Telomere maintenance mechanisms are activated in ganglioneuroblastoma and ganglioneuroma

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Abstract. Telomere maintenance mechanisms (TMM) have garnered attention as a mechanism associated with the treatment resistance and poor prognosis of neuroblastoma (NB). Ganglioneuroblastoma (GNB) and ganglioneuroma (GN) are histologically classified as neuroblastic tumors (NTs) along with NB; however, few reports have addressed TMM in GNB and GN. The present study analyzed 321 NTs diagnosed in Japan, including 255 NB cases, 48 GNB cases and 18 GN cases, using a quantitative PCR-based C-circle assay for alternative lengthening of telomeres (ALT) and a telomerase reverse transcriptase (*TERT*) mRNA expression assay. ALT was identified in 38 NB cases (38/255, 15%) and 6 GNB cases (6/48, 12.5%), but not in GN. High *TERT* expression was observed in 38% (64/169), 23% (7/31) and 14% (1/7) of NB, GNB and GN cases, respectively. TMM activation, defined as ALT(+) and/or high *TERT* expression, occurred in 12/48 GNB cases and 1/18 GN cases, particularly in the GNB-nodular type (10/21, 48%), which was similar to 39% (100/255) of NB cases. Furthermore, TMM(+) GNBs exhibited distinct features, including a high frequency of *ATRX* alterations and a lower frequency of *TERT* rearrangements. Chromosomal aberration analysis revealed frequent 7q gain, 17q gain and 11q loss in ALT(+) NTs (83%). Overall, TMM serves as a poor prognostic marker for high-risk NB and offers valuable insights for the risk classification of GNBs.

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Abbreviations: ALT, alternative lengthening of telomeres; CGH, comparative genomic hybridization; FISH, fluorescence *in situ* hybridization; GN, ganglioneuroma; GNB, ganglioneuroblastoma; HR, high-risk; INRG, International Neuroblastoma Risk Group; INSS, International Neuroblastoma Staging System; JCCG, Japan Childhood Cancer Group Neuroblastoma Committee; NB, neuroblastoma; NTs, neuroblastic tumors; TC, telomere content; TERT, telomerase reverse transcriptase; TMM, telomere maintenance mechanisms

Key words: TMM, NB, GNB, GN, NTs

Introduction

Neuroblastic tumors (NTs) histopathologically include neuroblastoma (NB), ganglioneuroblastoma (GNB), and ganglioneuroma (GN) (1-4). NB and GNB exhibit various clinical features, from cases with a good prognosis that may undergo spontaneous regression without treatment to those with a poor prognosis, where survival rates remain at 30-40%, even with intensive multimodal treatment (4,5). GN is generally considered a benign tumor; however, there have been reports of malignant transformation from GN to NB (6). Thus, NTs have diverse prognoses and treatment responses, which necessitate a risk classification system to guide effective treatment strategies for each tumor type. In 2009, the International Neuroblastoma Risk Group (INRG) criteria were proposed for NB tumor classification, incorporating seven potential prognostic factors: tumor stage, histology, *MYCN* amplification, age at diagnosis, 11q aberration, and DNA ploidy. These criteria stratify patients into four risk categories: very low, low, intermediate, and high risk (7).

Many tumor cells extend their telomeres to maintain proliferation and evade apoptosis by avoiding telomere shortening during cell division. Recently, a comprehensive genomic

analysis of NB samples has revealed that the telomere maintenance mechanisms (TMM) is frequently activated in high-risk (HR) NBs. Telomeric DNA is maintained through transcriptional activation of the telomerase reverse transcriptase (*TERT*) gene via *MYCN* overexpression in *MYCN*-amplified NBs, genomic rearrangement resulting in enhancer hijacking or promoter mutations of *TERT* in *MYCN*-not-amplified HR NBs (8-10).

An alternative TMM involves telomere elongation via the alternative lengthening of telomeres (ALT), which is strongly correlated with *ATRX* alterations and predominantly observed in *MYCN*-not-amplified HR NBs (8,11-13). Recent reports have indicated that TMM-activated NB have poor prognoses (14-16), even those with non-HR group (14). Therefore, TMM is considered a new prognostic marker for the next version of the INRG classification. However, only a few reports on TMM in GNB and GN (17-20), as well as NBs in Asian populations have been published. Therefore, further studies on TMM in NTs are required.

In this study, we aimed to examine 321 NTs diagnosed in Japan for TMM, including 48 GNBs and 18 GNs. *TERT* qPCR and C-circle assay demonstrated that TMM activation occurred in a subpopulation of GNB and GN, in addition to NB. Genomic characterization of ALT(+) and *TERT*-high NT tumors was conducted using array comparative genomic hybridization (CGH) and mutation analyses.

Materials and methods

Clinical samples. Written informed consent was obtained from the parents or legal guardians of all patients, and assent was obtained from the patients themselves when appropriate, at hospitals participating in the Japan Childhood Cancer Group Neuroblastoma Committee (JCCG-JNBSG). A total of 321 NT samples were collected from patients aged between 0 months and 9 years who underwent surgery or biopsy between November 2014 and April 2018. All tumor samples analyzed in this study were obtained at the time of initial diagnosis, prior to any treatment. These tumors were histopathologically diagnosed as NB, GNB, or GN by a pathological central review of the JCCG-JNBSG and staged according to the International Neuroblastoma Staging System (INSS) (7). The *MYCN* gene copy number and DNA ploidy were determined as part of the routine diagnostic procedures by the central molecular diagnosis team at Saitama Cancer Center (21,22). The study design was approved by the Ethics Committee of Saitama Cancer Center (approval nos. 1528 and 1529).

C-circle and telomere content assays. Genomic DNA from tumors and the ALT(+) neuroblastoma cell line SK-N-FI was extracted using the standard proteinase K digestion and phenol- chloroform extraction method. The assay was performed on each sample with and without phai29 polymerase. C-circle and telomere content (TC) assays using qPCR were performed in triplicate by QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems), as previously described (23-25). Primer sequences for qPCR are listed in Table SI. TC was relative to that of ALT+ cell line SK-N-FI with arbitrary value of 14. C-circle was relative to that of ALT+ cell line SK-N-FI with arbitrary value of 196. Telomere elongation and ALT were defined as TC >12 and C-circle level >7.5, respectively, based on previously established calculations and cut-off values (24,25). SK-N-FI cells were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM, FujiFilm) supplemented with 10% FBS, 100 μg/ml penicillin/streptomycin, and 1% MEM NonEssential Amino Acids (Fujifilm). Cultures were maintained at 37˚C under 5% CO_2 in air. We confirmed SK-N-FI was mycoplasma-free and had characteristic STR markers.

