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FULL-LENGTH ORIGINAL RESEARCH

STXBP1 mutations in early infantile epileptic encephalopathy with suppression-burst pattern

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Summary

Purpose: De novo *STXBP1* mutations have been found in individuals with early infantile epileptic encephalopathy with suppression-burst pattern (EIEE). Our aim was to delineate the clinical spectrum of subjects with *STXBP1* mutations, and to examine their biologic aspects.

Methods: *STXBP1* was analyzed in 29 and 54 cases of cryptogenic EIEE and West syndrome, respectively, as a second cohort. RNA splicing was analyzed in lymphoblastoid cells from a subject harboring a c.663 + 5G>A mutation. Expression of STXBP1 protein with missense mutations was examined in neuroblastoma2A cells.

Results: A total of seven novel *STXBP1* mutations were found in nine EIEE cases, but not in West syndrome. The mutations include two frameshift mutations, three nonsense mutations, a splicing mutation, and a recurrent missense mutation in three unrelated cases. Including our previous data, 10 of 14 individuals (71%) with *STXBP1* aberrations had the onset of spasms after 1 month, suggesting relatively later onset of epileptic spasms. Nonsense-mediated mRNA decay associated with abnormal splicing was demonstrated. Transient expression revealed that STXBP1 proteins with missense mutations resulted in degradation in neuroblastoma2A cells.

Discussion: Collectively, *STXBP1* aberrations can account for about one-third individuals with EIEE (14 of 43). These genetic and biologic data clearly showed that haploinsufficiency of *STXBP1* is the important cause for cryptogenic EIEE.

KEY WORDS: *STXBP1*, EIEE, West syndrome, Haploinsufficiency.

Early infantile epileptic encephalopathy with suppression-burst (EIEE), also known as Ohtahara syndrome (Ohtahara et al., 1976), is characterized by early onset of tonic seizures, seizure intractability, characteristic suppression-burst patterns on electroencephalography (EEG), and poor outcome with severe psychomotor retardation (Djukic et al., 2006; Ohtahara & Yamatogi, 2006). We recently found de novo mutations in *STXBP1* (encoding syntaxin binding protein 1, also known as MUNC18-1) in individuals with EIEE (Saitsu et al., 2008). The subjects with *STXBP1* aberrations, including four missense mutations and a 2-Mb microdeletion encompassing *STXBP1*, showed the characteristic feature of EIEE. A mutant protein with a missense change (p.C180Y) showed structural instability with significant thermolability and impaired binding to syntaxin-1A compared with the wild-type (Saitsu et al., 2008). These findings suggest that haploinsufficiency of *STXBP1* causes EIEE.

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West syndrome is one of the most common infantile epileptic syndromes and is characterized by epileptic spasms, arrest of psychomotor development, and hypsarrhythmia on EEG (Kato, 2006). Brain malformations and metabolic disorders were found as underlying causes of symptomatic West syndrome, but many cryptogenic cases remain etiologically unexplained (Kato, 2006). Two causative genes, *ARX* (aristaless related homeobox) and *CDKL5* (cyclin-dependent kinase-like 5), are mutated only in a subset of familial and sporadic cases of West syndrome (Stromme et al., 2002; Kalscheuer et al., 2003; Kato et al., 2003; Weaving et al., 2004; Guerrini et al., 2007; Bahi-Buisson et al., 2008). EIEE and West syndrome are considered as a continuum of epileptic encephalopathies because the majority (75%) of EIEE transit to West syndrome (Yamatogi & Ohtahara, 2002; Ohtahara & Yamatogi, 2006). Specific mutations of *ARX* have been also found in EIEE (Kato et al., 2007), further suggesting a common pathologic mechanism among two syndromes. *STXBP1* mutations would be possibly involved in West syndrome. However, it remains to be determined.

To delineate the clinical spectrum of *STXBP1* mutations, a second cohort consisting of EIEE and West syndrome cases was investigated. Novel *STXBP1* mutations have been found only in EIEE cases. We also characterized biologic aspects of *STXBP1* mutations by using lymphoblastoid cells derived from a patient, and by transient expression of mutant STXBP1 proteins in neuroblastoma2A cells.

Subjects and Methods

Subjects

A total of 29 and 54 Japanese individuals with EIEE and West syndrome, respectively, were newly recruited as a second cohort. Brain malformations were not found in all cases. The diagnosis was made on the basis of clinical features and characteristic patterns on EEG. Experimental protocols were approved by Institutional Review Boards for Ethical Issues at Yokohama City University School of Medicine and Yamagata University Faculty of Medicine. Informed consent was obtained from all individuals and/or their families in agreement with the requirements of Japanese regulations. Clinical aspects of subjects with *STXBP1* mutations are summarized in Table 1.

Mutation screening

Genomic DNA was obtained from peripheral blood leukocytes according to standard protocols. Mutation screening of 1st to 20th exons covering the coding region of *STXBP1* was performed by high-resolution melt analysis (HRM). Realtime polymerase chain reaction (PCR) and HRM were serially performed in a 12-μl mixture on RotorGene-6200 HRM (Corbett Life Science, Brisbane, Qld, Australia). For the 2nd to 20th exons, PCR mixture contained 1× ExTaq buffer, 0.2 mM each dNTP, 0.25 μM each primer, 1.5 μM SYTO9 (Invitrogen, Carlsbad, CA, U.S.A.), and 0.375 U Ex TaqHS polymerase (Takara Bio, Ohtsu, Japan). For the first exon, the PCR mixture contains 1× PCR Buffer for KOD FX, 0.4 mM each dNTP, 0.3 μM each primer, 1.5 μM SYTO9, and 0.3 U KOD FX polymerase (Toyobo, Osaka, Japan). PCR primers and conditions are shown in Table S1. PCR samples showing an aberrant melting curve pattern were sequenced as previously described (Saitsu et al., 2008). For all the families showing de novo mutations, parentage was confirmed by microsatellite analysis as described previously (Saitsu et al., 2008).

RNA analysis

Lymphoblastoid cells (LCL) derived from a subject harboring a c.663 + 5G>A mutation was grown in Roswell Park Memorial Institute 1,640 medium supplemented with 10% fetal bovine serum (FBS), 1× Antibiotic-Antimycotic (Invitrogen), and 8 μg/ml tylosin (Sigma, Tokyo, Japan) at 37°C in a 5% CO_2 incubator. After incubation with dimethyl sulfoxide (DMSO) (as vehicle control) or 30 μM cycloheximide (Sigma) for 4 h, total RNA was extracted using TRizol (Invitrogen). One μg total RNA was subjected to reverse transcription using PrimeScript 1st strand synthesis kit with random hexamers (Takara). Minus reverse transcriptase (RT) control with no reverse transcriptase was included in each experiment. PCR was performed in a 15-μl mixture, containing 1 μl cDNA, 1× PCR Buffer for KOD FX, 0.4 mM each dNTP, 0.3 μM each primer, and 0.3 U KOD FX polymerase (Toyobo). Primer sequences are 5′-CTTTGTGCCACCCTGAAGGAGTACC-3′ in ex7-F and 5′-CAGTGCTATCCACAGGTCGTCGTC-3′ in ex10-R. The control cDNA isolated from a normal LCL sample was used as a reference. PCR products electrophoresed in 2% agarose gel were stained with ethidium bromide, and were analyzed by quantitative densitometry on FluorChem 8,900 (Alpha Innotech, San Leandro, CA, U.S.A.). Experiments were repeated three times. Inhibition of nonsense-mediated mRNA decay (NMD) was estimated according to the density ratios of upper normal and lower aberrant bands with/without 30 μM cyclophosphamide treatment in the culture of the patient's LCL. Statistical analyses were done using the unpaired Student's *t*-test (two-tailed). Each PCR band was sequenced using purified DNA by QIAEXII Gel extraction kit (Qiagen, Tokyo, Japan).

