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Length of Telomeric Repeats in Neuroblastoma: Correlation with Prognosis and Other Biological Characteristics

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Telomeres are the physical ends of eukaryotic chromosomes. In view of reports of the reduction of telomeric repeats in human malignant tumors, we measured the lengths of telomeric repeats in 55 primary neuroblastomas. The average lengths of telomeric repeats in these tumors fell in a wide range (from 1.1 kb to more than 23 kb) relative to those in ganglioneuromas and normal peripheral mononuclear cells. The reduction of telomeric repeats was significantly correlated with advanced stages of tumor development, poor prognosis, and increased S-phase fractions in tumor cells. On the other hand, three cases of Stage IV-S tumors showed the reduction of telomeric repeats and low percentage of S-phase fractions. These Stage IV-S patients had a good prognosis with spontaneous regression of metastatic tumors.

Key words: Neuroblastoma — Telomere — Prognosis — Progression

Human chromosomes, like those of other eukaryotes, end in characteristic G-rich repeats called telomeres, which prevent end-to-end fusion and exonucleolytic degradation.[1,2] The average lengths of telomeric repeats in blood and sperm DNA are estimated to be 10 kb and 15 kb, respectively, and this difference is due to the variable number of TTAGGG repeats.[3–5] The reduction of telomeric repeats has been found to occur in human carcinomas[5,6] and with aging in normal tissues.[6,7] It is suspected that the length of telomeric repeats decreases after many cell divisions.

Neuroblastomas are one of the common solid tumors found in children and they are highly variable in malignancy. Although some neuroblastomas spontaneously mature or regress, others are highly aggressive and rapidly fatal. The clinical staging of the tumor is an imperfect indicator of a patient's prognosis, because the prognosis mainly depends upon the biological characteristics of the tumor cells. Recently, several prognostic factors that can be assessed at diagnosis have been identified; histopathological classification,[8] the amount of the tumor cell DNA measured by flow cytometry,[9–11] N-*myc* gene amplification in the tumor cell DNA,[12,13] and so on.

We hypothesized that the reduction of telomeric repeats is associated with tumor progression in neuroblastoma. When DNA is digested with a frequently cutting restriction enzyme such as *Hin*fI, *Rsa*I, or *Hae*III, which will not cut the telomeric repeats, subsequent probing with a labeled oligonucleotide for (TTAGGG)n will reveal the size of the fragment consisting mostly of telomeric DNA sequences. Therefore, we measured the length of telomeric repeats of neuroblastoma cell DNA and analyzed its association with the patients' prognosis, as well as other prognosis-associated factors.