TERT expression and genomic rearrangement analysis. Total RNA from human frozen tissue was extracted by ISOGEN II (NIPPON GENE) and cDNA synthesis was performed by ReverTra Ace® (TOYOBO) and random primers (Takara Bio) according to the manufacturer's instructions. RT-qPCR analysis was conducted in triplicate using QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems). *TERT* mRNA expression was measured using qPCR (Table SI). The median *TERT* mRNA expression in NB diagnosed at >18 months of age (arbitrary unit=0.31) was used to define the *TERT*-high (>0.31) and *TERT*-low (<0.31) expression groups. The genomic status of the *TERT* locus was assessed by break-apart fluorescence *in situ* hybridization (FISH) using custom SureFISH probes (Agilent Technologies cat#G110997R-8) following the manufacturer's protocol. Images were acquired using the BZ-X710 and BZ-X Analyzer (Keyence). Structural abnormalities of the *TERT* gene were defined as cases in which the signals were separated by more than 10% of the nuclear major axis length. In *TERT*-high tumors without *TERT* structural abnormalities, *TERT* promoter mutation analysis was performed by Sanger sequencing (Table SI) (26).

Array comparative genomic hybridization analysis and genomic subgrouping. Microarray-based CGH analysis was performed on 38 samples using the 8x60 K Human Genome CGH (Agilent Technologies #G4450A) or the Human Genome customized 8x60 K CGH + SNP Microarray Kit (Agilent Technologies #G4885A), and CytoGenomics software (Agilent Technologies) following the manufacturer's protocol. CNVs were identified using CytoGenomics 5.4.0.11 with the ADM-2 algorithm under default settings, with a minimal absolute average log ratio of 0.25 as the cut-off. Based on the chromosomal aberration profiles, including 1p loss, 11q loss, 17q gain, and *MYCN* amplification, each tumor was categorized into genomic subgroups (GGs; partial/segmental, GG-P; whole/numerical, GG-W) (21,27,28).

ATRX mRNA expression and mutation analysis. *ATRX* mRNA expression was analyzed using TaqMan qPCR (Table SI). ALT(+) NBs that showed <70% (<0.12) of the lowest *ATRX* mRNA expression in ALT(-) NBs were considered to have decreased *ATRX* mRNA expression. The copy number alteration of the *ATRX* gene was assessed by qPCR and/or customized 8 x 60 K CGH + SNP analysis (Agilent Technologies #G4885A). The relative value of *ATRX* genomic copy number in qPCR assay was calculated as the ratio of *ATRX*/*SMARCA1*: *ATRX* copy number 'loss' was defined as <0.66 in tumors. Mutation analysis was performed using Sanger sequencing for ALT(+) NB and GNB without *ATRX*