Expression vectors

A fragment containing internal ribosomal entry signal (IRES) and nuclear-localized Flag-DsRed was inserted into pEGFP-C1 vector (Clontech, Mountain View, CA, U.S.A.). Human *STXBP1* cDNA was fused to this vector, achieving dual expression of both N terminal EGFP-tagged STXBP1 and nuclear-localized Flag-DsRed. A wild-type *STXBP1* cDNA, four mutants (c.251T>A, p.V84D; c.539G>A, p.C180Y; c.1328T>G, p.M443R; c.1631G>A, p.G544D) and two normal variants (c.250G>A, p.V84I; c.1292A>T,

Table 1. Summary of clinical features of subjects with *STXBP1* mutations

Subject (age)	Sex	Mutation	Dx	Age at onset	Initial symptoms	Initial EEG	Age at onset of spasms	Age at onset of SB pattern	Age at transit to WS	Transition from spasms to other seizures	Transition to other EEG findings	Response to therapy	Development	Neurologic examination	Magnetic resonance imaging
1,751 (3 y)	M	c.1217G>A p.R406H de novo	EIEE	0 d	Bilateral convulsion	SB	3 w	1 m	5 m	No	Multifocal spike and slow wave complex	Intractable, hourly	No head control, No social contact	Profound MR, Severe spastic quadriplegia	Normal at 1 m, Mild brain atrophy at 3 y
1,989 (15 m)	M	c.1217G>A p.R406H *de novo*	EIEE	43 d	GTCS with upward eye gazing	SB	2 m	1 m	2 m	~48 d myoclonic seizure	No	Intractable, daily	No head control, No social contact	Profound MR, Severe spastic quadriplegia	Normal at 11 m
2,123 (20 m)	M	c.1217G>A p.R406H *de novo*	EIEE	15 d	Partial seizures (right hemiconvulsion)	Focal spike at P3	2 m	2 m	No	Tonic seizure to myoclonic seizure	Unknown	Intractable, daily	No head control, No social contact	Profound MR, Severe spastic quadriplegia	Normal at 1 m, Mild frontal brain atrophy at 20 m
1,792 (6 y)	F	c.157G>T p.E53X *de novo*	EIEE	2 d	Spasms	SB	2 d	1 w	6 m	Versive seizure after hypoxia at 2 y	Multifocal irregular spikes	Intractable, daily	No head control, No social contact	Profound MR, Severe spastic quadriplegia	Normal at 0 m, Mild ventricular dilatation at 14 m
1,694 (17 m)	M	c.388_389delCT p.L130DfsX11 *de novo*	EIEE	2 m	Secondary generalized seizures initiated from the right face	SB with fluctuated baseline	2 m	2 m	3 m	CPS	Multifocal spike and slow wave complex with desynchronization	Seizure-free after ACTH or VPA with KBr	No head control. No social contact	Profound MR, Severe spastic quadriplegia	Normal at 3 m
1,951 (6 m)	F	c.663 + 5G>A *de novo*	EIEE	5 d	Blinking to tonic seizures	SB with fluctuated baseline	1 m	1 m	3 m	Tonic seizure	Left temporal spike and slow wave complex	Seizure free with VB6 for spasms and ACTH for WS	Eye pursuit and smiling from 4 m, Head control and rolling over from 6 m	Moderate MR, Quadriplegia	Normal at 0 m, subdural effusion at 2 m
1,655 (6 m)	M	c.703C>T p.R235X *de novo*	EIEE	1 m	Spasms in cluster	SB	1 m	1 m	No	No	Occipital spikes	Seizure free from 6 m after high-dose PB	No head control, No social contact	Profound MR, Severe spastic quadriplegia	Delayed myelination and brain atrophy
2,103 (11 m)	F	c.747dupT p.Q250SfsX6 *de novo*	EIEE	3 d	Clonic convulsion	Focal spike at C3, Cz, Fz	31 d	1 m	10 m	Partial seizure (abnormal eye movement) and myoclonic seizures	Multifocal spikes	Intractable, hourly	No head control, Smiling from 5 m	Profound MR, Severe spastic quadriplegia	Normal at 1 m, left mild brain atrophy at 3 m
1,979 (10 y)	M	c.961A>T p.K321X *de novo*	EIEE	2 w	Partial seizures	SB	3 w	1 m	3 m	Partial seizure	Multifocal spikes with asymmetric background activity	Intractable, daily	No head control, No social contact	Profound MR, Severe spastic quadriplegia	Normal at 0 m, Brain atrophy and subdural hematoma at 7 m after ACTH

Dx, diagnosis; GTCS, generalized tonic–clonic seizures; SB, suppression-burst; WS, West syndrome; CPS, complex partial seizure; ACTH, adrenocorticotropic hormone; VPA, valproic acid; KBr, potassium bromide; VB6, vitamin B_6; PB, phenobarbital; MR, mental retardation; d, day(s); w, week; m, month(s); y, year(s); 0 w, 0–6 days; 0 m, 0–3 week.

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p.Q431L) were generated as described previously (Saitsu et al., 2008). c.250G>A was registered as single nucleotide polymorphism (SNP) (rs34830702). c.1292A>T was observed in one of 250 normal controls (allele frequency: 1/500), but not in EIEE or West syndrome.

Cell culture, transfection, and immunoblotting

Mouse neuroblastoma 2A (N2A) cells were grown as described previously (Saitsu et al., 2008). For transient expression experiments, N2A cells on glass cover slips (in 24-well plates for microscopic detection) and 3.5-cm culture dish (for immunoblotting) were transfected with 200 and 800 ng of plasmid DNA using FuGene6 reagent (Roche diagnostics, Tokyo, Japan), respectively. After 3 h, culture medium was changed to low serum medium (5% FBS) with 20 μM all transretinoic acid (Sigma) in order to induce neural differentiation, and cells were subsequently cultured for 2 days. For microscopic detection, N2A cells were washed in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde/PBS for 15 min. Cover slips were mounted using Vectashield with DAPI (Vector Laboratories, Youngstown, OH, U.S.A.) and images were visualized with an AxioCam MR CCD fitted to Axioplan2 fluorescence microscope (Carl Zeiss, Tokyo, Japan), and captured using Axio Vision 4.5 software (Carl Zeiss). The exposure time for enhanced green fluorescent protein (EGFP) and DsRed capture was fixed in a series of experiments to enable direct comparison between different experimental samples. For immunoblotting, N2A cells were washed twice in ice-cold PBS, and lysed in sodium dodecyl sulfate (SDS) sample buffer. Samples were size-fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to the polyvinylidene difluoride (PVDF) membrane, and analyzed with anti-Munc18 (for STXBP1 detection, 1:5,000 dilution) (BD Transduction Laboratories, Tokyo, Japan) or anti-Flag M2 (1:2,000 dilution) (Sigma) antibody. Secondary antibody was peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, U.S.A.). Blots were detected using the Supersignal West dura (Pierce, Yokohama, Japan). Chemiluminescence was evaluated by quantitative densitometry using a FluorChem 8,900 (Alpha Innotech). Experiments were repeated three times.

