The median age at diagnosis for those with tumours with low or undetectable activity was significantly younger than that with high telomerase activity ($P < 0.01$). The stages of tumours with high telomerase activity were significantly advanced compared with those with low or undetectable activity. Currently, 13 cases (57%) with high telomerase activity and 2 cases (2%) with low telomerase activity have died of disease ($P < 0.001$). In 3 of 78 cases in which the primary tumours showed low telomerase activity, a second-look operation was necessary 7–12 months after the initial operation because of residual tumour after chemotherapy. Interestingly, 2 of these 3 residual tumours became ganglioneuroma at histopathological examination, and telomerase activity was undetectable in both. The other case retained low telomerase activity and contained numerous and histologically variable neuroblasts. Three of 4 cases with undetectable telomerase activity (stage IVS) regressed as reported previously [13], and all 4 cases are alive and disease free.

Table 1. *Correlation between telomerase activity and other prognosis-associated factors in neuroblastoma cases*

Telomerase activity	Undetectable ($n = 4$)	Low ($n = 78$)	High ($n = 23$)
Age at diagnosis (range, month)	6–9	1–96	7–129
(median)	(8)	(8)	(24)
Stage			
I, II ($n = 51$)	0	48	3
III, IV ($n = 45$)	1	25	19
IV-S ($n = 9$)	3	5	1
Alteration of TRFs	3	11	17
(reduced TRFs/elongated TRFs)	(3/0)	(11/0)	(14/3)
MYCN amplification	0	0	15
MYCL LOH/informative cases	0/2	1/34	8/13
Trk-A expression			
high	2	47	4
low	1	17	2
Ha-*ras* p21 expression			
high	3	45	1
low	0	25	5
DNA ploidy			
aneuploid	1	47	5
tetraploid	0	2	2
polyploid	0	3	0

Alterations of terminal restriction fragments (TRFs) were present in 28 cases showing reduced TRFs (<8 kb) and 3 with elongated TRFs (>15 kb).

Telomere length of neuroblastoma

Terminal restriction fragments (TRFs), an indicator of telomere length, were estimated at the peak position of the hybridisation signal with a ^{32}P-labelled $(TTAGGG)_4$ probe. The lengths of TRFs in normal adrenal glands of neuroblastoma patients ranged from 8 and 15 kb [14, 23]. Thus, we considered that reduced TRFs were <8 kb and elongated TRFs were >15 kb. TRF analysis revealed that 28/105 (27%) of the untreated tumour samples had reduced TRFs (<8 kb) and 3/105 had elongated TRFs (>15 kb). The other 74 had TRF lengths indistinguishable from those of adjacent normal adrenal glands. While 14 (61%) and 3 (13%) of the 23 tumours with high telomerase activity showed reduced and elongated TRF lengths, respectively, only 14 of 82 tumours (17%) with low or undetectable telomerase activity showed reduced TRF lengths and no elongated TRF lengths were detected among these cases. These differences were significant ($P < 0.001$).

Correlation with other prognosis-predicting biological factors (Table 1)

In 23 tumours with high telomerase activity, 15 showed *MYCN* gene amplification by Southern blot analysis (3–250 copies). In contrast, all 82 tumours with undetectable or low telomerase activity contained a single copy of *MYCN* gene. Thus, *MYCN* amplification was significantly correlated with high telomerase activity ($P < 0.001$). LOH analysis at the *MYCL* locus (1p32) revealed that LOH was detected in 8 (62%) of 13 informative cases with high telomerase activity and only one (3%) of 34 informative cases with low telomerase activity. *Trk*-A and Ha-*ras* p21 expression was detected in 73 (70%) and 79 (75%) in 105 tumours, respectively. 67/82 (82%) tumours with undetectable or low telomerase activity expressed *trk*-A and 73 (89%) expressed Ha-*ras* p21. However, in 23 tumours with high telomerase activity, *trk*-A and Ha-*ras* p21 were detected in only 6 (26%) and 6 (26%), respectively. There was a significant correlation between undetectable or low telomerase activity and the expression levels of *trk*-A and Ha-*ras* p21 ($P < 0.0001$). Flow-cytometric analysis of cellular DNA content revealed that 53 tumours were aneuploid, 45 were diploid, 4 were triploid and 3 were polyploid. In 23 tumours with high telomerase activity, only 5 (22%) were aneuploid, while 48 (59%) of 82 tumours with undetectable or low telomerase activity were aneuploid. Thus, an aneuploid pattern were significantly correlated with undetectable or low telomerase activity ($P = 0.004$).