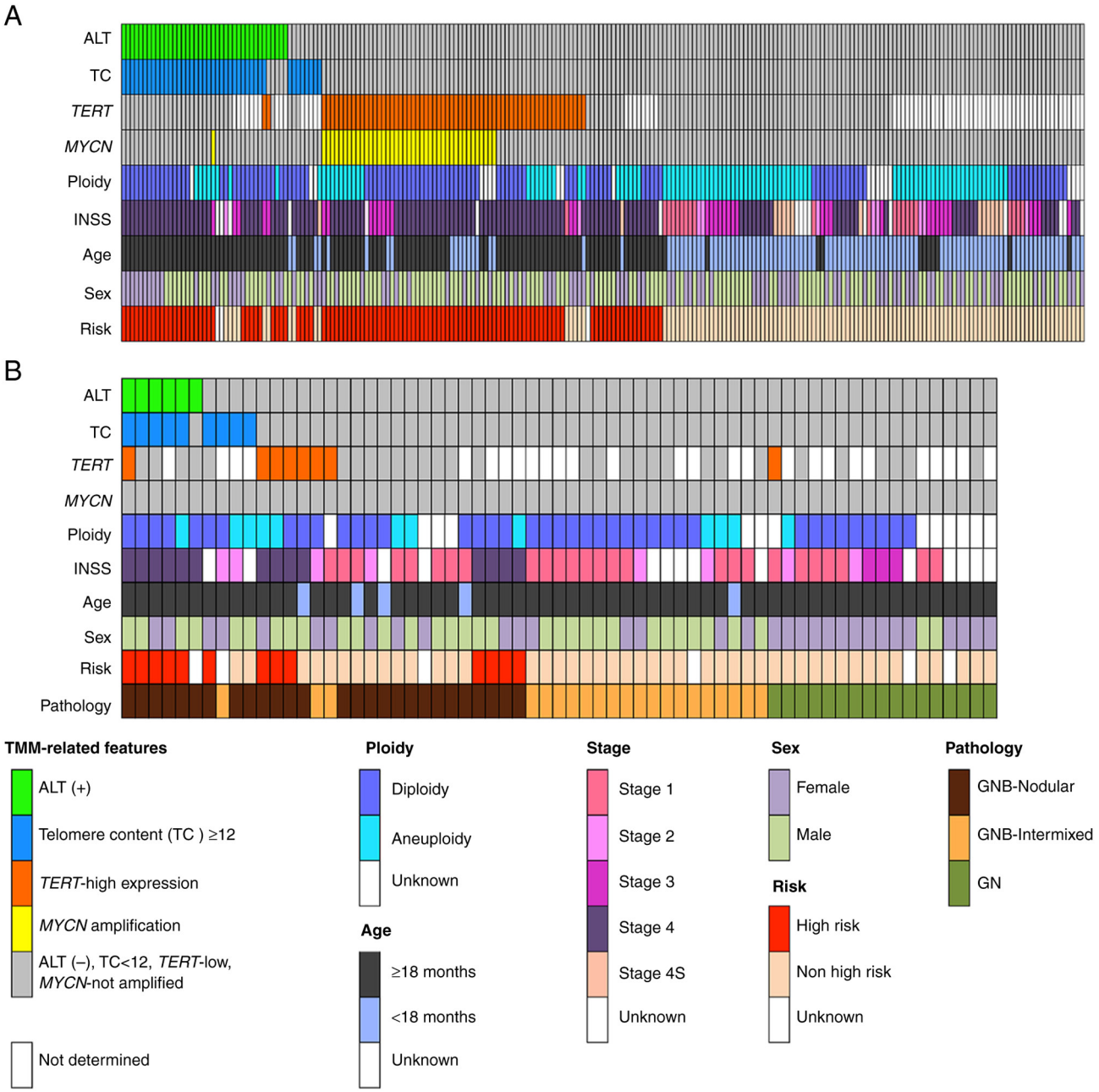

Figure 1. Landscape of TMM and clinical and pathological features of 255 NBs, 48 GNBs and 17 GNs. (A) NBs (n=255), (B) GNBs (n=48), and GNs (n=17). *MYCN* status, *TERT* mRNA expression, presence of C-circle (alternative lengthening of telomeres), telomere content, and clinical and pathological variables are shown. ALT, alternative lengthening of telomeres; TC, telomere content; INSS, International Neuroblastoma Staging System; TMM, telomere maintenance mechanisms; NB, neuroblastoma; GNB, ganglioneuroblastoma; GN, ganglioneuroma.

deletion (Table SI). RT-PCR products were generated using the following primer pairs: 1F/1R, 2F/2R, 3F/3R, and 4F/4R. For sequencing of each RT-PCR product, the following primers were used: RT-PCR 1F-1R product (RT-PCR1F, RT-PCR1R, Sequencing 1F-1, 1F-2, 1F-3, 1R-1, and 1R-2); RT-PCR 2F-2R product (RT-PCR2F, RT-PCR2R, Sequencing 2F-1, 2F-2, 2R-1 and 2R-2); RT-PCR 3F-3R product (RT-PCR3F, RT-PCR3R, Sequencing 3F-1, and 3R-1); RT-PCR 4F-4R product (RT-PCR4F, RT-PCR4R, Sequencing 4F-1, 4F-2, and 4F-3).

Statistical analyses. The patients were grouped according to various biological and clinical aspects of the disease. The significance of differences in characteristics between the groups was examined using the χ^2 or Fisher's exact test for categorical variables. Comparisons between two groups of continuous variables were performed using the Mann-Whitney U test. Comparisons among three or more groups were performed using the Kruskal-Wallis test followed by Dunn's multiple comparisons test. Statistical analyses were conducted using GraphPad Prism version 6 (Dotmatics). Two-sided $P<0.05$ was considered to indicate a statistically significant difference.

Results

Analysis of ALT, TERT mRNA expression, and TC in NB. The clinical information of NB (n=255), GNB (n=48), and

Table I. Patient characteristics of alternative lengthening of telomeres and TERT expression status in neuroblastoma cases.

	ALT (n=255)				*TERT* (n=169)			
Characteristic	ALT(+) (n=38)	ALT(-) (n=217)	ALT(+), %	P-value	*TERT*-high (n=64)	*TERT*-low (n=105)	*TERT*-high, %	P-value
Age								
<18 months	0	115	0.0	<0.0001	14	55	20.3	<0.0001
≥18 months	38	102	27.1		50	50	50.0	
Sex								
Male	19	129	12.8	n.s.	42	58	42.0	n.s.
Female	19	88	17.8		22	47	31.9	
INSS								
1, 2 or 3	6	79	7.0	0.0054	16	37	30.2	0.013
4	30	117	20.4		48	55	46.6	
4S	0	15	0.0		0	7	0.0	
Unknown	2	6	25.0		0	6	0.0	
MYCN statu								
Not amplified	37	146	20.2	<0.0001	23	93	19.8	<0.0001
Amplified	1	71	1.4		41	12	77.4	
Risk (INRG)								
Non-high risk	5	108	4.4	<0.0001	7	58	10.8	<0.0001
High risk	31	108	22.3		57	44	56.4	
Unknown	2	1	66.7		0	3	0.0	
Ploidy								
Aneuploidy	8	92	8.0	0.037	19	46	29.2	n.s.
Diploidy	29	102	22.1		39	50	43.8	
Unknown	1	23	4.2		6	9	40.0	

INSS, International Neuroblastoma Staging System; INRG, International Neuroblastoma Risk Group; ALT, alternative lengthening of telomeres; n.s., not significant.

GN (n=18) is presented in Table SII, and the TMM status, including ALT, *TERT* mRNA expression, and TC, for each tumor, is shown in Fig. 1. ALT(+) and *TERT*-high were mutually exclusive in all cases except for two NB samples and one GNB sample. ALT(+) or *TERT*-high cases were classified as TMM, excluding 'ever-shorter telomeres' characterized by long telomeres without an abundance of C-circle formation associated with TMM (29). The correlations between ALT and *TERT* mRNA expression levels and age, INSS, *MYCN* amplification status, risk classification, and DNA ploidy status were analyzed. Of the 255 NBs, 38 (14.9%) were ALT(+). ALT(+) cases were significantly more prevalent among patients aged ≥18 months (P<0.0001), those with INSS stage 4 (P=0.0054), those in the HR group (P<0.0001), and those with diploidy (P=0.0037) (Table I).

The number of patients with *TERT*-high NB was 64 of 169 (37.9%). Patients with *TERT*-high were significantly more prevalent among those aged >18 months (P<0.0001), those with INSS stage 4 (P=0.013), and those in the HR group (P<0.0001) (Table I).

Regarding *MYCN* status, ALT(+) was enriched in *MYCN*-not amplified NBs compared with *MYCN*-amplified cases (P<0.0001). In an ALT(+) NB with *MYCN* amplification, we observed the coexistence of *MYCN*-amplified cells (approximately 10% of the population) and non-amplified tumor cells on a FISH slide. This tumor was thought to be composed of a mixture of ALT(+) and *MYCN*-amplified clones because previous reports have described *MYCN* amplification and ALT(+) as mutually exclusive (8,12,30). *TERT*-high expression was more abundant in *MYCN*-amplified cases than in *MYCN*-not amplified cases (77.4% vs. 19.8%, P<0.0001), which is consistent with the fact that the *TERT* gene is a transcriptional target of MYCN.

To assess the difference in telomere length between ALT(+) and *TERT*-high NBs without *MYCN* amplification, we compared the distribution of TC in four NB subgroups, ALT(+) (n=35), *TERT*-high (n=21), TMM(-) HR group (n=10), and TMM(-) non-HR group (n=56), excluding two cases with both ALT(+) and *TERT*-high NB. The TC values in the ALT(+) NB group were significantly higher than those in the other groups [ALT(+) vs. *TERT*-high, TMM(-) HR, or TMM(-) non-HR, P<0.0001] and were significantly lower in the *TERT*-high NB group compared with the TMM(-) non-HR group (P=0.0015) (Fig. 2A).