Results

A total of seven novel heterozygous mutations found in six males and three females are presented together with four reported missense mutations in Fig. 1 (Saitsu et al., 2008). The recurrent p.R406H mutation occurred at evolutionary conserved amino acid (Fig. 1). All the mutations are novel and occurred de novo. Parentage was confirmed using several microsatellite markers (data not shown). All the mutations were found only in EIEE cases, but not in West syndrome.

Clinical features of *STXBP1* aberrations

Detailed clinical information of individuals with *STXBP1* mutations is summarized in Table 1. All nine individuals were born at term without asphyxia except for the subject 1,751 who had the short umbilical cord. Their body weight, height, and head circumference were normal at birth other than the subject 1,792 with mild microcephaly [30.5 cm, −2.0 standard deviation (SD)]. Epileptic spasms were preceded by other seizure types including partial seizures in seven subjects (other than 1,792 and 1,655), and initial EEG in two subjects showed focal epileptic discharges (2,123 and 2,103). Only one subject demonstrated suppression-burst pattern on EEG (Fig. 2A) in the neonatal period. Transition to West syndrome was observed in seven subjects with EIEE. Although seizures were intractable in six subjects, three subjects responded to medication, such as adrenocorticotropic hormone (ACTH) injection, vitamin B_6, high-dose phenobarbital, and valproic acid. All subjects demonstrated severe psychomotor developmental delay, and only two subjects had the social smile. Most subjects presented with normal brain at the first magnetic resonance imaging (MRI), and then showed mild brain atrophy, which might be influenced by ACTH injection, after 1 year (Fig. 2B).

Abnormal splicing and nonsense-mediated mRNA decay

To observe mutational effects of c.663 + 5G>A (intron 8), reverse transcriptase PCR was performed using total RNA extracted from LCL derived from the subject 1,951. PCR primers were designed to amplify exons 7 to 10 (Fig. 3A). Only one band (338 bp), corresponding to the wild-type *STXBP1* allele, was amplified using a cDNA template from a control LCL (Fig. 3B). In contrast, a smaller band was detected from the subject's cDNA (Fig. 3B). Direct sequencing of both fragments revealed that exon 8 was skipped in the abnormal band (Fig. 3B), resulting in the insertion of nine new amino acids followed by a premature stop codon at position 203. As intensity of the smaller band was significantly weak (Fig. 3B), NMD may be involved (Maquat et al., 1981; Shyu et al., 2008). Intensity ratio of mutant versus normal band was 29% in untreated condition. The ratio was raised up to 67% after 30-μM cycloheximide treatment preventing NMD (Fig. 3C), suggesting that the early truncated mutant mRNA underwent degradation by NMD.

Degradation of mutant STXBP1 proteins

We previously demonstrated that intense fluorescence signals in clusters likely representing protein aggregation were observed in approximately 20% of N2A cells transiently expressing mutant EGFP-STXBP1 (p.V84D, p.C180Y, p.M443R, and p.G544D), suggesting structural instability of STXBP1 proteins with missense mutations (Saitsu et al., 2008). The other 80% of cells showed diffuse

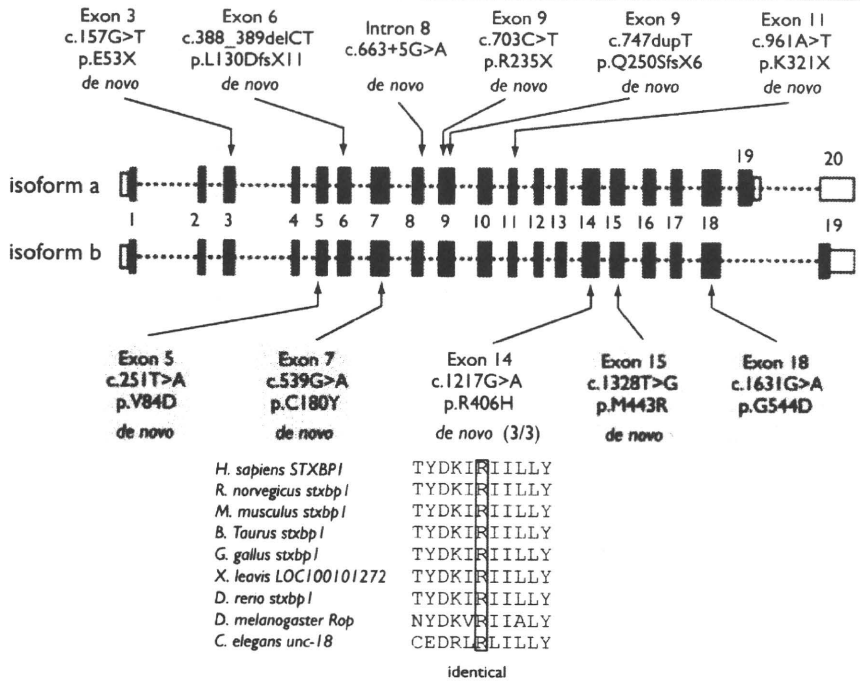

Figure 1.

Summary of *STXBP1* mutations found in Japanese individuals with EIEE. Schematic representation of *STXBP1* consisting of at most 20 exons (untranslated region and coding region are open and filled rectangles, respectively). There are two isoforms, a (GenBank accession number, NM_003165) with exon 19, and b (NM_001032221) without exon 19 of isoform a. The location of mutations is indicated by arrows. A total of 11 different mutations were presented: missense mutations below the gene scheme and the other types of mutations above the gene. Four mutations previously reported are in shadow (Saitsu et al., 2008), and seven without shadow are newly found in this study. All the seven novel mutations occurred de novo. The recurrent p.R406H missense mutation found in three unrelated subjects occurred at evolutionary conserved amino acids. CLUSTALW website (http://align.genome.jp/) was used to align homologs of different species.

cytosolic protein distribution similar to that expressing the wild-type, but the signal intensity was much weaker, implying possible protein degradation. To observe mutant protein degradation, a dual-expression vector of EGFP-STXBP1 and nuclear Flag-DsRed (EGFP-STXBP1-IRES-nuclear Flag-DsRed) was generated. Two days after transfection, the wild-type EGFP-STXBP1 was expressed in cytosol, but not in nucleus or plasma membrane as described previously (Saitsu et al., 2008). Notably, in mutant EGFP-STXBP1 transfected cells (p.V84D, p.C180Y, p.M443R, and p.G544D), EGFP signals were almost absent, whereas nuclear DsRed was expressed at comparable levels to that of wild-type (Fig. 4A). Two normal variants, p.V84I and p.Q431L, were expressed in a manner similar to that of the wild-type, with less intensity for a p.Q431L variant (Fig. 4A). Decreased level of mutant STXBP1 expression was confirmed by immunoblotting using Munc18 antibody (Fig. 4B, top). Transfection efficacy or amount of protein loading was similar in all cases based on the level of Flag-DsRed (Fig. 4B, bottom).

Discussion

We could successfully find a total of seven novel mutations: two frameshift mutations (2-bp deletion and 1-bp insertion), three nonsense mutations, a splicing mutation, and a recurrent missense mutation. Transcripts associated with frameshift, nonsense, and splicing mutations are likely to be degraded by NMD. In the case harboring c.663 + 5G>A, aberrant splicing associated with NMD was demonstrated in LCL derived from the subject. Moreover, mutated STXBP1 proteins underwent degradation in N2A cells. Variable expression of mutated STXBP1 proteins has been also reported in HeLa cells (Ciufo et al., 2005), suggesting that degradation mechanism of mutated STXBP1 proteins may be common in mammalian cells. Considering these genetic and biologic data presented here as well as a complete deletion of *STXBP1* in one EIEE case (Saitsu et al., 2008), haploinsufficiency of *STXBP1* consistently results in EIEE.