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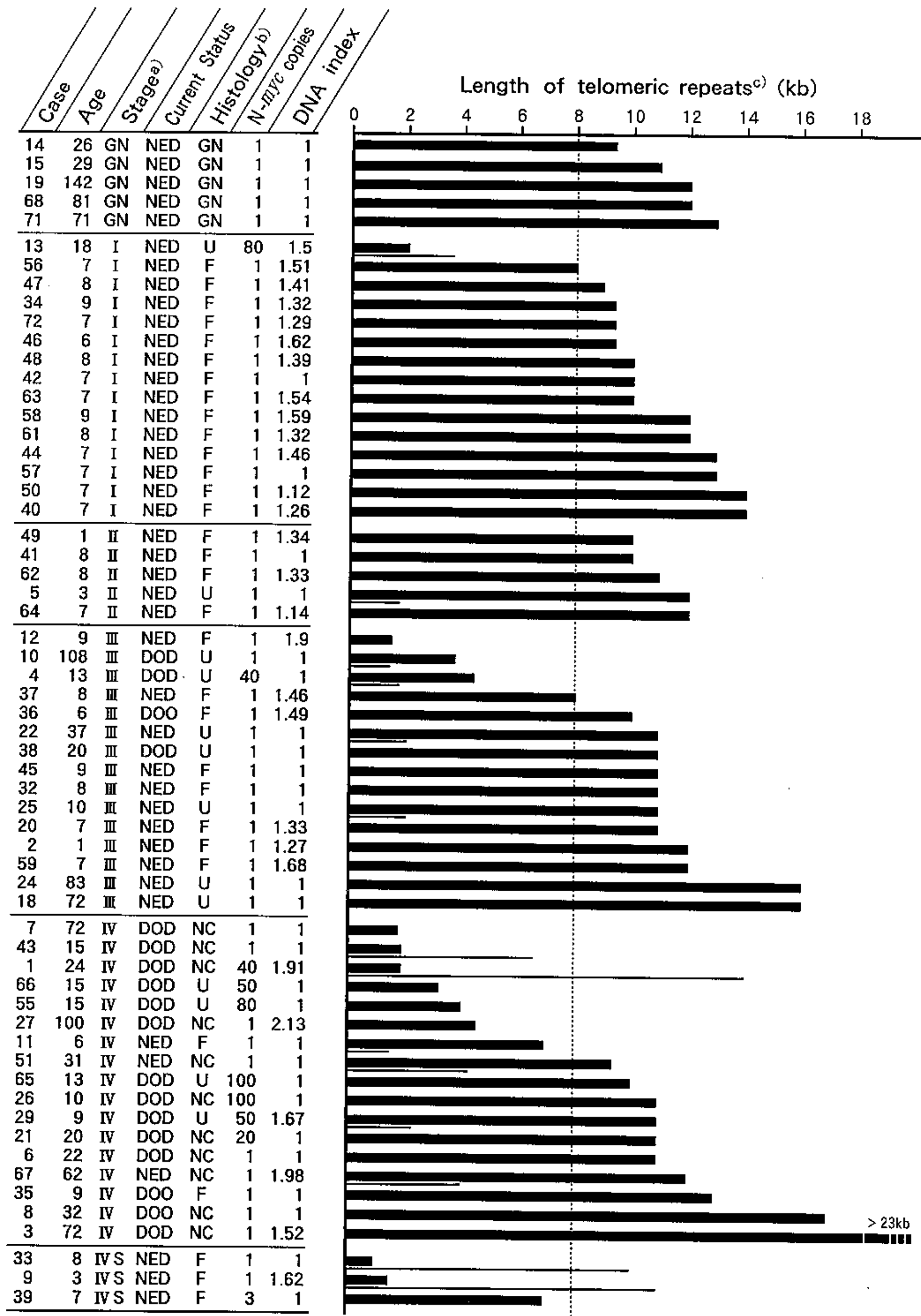
MATERIALS AND METHODS

Patients Fifty-five patients with neuroblastoma who were operated on between 1984 and 1990 in our hospital were enrolled in this study. Tumors were staged according to the standard clinical and pathological criteria of Evans *et al.*[14] This staging system divided patients with disseminated disease into two subgroups termed Stage IV and IV-S (S for 'special'). The Stage IV-S patients had Stage I or II primary tumors with disseminated disease restricted to the liver, skin, or bone marrow. The age at diagnosis, sex, clinical stage, and current status for each patient are summarized in Fig. 1. Histological types of tumors were classified according to the criteria of Shimada *et al.*[8] Patients at Stages I, II, and IV-S were treated with primary surgery and chemotherapy consisting of cyclophosphamide, vincristine, and doxorubicin and those at Stages III and IV were treated with primary, delayed primary, or second-look surgery and multidrug chemotherapy consisting of cyclophosphamide, vincristine, doxorubicin, *cis*-dichlorodiamine platinum, and etoposide.

Tumor samples and DNA analysis We stored the 55 primary neuroblastoma and 5 ganglioneuroma specimens immediately after the operations at −70°C until DNA isolation was performed. Genomic DNA from these