Analysis of ALT, TERT mRNA expression, and TC in GNB and GN. The C-circle assay (n=48) and *TERT* mRNA qPCR (n=31)

Table II. Patient characteristics of alternative lengthening of telomeres and TERT expression status in ganglioneuroblastoma cases.

	ALT (n = 48)				*TERT* (n = 31)			
Characteristic	ALT (+) (n=6)	ALT (-) (n=42)	ALT (+), %	P-value	*TERT*-high (n=7)	*TERT*-low (n=24)	*TERT*-high, %	P-value
Age								
<18 months	0	5	0	n.s.	1	2	33	n.s.
≥18 months	6	37	14		6	22	21	
Sex								
Male	4	27	13	n.s.	4	15	21	n.s.
Female	2	15	12		3	9	25	
INSS								
1, 2 or 3	0	30	0	0.0018	2	17	11	0.0302
4	5	9	36		5	5	50	
Unknown	1	3	25		0	2	0	
MYCN status								
Not amplified	6	42	13	-	7	24	23	-
Amplified	0	0	-		0	0	-	
Risk (INRG)								
Non-high risk	0	31	0	0.0012	3	17	15	n.s.
High risk	5	8	38		4	5	44	
Unknown	1	3	25		0	2	0	
Ploidy								
Aneuploidy	1	9	10	n.s.	2	5	29	n.s.
Diploidy	5	28	15		4	16	20	
Unknown	0	5	0		1	3	25	
Histology								
Nodular	6	21	22	0.0285	5	15	25	n.s.
Intermixed	0	21	0		2	9	18	

INSS, International Neuroblastoma Staging System; INRG, International Neuroblastoma Risk Group; ALT, alternative lengthening of telomeres; n.s., not significant.

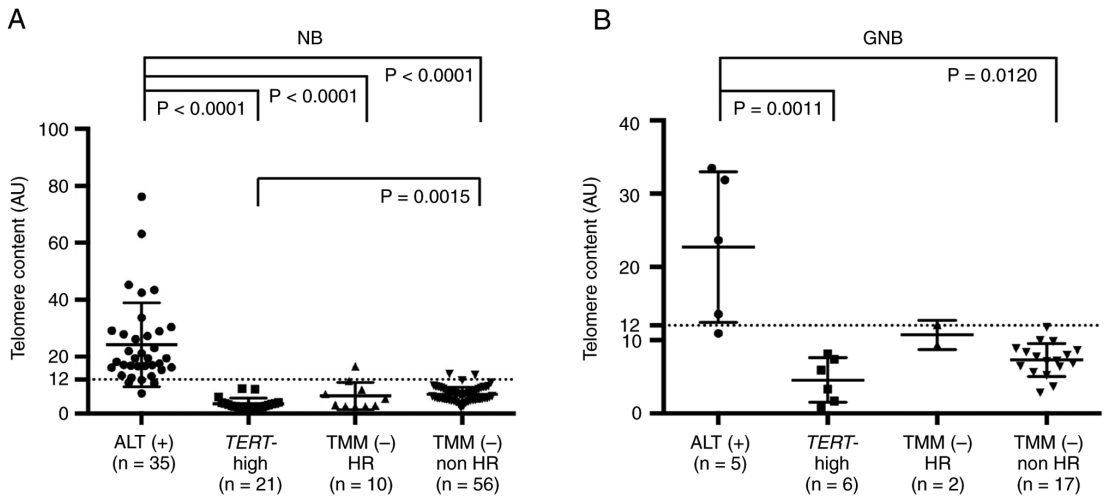

Figure 2. TCs in *MYCN*-not-amplified neuroblastic tumors. (A) Relative TC measured by qPCR in neuroblastomas with ALT(+) (n=35), *TERT*-high (n=21), TMM(-) HR (n=10), and TMM(-) non-HR (n=56). (B) Relative TC measured by qPCR in ganglioneuroblastomas in ALT(+) (n=5), *TERT*-high (n=6), TMM(-) HR (n=2), and TMM(-) non-HR (n=17). The horizontal dot line demarcates TC=12. AU, arbitrary unit; TC, telomere content; qPCR, quantitative PCR; ALT, alternative lengthening of telomeres; TMM, telomere maintenance mechanisms; HR, high risk; NB, neuroblastoma; GNB, ganglioneuroblastoma.

Table III. TERT abnormalities in MYCN-not-amplified, TERT-high neuroblastoma and ganglioneuroblastoma.

A, NB

Case no.	INSS stage	Age, months	*TERT* expression, *TERT*/*ACTB*	*TERT* genomic abnormality
3	4	18	0.37	Rearrangement
14	2	27	1.65	Rearrangement
61	4	66	0.51	Rearrangement
92	4	34	1.57	Rearrangement
93	4	47	1.35	Rearrangement
142	3	42	0.7	Rearrangement
156	4	48	0.62	Rearrangement
234	4	48	7.74	Rearrangement
237	4	76	1.45	Rearrangement
251	4	36	2.81	Rearrangement
285	4	40	0.48	Rearrangement
317	4	36	0.61	Rearrangement
320	4	69	7.35	Rearrangement
180	4	42	1.33	Amplification
144	4	42	0.43	Promoter mutation
31	4	22	0.55	-
178	4	17	2.36	-
246	4	37	1.7	-
292	3	29	0.43	-
295	3	56	1.59	-
196	4	62	0.64	ND
211	3	39	0.52	ND
240	1	19	0.44	ND

B, GNB

Case no.	INSS stage	Age, months	*TERT* expression, *TERT*/*ACTB*	*TERT* genomic abnormality
192	4	36	6.55	Rearrangement
9	4	57	0.71	-
151	4	14	0.49	-
205	1	44	0.41	-
230	4	39	0.76	-
267	4	66	0.58	-
277	2	32	1.24	-

INSS, International Neuroblastoma Staging System; NB, neuroblastoma; GNB, ganglioneuroblastoma; ND, not determined.

were performed in GNB, and the relationship between these measurements and age, INSS, risk classification, histology, and DNA ploidy status was analyzed. *MYCN* amplification was not observed in any of the GNBs. We identified 6 ALT(+) cases (12.5%) and 7 *TERT*-high cases (22.6%) among patients with GNB. Patients with ALT(+) GNB comprised a significantly higher proportion of those with INSS stage 4 (P=0.0018) and those in the HR group (P=0.0285) (Table II), whereas patients with *TERT*-high GNB made up a significantly higher proportion of those with INSS stage 4 (P=0.0302). Owing to the small sample size, no significant differences in age or ploidy status were observed in either C-circle or *TERT* mRNA measurements. Nodular tumor histology was significantly correlated with ALT(+) GNB (P=0.0285).