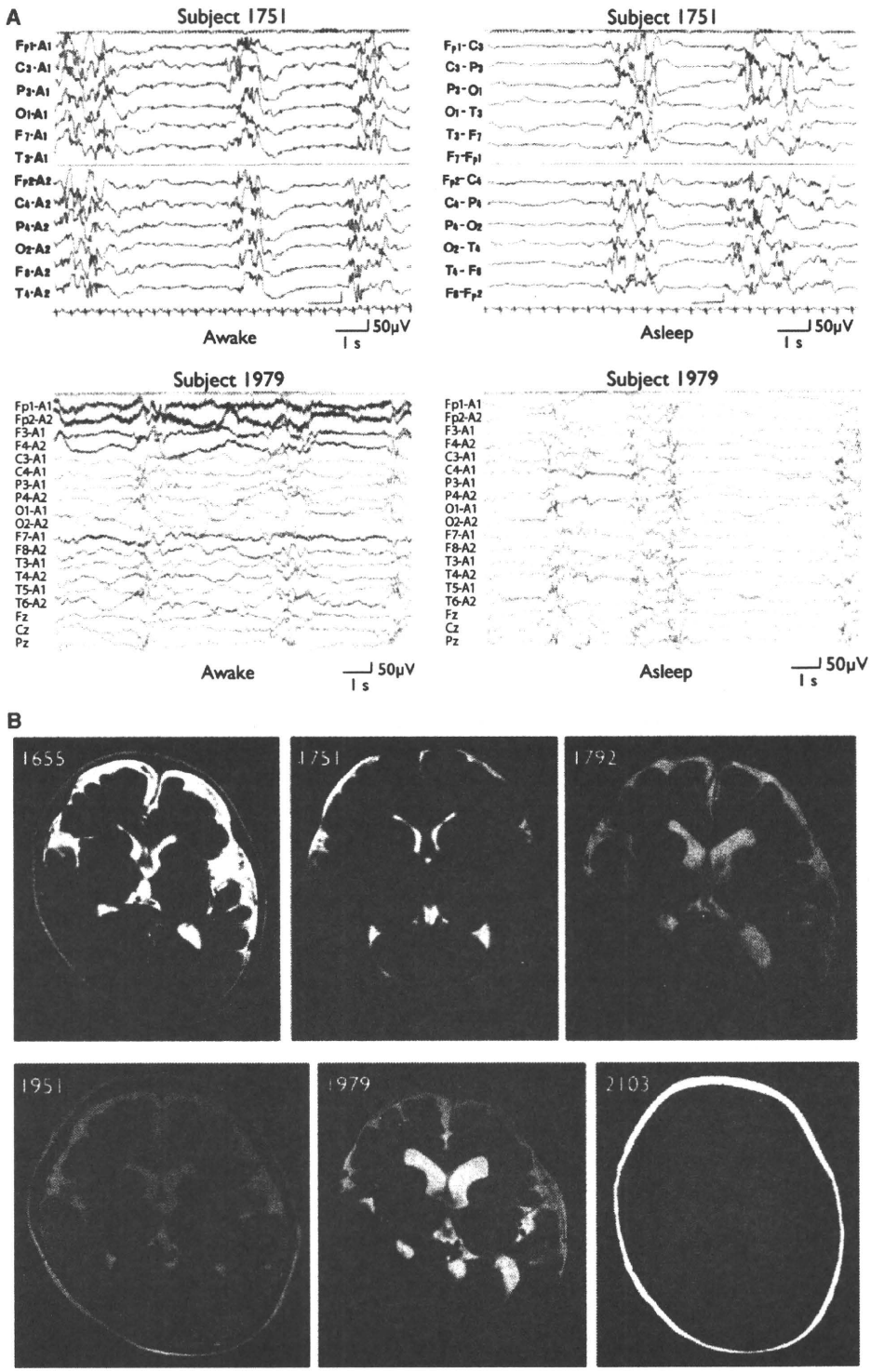

Figure 2.
EEG and brain MRI of subjects with *STXBP1* mutations. (**A**) EEG of subjects 1,751 and 1,979 at 1 month of age. Suppression-burst pattern characterized by diffuse bursts of irregular spikes and slow waves lasting 1 to 3 s with low-amplitude background lasting 2–5 s are seen during both sleep and wake. (**B**) T_2-weighted brain MRI scan of subjects 1,655 at 9 months of age, 1,751 at 1 month, 1,792 at 15 months, 1,951 at 1 month, and 1,979 at 15 months, and T_1-weighted MRI scan of subject 2,103 at 5 months. Mild dilation of lateral ventricles is seen in patients with 1,792, 1,951, 1,979, and 2,103, but none shows brain malformation.

Epilepsia, **(*):1–9, 2010
doi: 10.1111/j.1528-1167.2010.02728.x

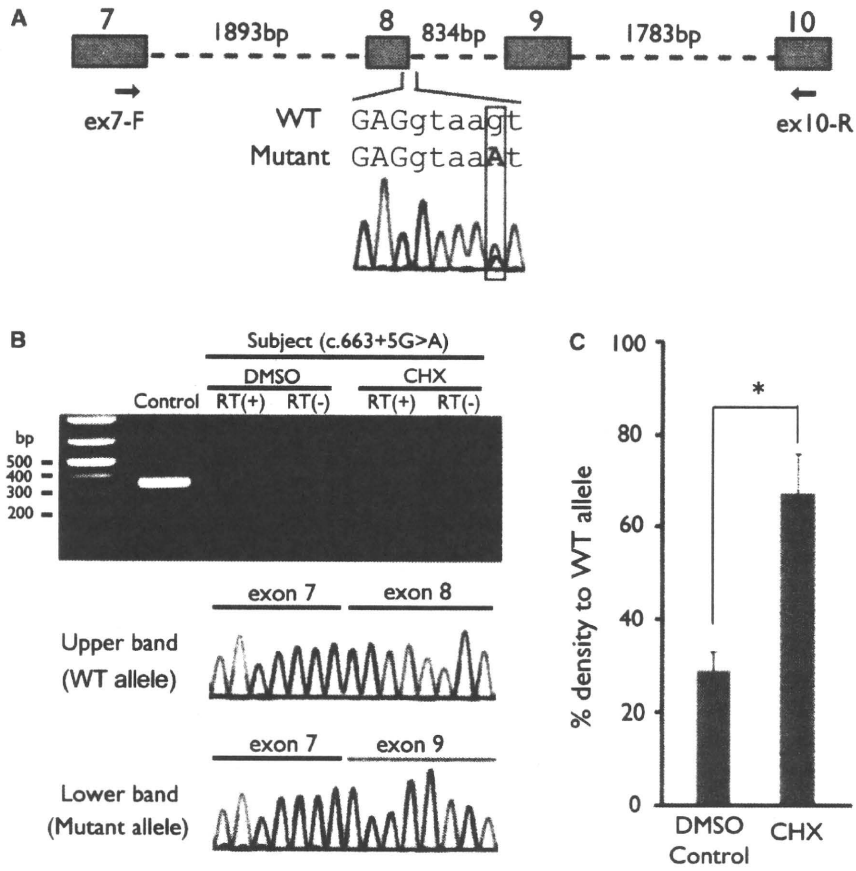

Figure 3.