Case	Age	Stage a)	Current Status	Histology b)	N-myc copies	DNA index
14	26	GN	NED	GN	1	1
15	29	GN	NED	GN	1	1
19	142	GN	NED	GN	1	1
68	81	GN	NED	GN	1	1
71	71	GN	NED	GN	1	1
13	18	I	NED	U	80	1.5
56	7	I	NED	F	1	1.51
47	8	I	NED	F	1	1.41
34	9	I	NED	F	1	1.32
72	7	I	NED	F	1	1.29
46	6	I	NED	F	1	1.62
48	8	I	NED	F	1	1.39
42	7	I	NED	F	1	1
63	7	I	NED	F	1	1.54
58	9	I	NED	F	1	1.59
61	8	I	NED	F	1	1.32
44	7	I	NED	F	1	1.46
57	7	I	NED	F	1	1
50	7	I	NED	F	1	1.12
40	7	I	NED	F	1	1.26
49	1	II	NED	F	1	1.34
41	8	II	NED	F	1	1
62	8	II	NED	F	1	1.33
5	3	II	NED	U	1	1
64	7	II	NED	F	1	1.14
12	9	III	NED	F	1	1.9
10	108	III	DOD	U	1	1
4	13	III	DOD	U	40	1
37	8	III	NED	F	1	1.46
36	6	III	DOO	F	1	1.49
22	37	III	NED	U	1	1
38	20	III	DOD	U	1	1
45	9	III	NED	F	1	1
32	8	III	NED	F	1	1
25	10	III	NED	U	1	1
20	7	III	NED	F	1	1.33
2	1	III	NED	F	1	1.27
59	7	III	NED	F	1	1.68
24	83	III	NED	U	1	1
18	72	III	NED	U	1	1
7	72	IV	DOD	NC	1	1
43	15	IV	DOD	NC	1	1
1	24	IV	DOD	NC	40	1.91
66	15	IV	DOD	U	50	1
55	15	IV	DOD	U	80	1
27	100	IV	DOD	NC	1	2.13
11	6	IV	NED	F	1	1
51	31	IV	NED	NC	1	1
65	13	IV	DOD	U	100	1
26	10	IV	DOD	NC	100	1
29	9	IV	DOD	U	50	1.67
21	20	IV	DOD	NC	20	1
6	22	IV	DOD	NC	1	1
67	62	IV	NED	NC	1	1.98
35	9	IV	DOO	F	1	1
8	32	IV	DOO	NC	1	1
3	72	IV	DOD	NC	1	1.52
33	8	IVS	NED	F	1	1
9	3	IVS	NED	F	1	1.62
39	7	IVS	NED	F	3	1

Fig. 1. Clinical profiles of neuroblastoma patients, other biological characteristics, and the length of telomeric repeats of tumors. NED, no evidence of disease; DOD, dead of disease; DOO, dead of other causes; REC, recurrent tumor; U, unfavorable; F, favorable; and NC, not classified because they were resected after chemotherapy. a) The classification by Evans *et al.*[14] b) The classification by Shimada *et al.*[8] c) When two signal peaks of telomeric repeats were observed, the lengths of the stronger signal peaks are shown as thick bars and those of the weaker signal peaks as thin bars. We determined that the reduction of telomeric repeats had occurred when the length was < 8 kb (dotted line).

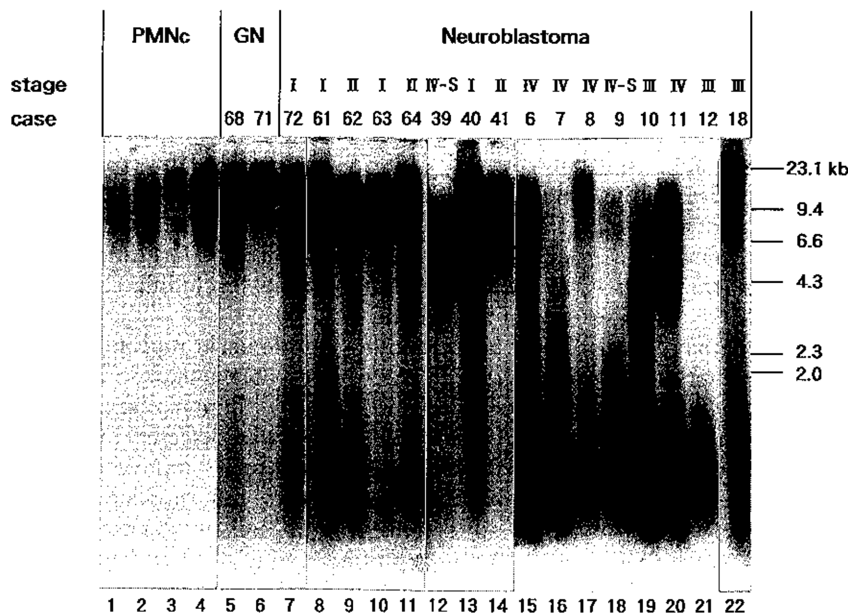


Fig. 2. Southern blot analysis of the length of the telomeric repeats in primary neuroblastoma samples. DNA was digested with the restriction enzyme *Hin*fI and hybridized with ^{32}P-labeled (TTAGGG)$_4$ probe. Lanes 1–4, PMNc from 4 unrelated healthy Japanese adults; lanes 5 and 6, ganglioneuroma; lanes 7–22, neuroblastoma. Lanes 18, 19, and 20 (Cases 9, 10, and 11) showed two peak fractions of telomeric repeats.