Similar to NB, four subgroups based on the status of ALT and *TERT* expression were established for analysis: ALT(+) (n=5), *TERT*-high (n=6), TMM(-) HR (n=2), and TMM(-) non-HR (n=17), excluding one case with both ALT(+) and *TERT*-high NB. Owing to the small sample size, the TC values in the TMM(-) HR group did not significantly differ from those in the ALT(+) group; however, TC values were significantly higher in the ALT(+) group compared with the *TERT*-high and TMM(-) non-HR groups [ALT(+) vs. *TERT*-high or TMM(-) non-HR, P=0.0011 or P=0.0120, respectively] (Fig. 2B).

All 18 GNs were ALT(-), but *TERT*-high was observed in one case diagnosed at 10 years and 7 months [1 of 8 cases (12.5%)].

Analysis of the mechanism of high TERT gene expression. Increased *TERT* expression is regulated by *MYCN* amplification, mutations in *TERT* promoter region, or *TERT* rearrangements, which result in super-enhancer hijacking. To further investigate the mechanism in each *TERT*-high tumor, 23 NBs, 7 GNBs, and 1 GN exhibiting *TERT*-high without *MYCN* amplification were analyzed by FISH to detect *TERT* rearrangements. Structural abnormalities of the *TERT* gene were identified in 14 NBs (60.9%, 13 rearrangements, and 1 amplification) and 1 GNB (16.7%, rearrangement), but not in GN. In one case of NB without *TERT* rearrangement, a mutation (C228T) in the promoter region of the *TERT* gene was observed using Sanger sequencing (Case #144; Table III; Fig. S1). No mutations in the *TERT* promoter region were observed in GNB and GN. These results are consistent with previous reports, suggesting that mutations or structural abnormalities in the *TERT* promoter region are the main mechanisms of *TERT*-high *MYCN*-not amplified NBs, whereas they are less frequent in *TERT*-high GNBs. This suggests that GNB utilizes other mechanisms to achieve *TERT*-high phenotype. Based on this notion, we further performed CGH analysis to explore whether common chromosomal aberrations occurred in 12 NTs (5 NB cases, 6 GNB cases, and 1 GN case) with *TERT*-high but no promoter mutations using sequencing or *TERT* rearrangement using FISH (Fig. S2). Among the 12 cases, 3 GNBs and 1 GN showed no chromosomal abnormalities, and no additional copy number changes in the *TERT* genomic region on chromosome 5 were detected in the remaining 8 tumors. A partial (segmental) gain of 17q was observed in 7 of the 12 cases, and a partial loss of 11q was observed in 6 of the 12 cases. Additionally, a gain of 7q was observed in 5 of the 12 cases (whole q-arm gain in 4 cases and partial gain in 1 case), and a partial gain of 11q was observed in 5 of the 12 cases. The genome group (GG) based on the previously proposed CGH signature showed that 7 of the 12 cases were classified as segmental chromosomal alterations (P3s and P1s), which are correlated with poor prognosis in patients with NB (21,27,28,31).

Table IV. ATRX status in alternative lengthening of telomeres (+) neuroblastoma and ganglioneuroblastoma.

A, NB

Case no.	INSS stage	Age, months	Sex	*ATRX* mRNA expression, *ATRX*/*ACTB*	Genomic DNA qPCR for *ATRX* exon 9	*ATRX* genomic abnormality by CGH	*ATRX* sequencing	*ATRX* aberration status
128	4	45	M	0.02	Loss	Loss	Normal	Mutation
75	4	68	M	0.02	Loss	Loss	ND	Mutation
125	4	50	M	0.02	Loss	Loss	Normal	Mutation
63	4	45	M	0.04	Loss	Loss	ND	Mutation
49	4	58	M	0.05	Loss	Loss	Normal	Mutation
157	4	86	M	0.07	Retain	Loss	Normal	Mutation
263	4	43	M	0.08	Loss	Loss	Normal	Mutation
47	4	41	M	0.13	Loss	ND	Normal	Mutation
64	4	41	M	ND	Loss	Retain	Normal	Mutation
105	4	30	M	ND	Loss	Loss	ND	Mutation
271	4	56	M	ND	Loss	Loss	ND	Mutation
80	4	51	M	0.1	Retain	Chr. X loss	Normal	Mutation
113	4	88	M	0.09	Retain	Retain	Normal	Low expression
13	4	121	M	0.1	Retain	Retain	Normal	Low expression
282	-	45	M	0.37	Retain	Retain	Normal	Normal
89	4	28	M	0.38	Retain	Retain	Normal	Normal
268	4	120	M	0.51	Retain	Retain	Normal	Normal
212	2	76	M	0.23	Retain	ND	Normal	ND
142	3	42	M	0.28	Retain	Retain	ND	ND
18	4	59	F	0.02	Loss	Chr. X loss/Loss	Normal	Mutation
217	4	71	F	0.06	Loss	Chr. X loss/Loss	Normal	Mutation
51	4	56	F	0.1	Loss	Chr. X loss/Loss	Normal	Mutation
295	3	56	F	ND	Retain	Chr. X loss/Loss	ND	Mutation
259	4	56	F	ND	Retain	Chr. X loss/Loss	ND	Mutation
79	4	91	F	0.02	Loss	Loss/Retain	ND	Mutation
171	3	73	F	0.14	Retain	Loss/Retain	Normal	Mutation
243	4	49	F	0.17	Retain	Loss/Retain	Normal	Mutation
36	4	45	F	0.49	Retain	Loss/Retain	Normal	Mutation
226	-	95	F	0.17	Retain	Chr. X loss/Retain	c.5162del	Mutation
311	3	41	F	ND	Retain	Chr. X loss/Retain	Normal	Mutation
257	3	49	F	0.08	Retain	Chr. X loss/Retain	ND	Mutation
207	4	39	F	0.3	Retain	Chr. X loss/Retain	Normal	Mutation
294	4	37	F	0.27	Retain	Retain	c.4356_4361del	Mutation
96	4	48	F	0.03	Retain	Retain	ND	Low expression
273	4	42	F	0.42	Retain	Retain	Normal	Normal
45	4	71	F	0.44	Retain	Retain	Normal	Normal
6	4	75	F	3.04	Retain	Retain	Normal	Normal
140	4	20	F	0.27	Retain	Chr.X gain	Normal	Normal