The c.663 + 5G>A mutation causing abnormal splicing associated with NMD. (**A**) Schematic representation of the genomic structure from exons 7–10 of *STXBP1*. Exons, introns, and primers are shown by boxes, dashed lines, and arrows, respectively. The mutation in intron 8 was colored in red. Sequences of exon and intron are presented in upper and lower cases, respectively. (**B**) RT-PCR analysis of subject 1,951 with c.663 + 5G>A and a normal control. Two PCR products were detected from the subject's cDNA: upper was the wild-type (WT) transcript and lower was the mutant. Only a single WT amplicon was detected in a control. The mutant amplicon was significantly increased by 30 μM cycloheximide (CHX) treatment compared to DMSO treatment as a vehicle control. RT (+): with reverse transcriptase, RT (−): without reverse transcriptase as a negative control. Sequence of WT and mutant amplicons clearly showed exon 8 skipping in the mutant allele. (**C**) Quantitative analysis of the NMD inhibition by CHX based on the data shown in (**B**). *p = 0.0023 by unpaired Student's *t*-test, two tailed. Averages of three repeated experiments are shown with error bars (SD).

The subjects with *STXBP1* mutations in this study showed distinctive features of EIEE, such as early onset seizures, typically frequent epileptic spasms, suppression-burst pattern on EEG, transition to West syndrome after a few to several months, and severe developmental delay, as described in our previous report (Saitsu et al., 2008). Taken together, 14 individuals with EIEE have been found to be associated with *STXBP1* aberrations. A detailed analysis of clinical features revealed that the age at onset of epileptic spasms is rather later in subjects with *STXBP1* aberrations compared to the 16 original subjects reported by Yamatogi and Ohtahara (2002). Only 29% of the subjects (4 of 14) in our series had the onset of spasms within 1 month in contrast to 75% (12 of 16) in the series of Yamatogi and Ohtahara (2002). Although the subjects with *STXBP1* aberrations should be diagnosed as EIEE, the late onset of spasms would suggest that *STXBP1* aberrations could cause an intermediate epileptic encephalopathy between EIEE and West syndrome. The presence of two subjects showing suppression-burst pattern with fluctuated baseline, which could be regarded as intermediate pattern between suppression-burst and hypsarrhythmia, may support this idea.

Another interesting feature is the presence of myoclonic seizures, which are thought to be rather rarely observed in EIEE cases, in three subjects with *STXBP1* mutations (1,989, 2,123, and 2,103). Myoclonic seizures are the main ictal symptom of early myoclonic encephalopathy (EME), which is another epileptic syndrome showing suppression-burst patterns on EEG (Engel, 2006). The prevailing initial seizure type is a main difference between EIEE and EME:

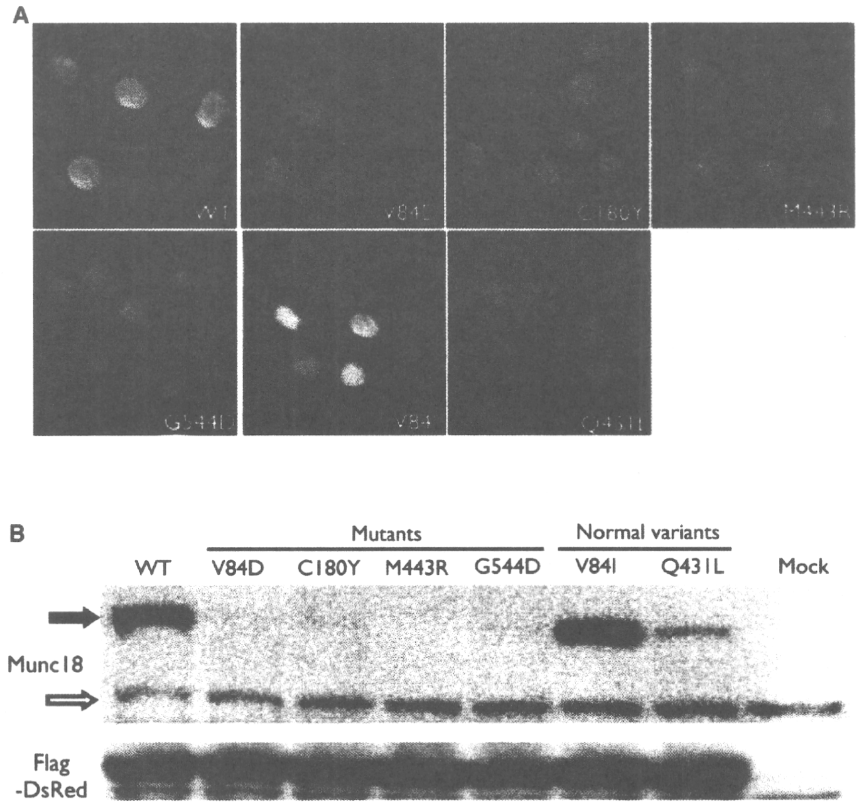

Figure 4.
Degradation of mutant EGFP-STXBP1 proteins in N2A cells. (**A**) The wild-type (WT) EGFP-STXBP1 was expressed in cytosol, but not in nucleus or in plasma membrane. In contrast, cells transfected with mutant EGFP-STXBP1 (p.V84D, p.C180Y, p.M443R, and p.G544D) showed almost absent EGFP signals, whereas nuclear DsRed was expressed at levels comparable to that of WT. The p.V84I variant registered as SNP (rs34830702) was expressed similarly to the WT. The p.Q431L variant found in a normal control was expressed in a similar pattern but weakly compared to the WT. Exposure time for EGFP and DsRed capture was fixed, enabling direct comparison between different samples. (**B**) Immunoblot analysis of mutant STXBP1 proteins by using a monoclonal Munc18 antibody (top). Upper and lower bands represent EGFP-STXBP1 and endogenous STXBP1 proteins, respectively. Expression of four mutant STXBP1 proteins was not detected, whereas WT and two normal variants could be detected. The observed differences in expression were not due to either transfection or loading differences, because the level of Flag-DsRed was similar in all cases (bottom). Mock, no transfection.

tonic seizures in EIEE and myoclonic seizures in EME. However, EIEE and EME have common features, and it is often difficult to distinguish between them. These three subjects can be diagnosed as EME when myoclonic seizures dominate. Therefore, it is possible that *STXBP1* could be also causative for EME. In terms of genotype–phenotype relationship, we found no difference in clinical data between seven subjects with missense mutations and seven subjects with microdeletion, premature termination codon, or splicing mutations. This finding is supported by our experimental data that both missense mutations and a splicing mutation resulted in haploinsufficiency of *STXBP1*: degradation of STXBP1 proteins with missense mutations and NMD associated with an aberrant splicing.

Recently, Hamdan et al. (2009) reported that two de novo *STXBP1* mutations, p.R388X and c.169 + 1G>A, were identified in 2 of 95 individuals with mental retardation and nonsyndromic epilepsy (2%), suggesting that clinical spectrum of *STXBP1* mutations may be broader. However, it is clear that EIEE is the core phenotype of *STXBP1* aberrations in this Japanese cohort as one-third of EIEE cases harbored its mutations. It is also noteworthy that none of West syndrome cases possessed *STXBP1* mutation, suggesting that subjects with initial West syndrome is rarely caused or not caused by *STXBP1* abnormality.