samples was prepared using proteinase K and SDS[5] followed by phenol/chloroform extractions. DNA samples from PMNc of 6 healthy Japanese aged 22–51 years were gifts from Dr. J. Asakawa and 4 PMNc DNA samples were extracted from these neuroblastoma patients. Southern blot analysis for N-*myc* gene amplification was done with an N-*myc* probe (PN-*myc*·1, Oncor Inc., Gaithersburg, Maryland), as described previously.[15] To estimate the length of telomeric repeats, DNA samples (5 μg) were digested to completion with *Hin*fI, electrophoresed on 1% agarose gels, and then blotted onto nitrocellulose filters. The filters were hybridized in 10×Denhart's, 1 *M* NaCl, 50 m*M* Tris-HCl (pH 7.4), 10 m*M* EDTA, 0.1% SDS, and 50 μg/ml denatured salmon sperm DNA at 50°C with a (TTAGGG)$_4$ probe[16] that had been labeled with [γ-^{32}P]-dATP by T$_4$-polynucleotide kinase (Takara, Kyoto). The filters were washed in 4×SSC (1× =0.15 *M* NaCl-0.015 *M* sodium citrate) and 0.1% SDS at 55°C and then autoradiographed.

Flow cytometric analysis of DNA contents Frozen samples were thawed at 20°C and cut into small pieces with scissors. Suspensions of single nuclei were prepared by using the detergent-trypsin procedure of Vindeløv *et al.*[17] and stained with propidium iodide (Becton Dickinson, Mountain View, California). Measurement of DNA cellular content parameters was performed with a FACScan flow cytometer (Becton Dickinson). The nuclei (2×10^4 nuclei per sample) were analyzed, and single-parameter histograms were used to evaluate the DNA content of the tumors. The DNA index of each tumor sample was determined by calculating the ratio of the modal channel number for tumor G$_0$/G$_1$-phase cells to that for normal diploid cells. Hence the DNA index of diploid neuroblastoma was 1.0. The S-phase fraction was determined using the two methods described by Milthorpe.[18]

Statistical methods Tumors were classified into two groups by the presence or absence of the reduction of telomeric repeats. Chi-square or Fisher's exact test, where appropriate, and Mann-Whitney-U tests were used for comparisons between the two groups. Kaplan-Meier disease-free survival curves were analyzed by the log-rank test.[19] Correlation coefficient was determined by linear regression analysis. A P value of <0.05 was considered significant.

RESULTS

The length of telomeric repeats in neuroblastomas Autoradiograms of Southern blot analyses performed on *Hin*fI-digested samples with a (TTAGGG)$_4$ probe are shown in Fig. 2, and the estimated lengths of telomeric repeats in primary tumor samples are summarized in Fig. 1. The same filters were also hybridized with β-globin probe and the possibility of partial digestion of DNA in Southern blot analyses was excluded (data not shown). For some tumor samples (including Case 3) and PMNc samples, we repeated the experiment with restriction

[5] The abbreviations used are: SDS, sodium dodecyl sulfate; SSC, standard saline citrate. PMNc, peripheral mononuclear cells.

enzyme *Rsa*I or *Hae*III and obtained very similar results to those obtained with *Hin*fI (data not shown), implying that these fragments consisted mostly of telomeric repeats. The effect of sample degradation was also excluded by the hybridization of undigested DNA with the $(TTAGGG)_4$ probe (data not shown). When two signal peaks were observed on Southern blots, we evaluated the stronger peak as the average length of telomeric repeats (Fig. 2, Cases 9–11). In all 10 PMNc DNA samples from healthy Japanese adults and neuroblastoma patients, the lengths of telomeric repeats ranged from 8 kb to 11 kb. In all five ganglioneuroma DNA samples, the lengths of telomeric repeats ranged from 9 kb to 13 kb, rather similar to those found in the normal PMNc DNAs. Based on these data, we considered that the reduction of telomeric repeats had occurred when their length was <8 kb. In the DNA samples from 55 neuroblastomas, a broad range of lengths of telomeric repeats was detected (from 1.1 kb to >23 kb), and 14 tumors (25.5%) showed the reduction of telomeric repeats. Four cases in advanced stages (Stages III and IV) showed long telomeric repeats, >23 kb in Case 3 and >15 kb in Cases 8, 18, and 24.