B, GNB

Case no.	INSS stage	Age, months	Sex	*ATRX* mRNA expression, *ATRX*/*ACTB*	Genomic DNA qPCR for *ATRX* exon 9	*ATRX* genomic abnormality by CGH	*ATRX* sequencing	*ATRX* aberration status
214	4	39	M	0.02	Loss	Loss	Normal	Mutation
258	-	40	M	0.05	Loss	Loss	Normal	Mutation
77	4	25	M	0.26	Loss	Loss	Normal	Mutation
230	4	39	M	0.79	Retain	Retain	c.2518dup	Mutation
86	4	53	F	0.09	Loss	Loss/Retain	c.2518dup	Mutation
118	4	43	F	0.11	Loss	Chr. X loss/Loss	ND	Mutation

INSS, International Neuroblastoma Staging System; qPCR, quantitative PCR; CGH, comparative genomic hybridization; M, male; F, female; NB, neuroblastoma; GNB, ganglioneuroblastoma; ND, not determined; Chr. X loss, loss of chromosome X.

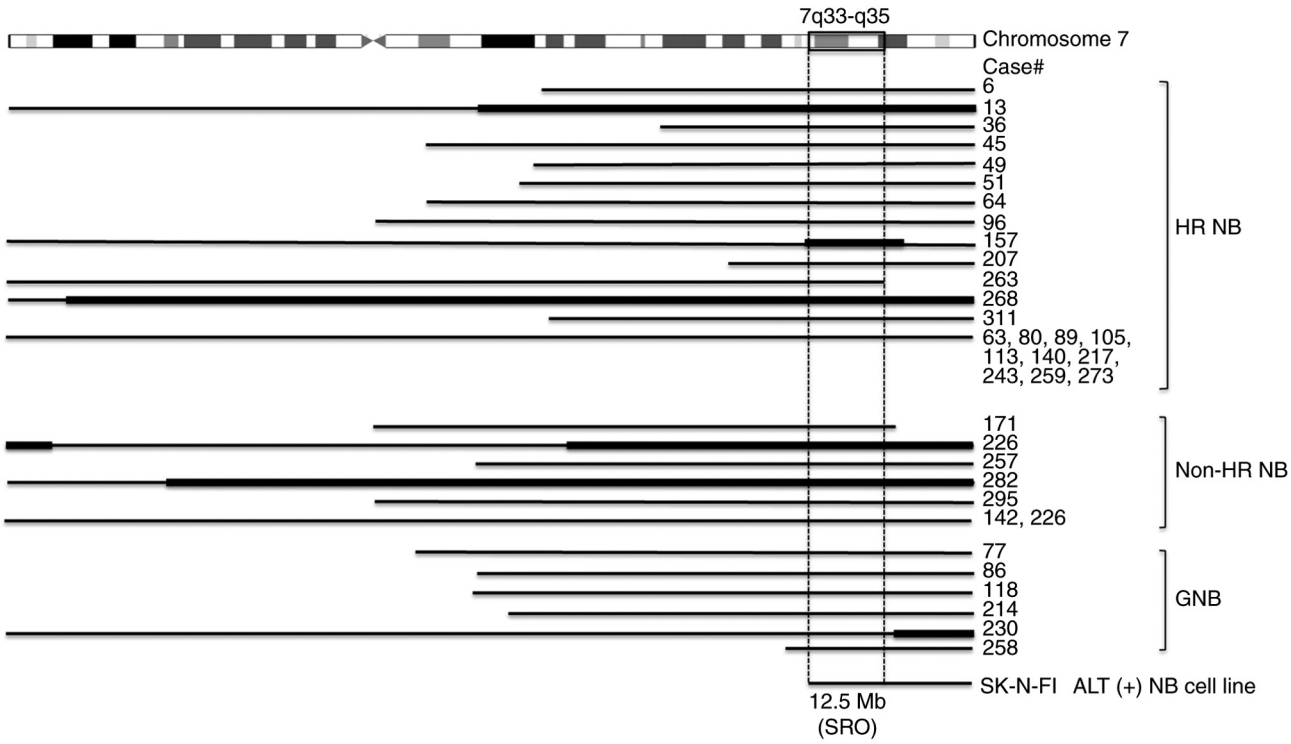

Figure 3. Chromosomal gains on chromosome 7 in ALT(+) neuroblastic tumors. Partial chromosomal gains in 36 ALT(+) neuroblastomas, six ALT(+) ganglioneuroblastomas, and SK-N-FI cell lines are shown. The SRO of the partial gain was defined on chromosome 7q (hg19: chr. 7 127892164-144419786, approximately 12.5 Mb). The line represents the chromosome gain region. The bold line indicates the additional copy number gain. ALT, alternative lengthening of telomeres; HR, high risk; NB, neuroblastoma: GNB, ganglioneuroblastoma; SRO, smallest region of overlap.

Analysis of ATRX genetic alterations in ALT (+) NB and GNB. ALT(+) NBs frequently exhibit *ATRX* gene alterations (15,16). To assess *ATRX* status, we performed genomic qPCR targeting *ATRX* exon 9, array CGH using a custom CGH + SNP array, and TaqMan qPCR for *ATRX* mRNA expression. Copy number loss of *ATRX* was detected in at least 25 of 38 ALT(+) NBs (65.8%, Table IV). Additionally, 16 of the 32 ALT(+) NBs (50%, Table IV) showed decreased *ATRX* mRNA expression (relative expression value <0.12). Among the 25 cases with *ATRX* deletion, 13 (13/19, 68.4%) exhibited decreased *ATRX* mRNA expression, whereas 5 were not analyzed owing to the unavailability of frozen samples. Three NBs showed decreased *ATRX* mRNA (exon 9) expression despite retaining the *ATRX* (exon 9) region. Sanger sequencing of the *ATRX* region revealed at least two deletion mutations, c.5162del and c.4356_4361del, in both cases (Fig. S3A and B). Collectively, 29 of 36 ALT(+) NBs (80.5%) exhibited *ATRX* genetic alterations in the sample set.