How haploinsufficiency of *STXBP1* leads to infantile epileptic encephalopathy remains to be elucidated. *STXBP1* abnormalities suggest a novel story in which impaired synaptic vesicle release is involved in pathogenesis of epilepsy. Although no seizures have been reported in *Stxbp1* heterozygous knockout mice (Verhage et al., 2000), they showed impaired synaptic function due to reduced size and

replenishment rate of readily releasable vesicles (Toonen et al., 2006), suggesting that heterozygous deletion of *Stxbp1* indeed affected synaptic function in mice. It is possible that the absence of seizures in *Stxbp1* heterozygous knockout mice might be due to the different genetic background. Because *Stxbp1* mutants have been backcrossed for at least six generations to a C57BL/6 background (Toonen et al., 2006), it would be interesting to examine whether seizures would occur in other genetic background. Alternatively, effect of gene dosage alterations of *STXBP1/Stxbp1* may vary between humans and mice: Humans might be more susceptible than mice; therefore, loss of function of one allele could cause seizures only in humans but not in mice. Appropriate mice models by neatly manipulating gene dosage of *Stxbp1* may mimic human phenotype and enable detailed analysis of pathogenesis of infantile epileptic encephalopathy in relation to impaired synaptic function.

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Disclosure

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines. None of the authors has any conflict of interest to disclose.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. PCR conditions and primer sequences.

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Telomere Maintenance as Therapeutic Target in Embryonal Tumours

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Abstract: Embryonal tumours most commonly occur in the first few years of life and account for approximately 30% of childhood malignancies. Knowledge of these tumours' genetics has already impacted on their clinical management and further knowledge of their cellular immortalization will hopefully result in novel therapies. The ends of human chromosomes are capped and protected by telomeres; cellular replication, however, causes their loss. A critical length of telomere repeats is required to ensure proper telomere function and avoid the activation of DNA damage pathways that result in senescence and cell death. To proliferate beyond the senescence checkpoint, cells must restore their telomere length. Hence stabilization of telomere is an important step in cell immortalization and carcinogenesis. Telomere maintenance is evident in virtually all types of malignant cells, including embryonal tumours, where either a telomerase-dependent or alternative lengthening of telomeres (ALT) mechanism is employed in order to ensure their limitless replicative potential. For this reason effective strategies targeting telomere maintenance in cancer cells require a combination of telomerase and ALT inhibitors. In this review, we are giving an overview about telomere maintenance in childhood tumours and discussing its potential as a new therapeutic target.

Keywords: Cancer, child, telomere, novel therapies.

INTRODUCTION

The term embryonal tumours refers to a broad heterogeneous group of childhood malignancies. This includes medulloblastoma (MB), neuroblastoma (NB), soft tissue sarcomas, nephroblastoma (Wilms' tumour), bone tumours, retinoblastoma, hepatoblastoma (HB), germ-cell tumours and various other rare subtypes. Embryonal tumours differ fundamentally from adult onset cancers, both in their cell biology and their tissue environment. It is therefore likely that improved prognosis and effective treatments for these cancers will only be possible when the molecular events that are specific to the tumour's development are better understood [1].

The conceptual framework of cancer development has been redrawn in the current decade. There is gathering acceptance that embryonal tumour formation is a phenotypic outcome of dysregulated organogenesis with tumours viewed as abnormally differentiated tissue. There is accumulating evidence that embryonal solid tumours, similar to leukaemia, are organized as a developmental hierarchy which is maintained by a small fraction of cells endowed with many shared properties of tissue stem cells [2, 3].

The high proliferative ability of embryonal cells during development requires the establishment of a safe telomere maintenance mechanism for counteracting the shortening of chromosomal termini. Dysregulated, unlimited proliferation and the ability to bypass senescence are two of the acquired capabilities of cancer cells. Loss of telomere function is associated with loss of cancer cell viability through induction of apoptosis [4]. Therefore telomeres - symbolically supposed to be cancer's Achilles heel - and chromosome end biology have become highly attractive therapeutic targets against malignant tumours [5]. The erratic clinical behaviour of paediatric embryonal tumours suggests variable proliferative potential, thus making them attractive candidates for the study of telomere maintenance as a possible prognostic marker and/or therapeutic target. Elevated telomerase and telomere shortening could be signs of the excessive cell divisions experienced by cancer cells and could reflect the stage of malignancy and disease prognosis [6]. Down-regulation of telomerase activity has been shown to induce growth arrest and differentiation, which might predict a close correlation between telomerase activity levels and clinical outcome, while tumours with sustained telomerase activity might therefore become choice targets for telomerase directed therapy [7-10]. This review will provide a perspective on our current knowledge of embryonal tumours' telomere biology with respect to its developmental, diagnostic and therapeutic relevance.

TELOMERE DYNAMICS

Telomeres are specialized nucleoprotein complexes that cap and protect the ends of human chromosomes [11]. Telomeric chromatin is formed by tandem TTAGGG repeats together with specific telomere-binding proteins that play essential roles in protecting the chromosome from damage and degradation. Telomere repeats span ~10–15 Kb in length in humans and end in a 150–200 nucleotide single-stranded G-rich overhang that folds back and anneals with the double-stranded region of the TTAGGG repeats to form a large telomeric loop, known as the T-loop [12]. As a consequence, a portion of the strand along the length of the overhang-invasion is displaced, forming a single-strand DNA region called a D-loop [13]. The groups of telomere-associated proteins that form and stabilize the T-loop secondary structure are collectively called shelterin (Fig. 1). These shelterin proteins comprise the telomere repeat factor 1 and factor 2 complexes (TRF1 and TRF2) that bind to double-stranded telomeric DNA and the protection of telomeres 1 protein (POT1) that binds the single-stranded 3` G-rich overhang. Three other interconnecting proteins (TIN2, TPP1, and RAP1) protect the telomere integrity by assisting in the T- and D-loop formation [11, 14]. Besides determining the structure of the telomeric sequences, shelterin proteins have also been found to play a significant role in controlling the synthesis of telomeric DNA [11]. For more details on shelterin proteins binding sites and function see (Table 1)

Telomerase is a cellular ribonucleoprotein enzyme that stabilizes telomere length by adding TTAGGG repeats to the telomeric ends of the chromosomes (Fig. 2), thus compensating for the continued erosion of telomeres that occurs in the absence of telomerase. Telomerase is composed of two main components, human telomerase RNA (hTR) and human telomerase reverse transcriptase TERT [15-18]. This enzyme utilizes its own RNA as a template to synthesize telomeric DNA. Together with telomere-binding proteins, telomerase confers stability on the chromosomes and counteracts the telomere-dependent pathways of cell mortality [19]. Telomerase activity changes through life, going from a peak of activity during the first trimester *in utero*, where virtually all the tissues have active telomerase [20], to undetectable levels after birth in

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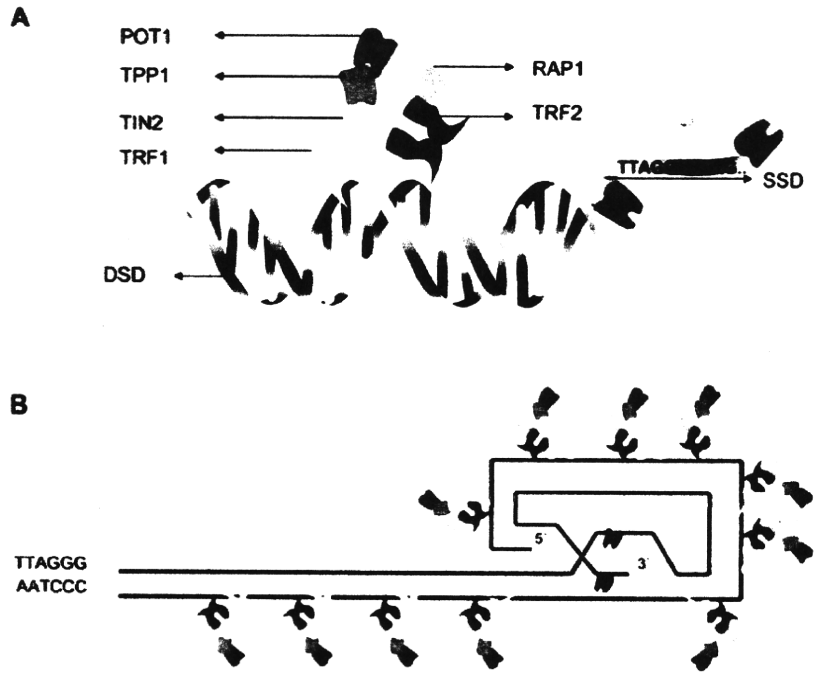

Fig. (1). Shelterin complexes.
(A) TRF1 and TRF 2 binds double-stranded telomeric DNA while POT1 protein bind the single-stranded DNA, resulting in a higher-order complex (t-loop) **(B)** Schematic of shelterin on telomeric DNA: The single-stranded G-rich overhang is protected by invasion within the double-stranded telomere. DSD: Double stranded DNA, SSD: Single stranded DNA.