Relation of the reduction of telomeric repeats in neuroblastomas to age at diagnosis, clinical stage, and prognosis There was no significant difference in the ages at diagnosis between the patients with the reduction of telomeric repeats and those without the reduction (mean 29.5 [SE 9.6] vs. 16.6 [3.2] months). There was a statistically significant correlation between the occurrence of the reduction of telomeric repeats and the stage at diagnosis (χ^2= 15.89, df=4, P<0.01). The reduction of telomeric repeats was detected in only 1 of 20 tumors at early stages at diagnosis (Stages I and II), and this single case (Case 13) was unique in having amplified N-*myc* genes at Stage I. On the other hand, the reduction of telomeric repeats was detected in 7 of 17 Stage IV tumors and in all 3 Stage IV-S tumors. Analysis of disease-free survival rates in two groups of patients with or without the reduction of telomeric repeats demonstrated a significant correlation between their reduction and poor prognosis (P<0.05; Fig. 3). However, among the patients with the reduction of telomeric repeats, all 3 Stage IV-S cases, whose tumors were diagnosed at <1 year old, have survived free of disease. The difference of survival rates in these two groups excluding stage IV-S cases was more striking (P<0.01).

Relation of the reduction of telomeric repeats to other biological characteristics in neuroblastomas In the histological classification scheme by Shimada *et al.*,[8] the reduction of telomeric repeats was detected in 5 of 31 favorable-type tumors and in 5 of 13 unfavorable-type tumors (P=0.11, Fisher's exact test; not significant). The reduction of telomeric repeats was detected in 6 (60%) of 10 primary tumors which had multiple copies of the N-*myc* gene, and in 8 (18%) of 45 tumors which had a single copy of the N-*myc* gene (P=0.01, Fisher's exact test). The DNA indexes of tumors measured by flow cytometry did not seem to correlate with the reduction of telomeric repeats. Yet the percentage of S-phase fractions in tumor cells with the reduction of telomeric repeats was significantly higher than that of S-phase fractions without their reduction (mean 24.7 [SE 2.7]% vs. 18.3 [1.0]%; P<0.001; see Fig. 4).

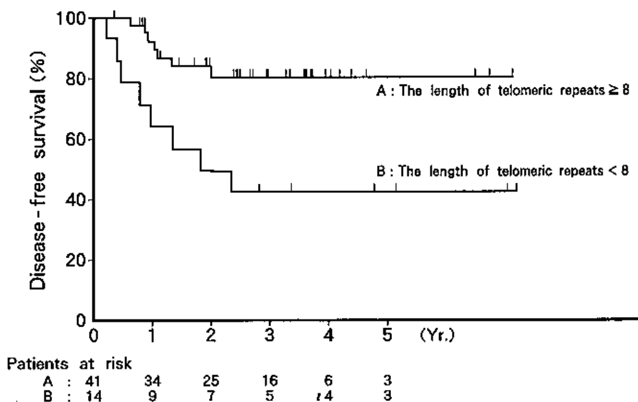


Fig. 3. Progression-free survival rate of patients with and without the reduction of telomeric repeats.

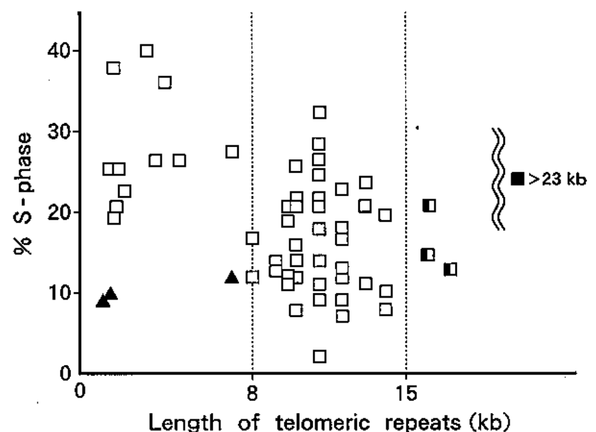


Fig. 4. Relationship between the length of telomeric repeats and the percentage of S-phase fraction in tumor cells. Every case with the reduction of telomeric repeats except for Stage IV-S cases (▲) had a high percentage of S-phase fraction (linear regression, γ= −0.55, P<0.001). On the other hand, all three of the Stage IV-S cases had a low percentage of S-phase fractions despite showing the reduction of telomeric repeats. ■, Cases 3 with >23-kb length; ◧, Cases 8, 18, and 24 with >15-kb length of telomeric repeats; ▲, Cases 9, 33, and 39 in Stage IV-S.