To date, no *ATRX* gene abnormalities have been reported in GNBs; therefore, we performed a detailed genome analysis of GNBs. We searched for *ATRX* mutations in all six ALT(+) GNBs using genomic qPCR, CGH + SNP array, and Sanger sequencing. Because *ATRX* is located on the X chromosome, boys generally have only one copy of the *ATRX* gene. In three of the four boys, copy number loss of *ATRX* was identified by both qPCR and CGH +SNP array, and expression analysis confirmed a marked decrease in *ATRX* mRNA expression in two cases (boys #214 and #258). In the case without *ATRX* deletion (boy #230), c.2518dup was identified (Fig. S3C). In one girl with GNB, a deletion of one X chromosome and an internal deletion of *ATRX* (exon 8-12 deletion) in the remaining allele were detected using qPCR and CGH+SNP array analysis (girl #118). In another girl with GNB, *ATRX* deletion in one allele (c.178_2220del2023) was confirmed by qPCR and CGH+SNP array analysis, and c.2518dup was detected by Sanger sequencing (girl #86) (Fig. S3D). Thus, *ATRX* abnormalities were identified in all six ALT (+) GNBs (Table IV).

Chromosomal aberrations in ALT(+) NTs. Copy number analysis was subsequently performed on 36 ALT(+) NB cases and 6 ALT (+) GNB cases to identify the characteristic chromosomal aberrations in ALT(+) NTs. A whole and/or partial chromosomal gain of 17q was observed in 41 (97.6%) cases, and a partial loss of 11q was observed in 37 (88.1%) cases (Fig. S3). Other frequently observed chromosomal aberrations included partial and overall gains of 7q (n=35, 83.3%), 7p (n=21, 50.0%), 11p (n=19, 45.2%), and 18q (n=23, 54.8%) and a partial gain of 2p (n=23, 54.8%). Based on the CGH-based genome group classifications (21,26,27,30), 41 (97.6%) tumors were classified as segmental/partial types (P3s, P2s, and P4s), and 1 was classified as numerical/whole type (W5s) (Fig. S4). Compared with *TERT*-high ALT(-) tumors, ALT(+) NTs appeared to exhibit a higher frequency of copy number alterations (Figs. S2 and S4). Furthermore, we analyzed the 7q gain region in ALT(+) NTs, which was the third most frequent alteration after 17q gain and 11q loss. We also observed a 7q gain in *TERT*-high ALT(-) NTs; however, most of these cases exhibited a whole-arm gain of 7q, rather than the partial (segmental) gains observed in ALT(+) NTs. We identified a common 12.5 Mb region of

overlap (hg19: chr7:131,876,751-144,419,786) involving at least 115 gene IDs from the University of California Santa Cruz database (Fig. 3).

Discussion

In the present study, high expression of *TERT* mRNA and ALT as TMM in NB was observed more frequently in the older age group (>18 months), INSS stage 4, and the HR group, with statistical significance, as in previous reports (15,16). There have been no reports analyzing TMMs in multiple GNB and GN samples.

Multiple approaches have been proposed for detecting ALT, including the C-circle assay, telomere length heterogeneity (TLH), and ALT-associated PML bodies (APBs), and ongoing debate remains as to which individual marker, or combination of markers, most accurately reflects ALT activation and its clinical relevance. Although the C-circle assay captures only extrachromosomal C-circle structures and does not encompass other ALT-associated features such as TLH or APBs, it provides high sensitivity and specificity and is widely used across tumor types to quantitatively assess ALT activity. Likewise, although *TERT* mRNA levels do not directly measure telomerase enzymatic activity as the TRAP assay does, TRAP analysis requires fresh tumor tissue, limiting the number of evaluable cases. For this reason, *TERT* mRNA expression has been widely adopted as a practical surrogate in TMM studies.

In this study, 6 of the 48 (12.5%) GNB cases were ALT(+) and 7 of the 31 (22.6%) cases were *TERT*-high. In total, at least 25% (12/48) of the GNBs were TMM(+), and 10 (83.3%) were nodular, a subgroup with aggressive histological features. As additional reference, a recently published meta-analysis that aggregated GNB cases extracted from multiple reports (total n=12, with heterogeneous ALT assessment methods) reported that 75% (9 of 12) of nodular GNBs were ALT-positive (32). Our study represents the first systematic analysis of ALT in a sizable GNB cohort using a single standardized assay (the C-circle method). GNB and GN are extremely rare tumors, and the numbers of GNB and GN cases analyzed in this study were relatively limited. This limitation may reduce the statistical power, particularly in subgroup analyses. Therefore, further studies with larger sample sizes will be required to more robustly determine the clinical and biological significance of TMM in GNB and GN.

This study also showed a high frequency of *ATRX* gene alterations in ALT(+) GNB with a nodular subtype, although *ATRX* gene alteration is not the only mechanism of ALT. Analysis of 16 cases of GN revealed no ALT(+) cases and only one case exhibited *TERT*-high expression.

Given that TMM activation has been strongly associated with poor prognosis in both HR and non-HR-NB (8,11,12,14-16), it will be important to investigate whether TMM-positive GNB and GN cases also exhibit adverse clinical outcomes. Because the tumors analyzed in this study were primary specimens obtained at initial diagnosis, a solid evaluation of the clinical significance of TMM status in GN and GNB will require several additional years of long-term follow-up. Further analyses with larger sample sizes and more complete prognostic data, including GNB and GN cases, will be essential to determine the clinical implications of TMM positivity in these tumors.

In NB, *TERT*-high is triggered by *MYCN* amplification and *TERT* gene rearrangement (8). In this study, genomic abnormalities in the *TERT* gene were observed in 15 of 23 NBs with *TERT*-high, indicating that this is a responsible mechanism. In contrast, among GNB cases, six out of seven with *TERT*-high showed no genomic abnormality of the *TERT* gene, suggesting that *TERT*-high is induced by an alternative mechanism distinct from that in NB. The expression of *TERT* is regulated by multiple transcriptional and epigenetic mechanisms beyond genomic alterations (33,34). Among these, c-MYC has been reported to directly bind to the *TERT* promoter and activate its transcription (35). In addition, NF-κB signaling can enhance *TERT* transcription through promoter binding and cooperation with c-MYC under stress or inflammatory conditions (36). BRD4, a bromodomain-containing chromatin reader, also contributes to TERT activation by maintaining an open chromatin structure at super-enhancer regions (37). These findings raise the possibility that, in NT, *TERT* upregulation is driven predominantly by epigenetic activation and transcriptional deregulation rather than by structural alterations of the *TERT* locus. Further integrative analyses incorporating chromatin accessibility and histone modification profiling will be necessary to elucidate these alternative *TERT* regulatory mechanisms.