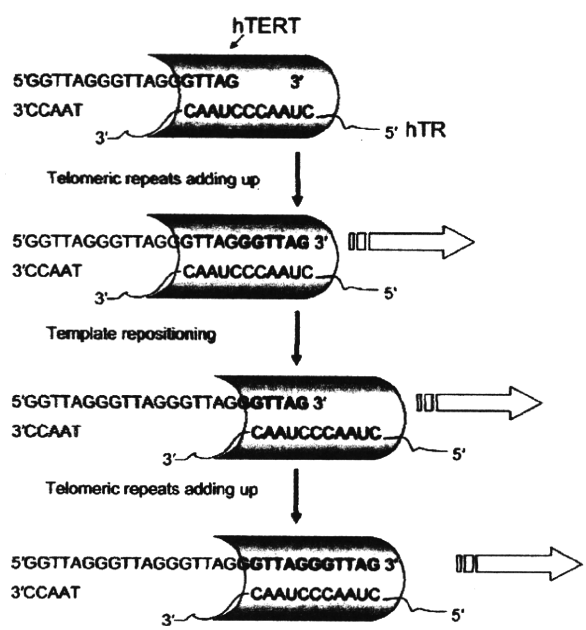

Fig. (2). Telomere elongation by telomerase.
TERT adds TTAGGG repeats to the telomereic ends utilizing the telomerase internal template hTR to specify the sequence.

most somatic tissues with the exception of highly proliferative cells such as germ cells and stem cell compartments [21].

Beside telomerase, in some tumours, particularly those of neuroepithelial and mesenchymal origin, telomeres are maintained by an alternative lengthening of telomere (ALT) mechanism [22-24]. In this process telomeres are usually longer and more heterogeneous than in telomerase-positive cells. In ALT cells, telomeric sequences and telomeric proteins are associated in large nuclear complexes to form ALT-associated promyelocytic leukaemia (PML) bodies that also contain recombination factors [25-27]. However, the exact mechanisms involved in recombination at telomeres are poorly understood.

TELOMERE/TELOMERASE BIOLOGY DURING DEVELOPMENT

Highly proliferative cell types such as embryonic cells, require active and controlled telomere maintenance strategies in order to protect the integrity of their genomes effectively. Most studies aimed at understanding the dynamics of the embryonic cells with respect to self-renewal, proliferation, and differentiation focus on genetic profiles and/or external signals such as growth factors, while the role of telomere and telomerase dynamics during embryonal development has barely been addressed. In germ line cells human telomeres are balanced between shortening processes with each cell division and elongation by telomerase, but once the cell is terminally differentiated or mature the equilibrium is shifted to gradual telomere shortening by repression of the telomerase enzyme [28-34]. Although telomerase activity is not high in non-proliferating sperms and ova, it is highly activated after fertilisation and maintained in embryonal stem cells up to blastocyst stage (Fig. 3) [30, 35, 36]. The absence of telomerase activity in some abnormal human oocytes has been linked to shortened telomeres and associated with reproductive chromosomal abnormalities such as translocations and therefore it could be used as a marker for the health status of the oocyte and the future embryo [37-39].

Table 1. Shelterin Related Proteins

Schelterin Protein	Function	Binding Site	References
TRF1 (Homodimer)	• Regulation of telomere length synthesis • Functional telomere structure	Double stranded telomeric DNA	[214]
TRF2 (Homodimer)	• Telomere length regulation • Telomere protection • TRF2 inhibition leads to telomere dysfunction	Double stranded telomeric DNA	[183]
POT1	• Telomere length regulation • Telomere capping	Single stranded telomeric DNA	[215]
TIN2	• Mediates the telomere architectural role of TRF1	Function as scaffold and binds TRF1 with its C-terminus	[216]
TPP1	• Negative regulator of telomere length • Stabilize the interactions between TRF1, TIN2 and TRF2	Function as scaffold and binds directly to POT1 and TIN2	[217]
RAP1	• Negative regulator of telomere length maintenance	TRF2 interacting	[218]

Embryonic stem cells are capable of indefinite self-renewal together with the ability to produce any cell type in the body. They display high levels of telomerase activity and TERT expression, both of which are rapidly down-regulated during differentiation [40] and are much lower or even absent in somatic cells. Moreover, increased telomerase activity enhanced self-renewal ability, proliferation and differentiation efficiency in TERT-overexpressing embryonic stem cells [40]. Although telomerase activity is supposed to be crucially important in telomere length setting during development, no direct correlation could be found between telomere length setting and telomerase activity within embryonic cells [41, 42]. Whether telomerase per se plays a direct role in early development is not certain, but its counterbalancing function against telomere shortening has been shown to be vital for stem cell viability [41, 42]. In adult stem cells the level of telomerase activity is low or undetectable and is upregulated in committed progenitor cells which have high reproducible activity in each tissue but insufficient to maintain their telomere length stably [40, 43, 44].

High levels of TERT mRNA and telomerase RNA component (TERC) have been detected in different regions of the developing murine central nervous system and this correlates with the proliferation of neural progenitors as early as embryonal day 10.5 of gestation [45-47]. A role for TERT in regulation of developmental death of neurons is suggested by a decrease in TERT expression that coincides with the period of neuronal death and by data showing that TERT promotes survival of developing brain neurons [48, 49]. Telomere length was found to be regulated during human, bovine and mouse embryogenesis by a telomerase-dependent mechanism [50]. In 20 week old human foetuses after the embryonic period and organogenesis are finished, telomerase has been reported to be expressed only in tissue-specific stem cells (Fig. **3**) [38, 41, 51, 52]. In contrast to embryonic cells, studies on tissue stem cells have reported low levels of telomerase activity [53-55]. It is difficult to argue, therefore, that this phenomenon is not a primitive tumour-suppressor mechanism. Whether telomerase per se plays a direct biological role in normal tissue or cancer development is not certain, but its counterbalancing function against telomere shortening has been shown to be vital for organ and tissue homeostasis and cell viability. A comprehensive understanding of how telomere/telomerase biology is regulated during development is crucial for determining how cancer may arise when embryonal cell regulation is disturbed. This invokes the embryonal tumour cell of origin, as telomere dynamics reflect the mitotic history of highly proliferating cells [2, 56]. Knowledge about factors that contribute to telomere length variability during intrauterine life will help to elucidate the presumed causal connections between early development and abnormal phenotypes, such as cancer, that appear later during childhood [42, 57-59]. These areas of investigative biology should help to determine the cellular culprit of particular embryonal cancer types, both with respect to the cell type in which neoplastic transformation occurs and to the molecular events that permit it. However, the limited number of studies available and the shortage of patient samples represent a challenge in investigating the real impact of telomere maintenance on childhood cancer development [42].