DISCUSSION

To define the reduction of telomeric repeats in neuroblastoma, their length should be compared to that of normal cells of adrenal gland or paraspinal ganglia in each patient. But these normal tissues are unavailable. So, in this study, the length of telomeric repeats in neuroblastoma was tentatively compared to that of ganglioneuroma, which is a benign tumor of the same origin as neuroblastoma, and that of PMNc of healthy Japanese adults and neuroblastoma patients. The peak fractions of telomeric repeats in the PMNc DNAs were more than 8 kb. These findings are similar to those of other investigators.[3-5] The lengths of telomeric repeats in all of the ganglioneuroma samples also fell in this range, while 25% of the neuroblastoma samples showed the reduction of telomeric repeats, particularly in advanced tumors and stage IV-S tumors. The reduction of telomeric repeats was significantly correlated with a poor prognosis and high percentage of S-phase fraction in flow cytometric DNA analysis except for stage IV-S tumors. These findings suggest that the reduction of telomeric repeats is related to the proliferative activity of neuroblastoma cells and seems to be a useful indicator of the aggressiveness of neuroblastoma. It is well known that N-*myc* gene amplification and unfavorable types of Shimada's histological classification are correlated with the aggressiveness of the neuroblastoma and a poor prognosis.[8, 12, 13] In our study, all tumors with N-*myc* gene amplification except for Stage I and IV-S tumors showed unfavorable histological types and were correlated with a poor prognosis. However, only 60% of neuroblastomas with N-*myc* gene amplification and 50% of unfavorable histological type tumors showed the reduction of telomeric repeats. The reduction of telomeric repeats may be a different biological characteristic from N-*myc* gene amplification and histological type. Clinically, N-*myc* gene amplification is correlated with a poor prognosis but the tumors without N-*myc* gene amplification are not always correlated with a good prognosis.[15, 20] In our 32 advanced neuroblastomas, 12 of 14 tumors with N-*myc* gene amplification and/or the reduction of telomeric repeats showed poor prognosis, while 15 of 18 other tumor showed good prognosis ($P=0.0001$, Fisher's exact test). We think that the combination of these two factors is a more accurate indicator to predict the prognosis of neuroblastoma patients.

It is known that neuroblastomas sometimes regress spontaneously. In particular, about 80% of Stage IV-S neuroblastomas have been reported to regress spontaneously.[21, 22] In this study, all three Stage IV-S neuroblastomas showed the reduction of telomeric repeats and their metastatic tumors regressed spontaneously. The discrepancy in prognosis between Stage IV-S tumors and other tumors with the reduction of telomeric repeats can not be clearly explained at present. However, the flow cytometric DNA analysis data, which revealed the low percentage of S-phase fractions in Stage IV-S tumors (see Fig. 4), signifies that the reduction of telomeric repeats in these tumors does not reflect proliferative activity. Recently, Harley *et al.*[7] reported that the number and length of telomeric repeats in human fibroblasts decreased as a function of serial passage during aging and that the reduction of telomeric repeats might be related to cellular senescence. These findings suggest that the reduction of telomeric repeats in Stage IV-S neuroblastoma might be related to spontaneous regression. We speculate that the reduction of telomeric repeats in neuroblastoma might be an indicator of two different characteristics; i.e., proliferative activity and cellular senescence.

One Stage IV neuroblastoma had long telomeric repeats (>23 kb) and 3 advanced tumors (Cases 8, 18, and 24) also had rather long telomeric repeats (>15 kb, i.e., longer than the sperm telomeres[4]). It is interesting that these four tumors were diagnosed at relatively older ages (>32 months old), when spontaneous regression is reported to be rare.[23] In immortal cells, loss of telomeric repeats due to degradation or incomplete replication may be balanced by the elongation of telomeric repeats with telomerase, which was recently identified in Hela cells,[24] or with telomere recombination.[1, 2] It is possible that these tumor cells possessed a telomerase-like activity that might be related to their immortality.

In conclusion, we found that the length of telomeric repeats was closely related to the biological phenotype in neuroblastoma. Although we do not know the mechanism of the reduction and the elongation of telomeric repeats in neuroblastoma, we can at least say that the length of telomeric repeats may be related to the progression and/or regression of neuroblastoma.

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