DISCUSSION

It is apparent that neuroblastoma has at least two disparate clinical outcomes: an excellent prognosis for patients who usually have early stages of disease and/or young age (favourable neuroblastoma) and a very poor prognosis who usually have advanced stages and/or old age (unfavourable neuroblastoma) [1]. However, some disseminated tumours regress (stage IVS) and some early tumours show poor prognosis. Thus, the clinical behaviour of neuroblastomas depend mainly on the characteristics of tumour cells as well as stage of disease. Efforts to understand these diverse clinical behaviours have been spent to identify the genetic events that characterise these subsets of neuroblastoma. The most frequent abnormalities in neuroblastoma are *MYCN* gene amplification [2] and deletion of the short arm of chromosome 1 [22, 23]. Although *MYCN* amplification is a well-known critical gene affecting poor prognosis, the tumours without *MYCN* amplification are not always favourable neuroblastomas [24]. Aneuploidy, especially hyperdiploidy, is reported to be associated with a favourable outcome in infant neuroblastoma [5]. The expression of *trk*-A, high-affinity nerve growth factor (NGF) receptor [3] and Ha-*ras* p21 [4] have also been reported to be associated with a favourable outcome in neuroblastoma patients.

In human germline cells, telomerase is present to maintain telomere length, while in embryonal somatic tissues telomerase is likely to be repressed gradually as cells differentiate. In immortal cells, it has been reported that telomerase is re-expressed and telomere lengths are stabilised to allow continued proliferation [25]. From our previous results [13, 14], we proposed two different pathways leading to the development of neuroblastoma: one involves a failure to repress telomerase activity during development (retention of telomerase activity in the germ line cell), and the other involves the reactivation of telomerase accompanied by other genetic alterations, similar to adult cancers. Two disparate clinical outcomes in neuroblastoma are considered to be derived from these two different pathways. In the present study, genetic alterations such as *MYCN* amplification and deletion of chromosome 1p32 (*MYCL* locus) were almost always detected in the tumours with high telomerase activity, except for one case with low telomerase activity and LOH at the *MYCL* locus. In fact, TRF length analysis revealed that telomere lengths are likely to be stabilised at various lengths in the tumours with high telomerase activity, while those in the tumours with undetectable or low telomerase activity are not likely to have stabilised, but demonstrate similar or shorter TRF lengths compared to those in normal tissues. Thus, the tumours with high telomerase activity are considered to have acquired immortality accompanied by several genetic alterations.

However, most tumours with undetectable or low telomerase activity showed high *trk*-A and Ha-*ras* p21 expression without genetic alterations. Developing neuroblasts require NGF for survival [26]. *Trk*-A is essential in the mediation of NGF stimuli that regulate the differentiation or programmed cell death of neuroblasts [27] and Ha-*ras* p21 is likely to be one of the participants in NGF signal transduction and cellular responsiveness [28]. The hypothesis is that the neuroblasts in tumours with undetectable or low telomerase activity may have the potential to respond to neurotrophic factors leading to regression or maturation concomitant with repression of telomerase activity.

Aneuploidy, especially hyperdiploidy, is reported to be associated with a favourable outcome of infant neuroblastoma [5]. Indeed, in our series, most aneuploid tumours in infants showed low telomerase activity, which may indicate that most aneuploid neuroblastomas consist of developing neuroblasts. However, some aneuploid tumours which were diagnosed at an older age showed high telomerase activity and genetic alterations. Approximately half the tumours with low telomerase activity, three regressed tumours without detectable telomerase activity and all ganglioneuromas were diploid. Thus, the aneuploid pattern is not a clear prognosis-predicting factor.

In a previous study [13], it appeared that in most stage IVS neuroblastomas, telomerase activity is insufficient to maintain telomere length and the cells eventually stop proliferating with complete repression of telomerase. This resembles

telomerase activity in normal fetal neuroblasts which is repressed before birth. In the present study, 2 infant neuroblastomas with low telomerase activity matured. Maturation of neuroblastoma might occur concomitant with telomerase repression as observed in HL-60 human promyelocytic leukaemia cells [29, 30].

In summary, telomerase activity in neuroblastomas can predict clinical behaviour which may be caused by two different pathways leading to the development of neuroblastoma. The correlation between telomerase activity and other prognosis-predicting factors indicates that the levels of telomerase activity reflect the distinct biological characteristics of neuroblastoma cells. More detailed analysis will be needed to identify the most valuable prognostic factor in neuroblastoma and a more thorough understanding of the regulation of telomerase may eventually lead to therapeutic applications against unfavourable neuroblastomas.