We performed CGH analysis based on the idea that unknown genomic abnormalities in the region encoding regulators of *TERT* gene expression may occur in NTs that exhibit *TERT*-high expression in the absence of *TERT* gene abnormalities and *MYCN* amplification. Despite the high frequency of 11q deletions and 17q gains, we were unable to identify appropriate candidates because of similar findings in ALT(+) tumors exhibiting *TERT*-low expression. Comprehensive analyses involving larger sample sizes are necessary to elucidate the mechanisms underlying *TERT*-high NBs/GNBs independent of *TERT* gene abnormalities and *MYCN* amplification.

CGH analysis of ALT(+) NTs showed a gain of 17q and a partial loss of 11q in many cases. These alterations were also frequently detected in ALT(-) NTs, suggesting that ALT occurs after 17q gains and 11q partial deletions. Although 1q42.2-1 qter deletions have been previously reported in some ALT(+) tumors (20), they were rare in our series (2 of 35 tumors). We specifically focused on 7q gains, which were the third most frequent alteration after 17q gain and 11q loss in ALT(+) NTs. It has been observed that 7q gain, but not entire chromosome 7 gain, contributes to either tumorigenesis or progression in NBs (38). Moreover, recurrent gain at 7q21.2-tel was observed in *MYCN*-not amplified NB metastases compared with primary tumors (39). These reports may explain the poor prognostic nature of ALT(+) tumors, which are susceptible to segmental gain in the 7q region. The 12.5 Mb region of the common 7q gains may serve as a useful prognostic marker in ALT(+) NTs.

Regarding the clinical implications of TMMs, activation of the TMM by *TERT*-high has been reported in various cancers, leading to the development of molecularly targeted drugs for telomerase inhibition. Recent studies have demonstrated that 6-thio-2´deoxyguanosine, BET bromodomain inhibitors (such as JQ1, AZD5153, and OTX015), dinaciclib, and carfilzomib inhibit the growth of NB cells and/or NB xenografts exhibiting

TERT overexpression or high telomerase activity. These findings suggest the need for the initiation of clinical trials targeting NTs with *TERT* overexpression or high telomerase activity (15,40-42).

In conclusion, the development of molecularly targeted drugs for ALT(+) tumors is important. In ALT(+) NB, the synergistic effect of AZD0156, an ATM inhibitor, used in combination with temozolomide and irinotecan, was reported both *in vitro* and *in vivo* (43). Previous studies have demonstrated the sensitivity of tumors with ALT(+) or *ATRX* mutations to PARP inhibitors (44,45).

From a translational perspective, assay selection must also take into account the availability of clinical specimens and methodological reproducibility across laboratories. While further comparative studies are needed to establish the most comprehensive and reliable TMM evaluation strategies, the combined use of the C-circle assay and *TERT* mRNA analysis currently represents a robust and widely utilized framework for molecular TMM assessment in NTs. Importantly, by applying this standardized approach to a large cohort, including in GNB and GN, our study provides new biological insights and contributes foundational data that may inform future refinements of INRG risk stratification and the clinical integration of TMM markers.

In conclusion, by expanding TMM analysis, whose role as a high-risk biomarker in NB is now supported by increasingly strong evidence, to GNB and GN, our findings may support a more precise update of the INRG risk classification across the full spectrum of NTs, including non-high-risk types. In addition, given that several therapeutic approaches have been proposed for TMM-positive tumors, incorporating TMM status into the molecular diagnostic framework for NTs, including GNB and GN, may help guide more tailored and effective treatment strategies in future clinical practice.

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Availability of data and materials

Sanger sequencing data generated in the present study may be found in the DNA Data Bank of Japan and ClinVar under accession numbers LC911903-LC911906 and SCV007329794-SCV007329796, respectively, or at the following URLs: LC911903, https://getentry.ddbj.nig.ac.jp/getentry/na/LC911903/?format=flatfile&filetype=html&trace=true&show_suppressed=false&limit=10; LC911904, https://getentry.ddbj.nig.ac.jp/getentry/na/LC911904/?format=flatfile&filetype=html&trace=true&show_suppressed=false&limit=10; LC911905, https://getentry.ddbj.nig.ac.jp/getentry/na/LC911905/?format=flatfile&filetype=html&trace=true&show_suppressed=false&limit=10; LC911906, https://getentry.ddbj.nig.ac.jp/getentry/na/LC911906/?format=flatfile&filetype=html&trace=true&show_suppressed=false&limit=10; SCV007329794, https://www.ncbi.nlm.nih.gov/clinvar/variation/4685457/?oq=SCV007329794&m=NM_000489.6(ATRX):c.2518dup%20(p.Arg840fs); SCV007329795, https://www.ncbi.nlm.nih.gov/clinvar/variation/4685458/?oq=SCV007329795&m=NM_000489.6(ATRX):c.5162del%20(p.Gly1721fs); SCV007329796, https://www.ncbi.nlm.nih.gov/clinvar/variation/4685459/?oq=SCV007329796&m=NM_000489.6(ATRX):c.4353GGA%5B1%5D%20(p.Glu1463_Glu1464del). Other data generated in the present study may be requested from the corresponding author.

Authors' contributions

TKa conceptualized and designed the study. MS, MH, MO, JA, RS, RO, KA, AN and HO performed the laboratory experiments and collected and analyzed the data. TT coordinated the collection and provided clinical data through the data center. TI, TKo and SU contributed to the provision of clinical samples and clinical information as members of the clinical research group. TKa provided resources. MS and MH drafted the original manuscript, with input from TKa. MH, MO, TI, TKo, SU and TKa critically reviewed and edited the manuscript for important intellectual content. MH and TKa confirm the authenticity of all the raw data. All authors read and approved the final manuscript, and agree to be accountable for all aspects of the work.

Ethics approval and consent to participate

Written informed consent was obtained from patients and/or their guardians at hospitals participating in the Japan Childhood Cancer Group Neuroblastoma Committee (JCCG-JNBSG). The study design was approved by the Ethics Committee of Saitama Cancer Center (approval nos. 1528 and 1529).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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