1. Woods WG, Lemieux B, Tuchman M. Neuroblastoma represent distinct clinical-biologic entities: a review and perspective from Quebec neuroblastoma screening project. *Pediatrics* 1992, **88**, 114–118.
2. Brodeur GM, Seeger RC, Schwab M, Varmus HE, Bishop JM. Amplification of N-*myc* in untreated human neuroblastoma correlated with advanced stage. *Science* 1984, **224**, 1121–1124.
3. Nakagawara A, Arima-Nakagawara M, Scavarda NJ, *et al.* Association between high levels of expression of the trk gene and favorable outcome in human neuroblastoma. *N Engl J Med* 1993, **328**, 847–854.
4. Tanaka T, Salmon DJ, Shimada H, *et al.* A significant association of Ha-ras p21 in neuroblastoma cells with patient prognosis. *Cancer* 1991, **68**, 1296–1302.
5. Look AT, Hayes FA, Nitschke R, McWilliams NB, Green AA. Cellular DNA content as predictor of response to chemotherapy in infants with unresectable neuroblastoma. *N Engl J Med* 1984, **311**, 231–235.
6. Blackburn EH. Telomerases. *Annu Rev Biochem* 1992, **61**, 113–129.
7. Watson JD. Origin of concatemeric T7 DNA. *Nature New Biol (London)* 1972, **239**, 197–201.
8. Hiyama K, Hirai Y, Kyoizumi S, *et al.* Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. *J Immunol* 1995, **155**, 3711–3715.
9. Taylor RS, Ramirez RD, Ogoshi M, Chaffins M, Piatyszek MA, Shay JW. Detection of telomerase activity in malignant and nonmalignant skin condition. *J Invest Dermatol* 1996, **106**, 756–765.
10. Hiyama E, Hiyama K, Tatsumoto N, Kodama T, Shay JW, Yokoyama T. Telomerase activity in human intestine. *Int J Oncol* 1996, **9**, 453–458.
11. Harley CB. Telomere loss: mitotic clock or genetic time bomb? *Mutat Res* 1991, **256**, 271–282.
12. Kim NW, Piatyszek MA, Prowse KR, *et al.* Specific association of human telomerase activity with immortal cells and cancer. *Science* 1994, **266**, 2011–2015.
13. Hiyama E, Hiyama K, Yokoyama T, *et al.* Correlating telomerase activity levels with human neuroblastoma outcomes. *Nature Med* 1995, **1**, 249–255.
14. Hiyama E, Hiyama K, Yokoyama T, Ichikawa T, Matsuura Y. Length of telomeric repeats in neuroblastoma: correlation with prognosis and other biological characteristics. *Jpn J Cancer Res* 1992, **83**, 159–164.
15. Evans AE. A proposed staging for children with neuroblastoma. *Cancer* 1971, **27**, 374–378.
16. Piatyszek MA, Kim NW, Weinrich SL, *et al.* Detection of telomerase activity in human cells and tumors by a telomeric repeat amplification protocol (TRAP). *Methods Cell Sci* 1995, **17**, 1–15.
17. Wright WE, Shay JW, Piatyszek MA. Modification of a telomeric repeat amplification protocol (TRAP) result in increased reliability, linearity and sensitivity. *Nucleic Acid Res* 1995, **23**, 3794–3795.
18. Mäkelä TP, Hellsten E, Vesa J, Alitalo K, Peltonen L. An Alu variable poly A repeat polymorphism upstream of L-*myc* at 1p32. *Human Mol Genet* 1992, **1**, 217.
19. Kaplan DR, Hempstead BL, Martin-Zanka D, Chao MV, Parada LF. The trk proto-oncogene product: a signal transducting receptor for nerve growth factor. *Science* 1991, **252**, 554–558.
20. Vindeløv LL, Christensen IJ, Nissen NI. Detergent-tripsin method for the preparation of nuclei for flow cytometric DNA analysis. *Cytometry* 1983, **3**, 323–327.
21. Hiyama E, Yokoyama T, Hiyama K, *et al.* Alteration of telomeric repeat length in adult and childhood solid neoplasias. *Int J Oncol* 1995, **6**, 13–16.
22. Fong CT, Dracopoli NC, White PS, *et al.* Loss of heterozygosity for the short arm of chromosome 1 in human neuroblastoma: correlation with N-*myc* amplification. *Proc Natl Acad Sci USA* 1989, **86**, 3753–3757.
23. Takeda O, Homma C, Maseki N, *et al.* There may be two tumor suppressor genes on chromosome arm 1p closely associated with biologically distinct subtypes of neuroblastoma. *Genes Chrom Cancer* 1994, **10**, 30–39.
24. Hiyama E, Hiyama K, Yokoyama T, Ishii T. Immunohistochemical analysis of N-myc protein expression in neuroblastoma: Correlation with prognosis of patients. *J Pediatr Surg* 1991, **26**, 838–843.
25. Shay JW. Aging and cancer: are telomeres and telomerase the connection? *Mol Med Today* 1995, **1**, 378–384.
26. Chen J, Chattopadhyay B, Venkatakrishnan G, Ross AH. NGF-induced differentiation of human neuroblastoma and neuroepithelioma cell line. *Cell Growth Differ* 1990, **1**, 79–85.
27. Matsushima H, Bogenmann E. Expression of trk A cDNA in neuroblastomas mediated differentiation *in vitro* and *in vivo*. *Mol Cell Biol* 1993, **13**, 7447–7456.
28. Wood KW, Sarnecki C, Roberts TM, Blenis J. Ras mediated nerve growth factor receptor modulation of three signal-transducting protein kinase: MAP kinase, Raf-1, and RSK. *Cell* 1992, **68**, 1041–1050.
29. Sharma HW, Sokoloski JA, Perez JR, *et al.* Differetiation of immortal cells inhibits telomerase activity. *Proc Natl Acad Sci USA* 1995, **92**, 12343–12346.
30. Zhang W, Piatyszek MA, Estey E, *et al.* Telomerase activity in acute myelogenous leukemia: inhibition of telomerase activity by differentiation-inducing agent. *Clin Cancer Res* 1996, **2**, 799–803.

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