TELOMERE MAINTENANCE DURING TUMOUR DEVELOPMENT

Despite the impressive advances that have been made in cell and molecular biology, how tumour cells are actually initiated and progress is still widely debated. The concept that the incidence of cancer rises exponentially in the final decades of life due to the sequential accumulation of the somatic mutations does not really fit the onset of paediatric cancers of mesenchymal or haematopoietic origin that develop and manifest early in childhood. Identification of the cells that mediate tumour initiation in childhood cancer and discovering the information that is necessary for the cell to transform itself into a neoplastic cell will provide an important baseline for genomic and proteomic analyses of embryonic tumours [60, 61].

Different hypotheses have been postulated in the literature; one assumes that a somatic differentiated cell can dedifferentiate or reprograme to regain properties associated with cancer cells whereas others claim that a stem cell is needed to initiate the carcinogenic process [62]. The first model scenario depends on the hypothesis that rapid proliferation of the telomerase negative dedifferentiated somatic cells can lead to shortened telomeres that may promote chromosomal and genomic instability which then primes the cell to become cancerous. In a later stage telomerase is then activated and stabilizes the previously shortened telomeres, thereby prolonging the lifespan of cancer cells. This hypothesis has been supported experimentally by the observation that almost all malignant cancers have telomerase activity, despite their shortened telomeres [28, 63-66]. Indirect support for this view comes from the observations that benign or pre-cancerous lesions are telomerase silent [63] while high telomerase levels are found to correlate with other molecular markers of progression, such as *MYCN* amplification in NB and with worse clinical outcomes [67]. This model implies that telomerase activation in cancer is an induced or aberrant function in otherwise enzyme-deficient somatic cells destined for senescence [43]. An alternative view is that telomerase may either contribute to tumour development through mechanisms unrelated to telomere length maintenance [68] or on the contrary TERT may exert a cell survival-promoting function [69, 70]. The second interesting hypothesis, which might apply in neoplasms derived during development, is that the tumour cells are telomerase positive not because of TERT expression under a selective pressure, but because they are derived from the oncogenic transformation of a stem cell or a pluripotent early precursor cell which has retained its telomerase

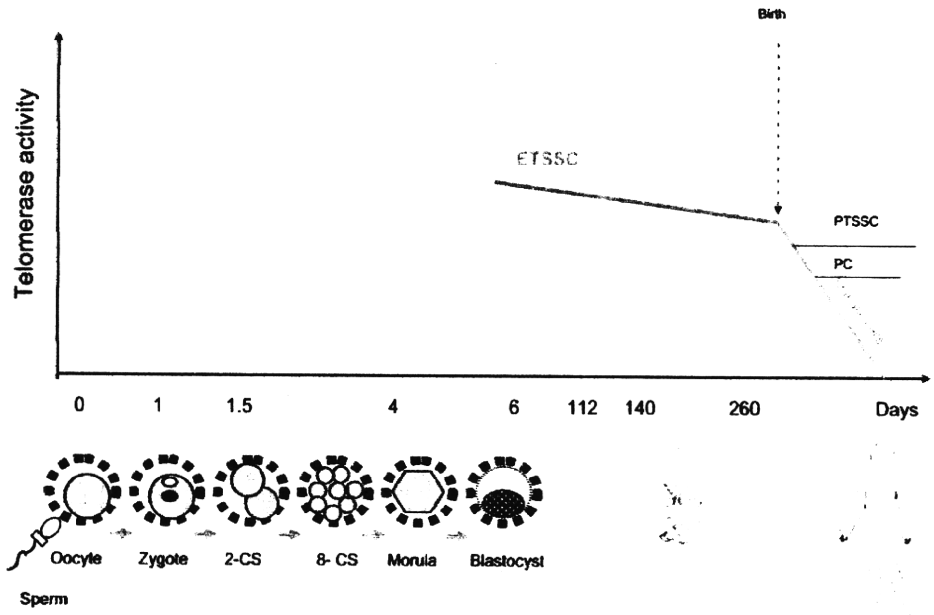

Fig. (3). Diagram depicting the telomerase activity during human embryonal development.
Telomerase activity is detected in all early developmental stages from oocytes through to blastocyst stage embryos. Telomerase activity is found to be relatively low in matured oocytes, increases after fertilization and then decreases gradually until the eight-cell stage. From the eight-cell stage onward, telomerase activity increases progressively with advancing embryo stage and reaches its highest level at the morula and blastocyst stage embryos and then decreases in the inner cell mass stage. In human fetuses (when the embryonic period and organogenesis are finished), telomerase is expressed in tissue-specific stem cells. Just after birth, telomerase activity is downregulated with the exception of dividing cells [38, 41, 42, 52].
CS: Cell stage, ETSSC: Embryonic tissue special stem cell, PTSSC: Postnatal tissue special stem cell, PC: Proliferating cells.

activity [40, 43, 44]. This concept has been proposed for several tumours [71] and supported by a number of reports demonstrating the presence of cancer stem cells in different adult cancers [72, 73]. In paediatric malignancies the cancer stem cell hypothesis was recently described in studies performed on leukaemia, where it was shown that a single cell with stem cell markers had the capacity of inducing the disease in mice [74]. More recently, cancer stem cells have also been isolated from solid embryonal tumours such as MB, NB, Ewing's sarcoma, RMS and HB [75-80]. However, much larger numbers of tumour samples and prospective multiparameter cell sorting experiments are needed for the cancer stem cell hypothesis to become widely accepted.

There is good evidence to suggest that telomere length maintenance in stem cell populations is important in facilitating the cell division required for tissue homeostasis. However, there has to be a balance between maintaining regenerative potential, on one hand tumour suppression, on the other. One mechanism that may contribute to adjust this balance may be telomere length, whereby stem cells may need to maintain telomeres at a length that provides sufficient replicative capacity for tissue homeostasis, versus the requirement to minimise telomere length and replicative capacity as a tumour suppressive mechanism. The dynamics of telomeres in stem cell populations may thus be crucial in the balance between tumour suppression and tissue homeostasis [81].

Telomerase in germ and stem cells continuously counteracts telomere shortening during cellular proliferation and permits embryonic stem cells to escape senescence [82, 83]. During early tumour formation, however, the timing of telomerase activation and telomere shortening may not be coordinated in a similar fashion. There is a suggestion that during the tumourigenesis process, prior to telomerase activation, telomere erosion may have evolved to a level where telomeric repeat sequences are too short to provide a functional substrate for telomerase enzyme activity [84]. In this scenario, as telomeres shorten with each cell cycle the "sticky" ends of chromosomes become prone to fusions [85], leading to subsequent chromosomal instability [86-88] and offering a mechanism for a continuous rearrangement of chromosome structure that might contribute to oncogene amplification and tumour suppressor gene deletion [89 , 90]. In fact, concurrent telomere shortening and genomic instability have been observed in the majority of embryonic tumours investigated including: Wilms' tumour [91-95], MB [96-99], NB [100-103] and rhabdomyosarcoma [104-106]. The view represented by the stem cell origin of embryonal tumours implies that the genetic alterations which lead to cancer accumulate in embryonal stem cells rather than mature cells. However, an alternative opinion holds that it is important to separate tumour-initiation and tumour-propagation; this may not involve the same cell type as the tumour-propagating cell may be a much differentiated progeny of the tumour-initiating cell. Hence improved therapeutic efficacy may be achieved by targeting both cell types which drives malignant progression as well as that which initiates – and maintains the stem cell pool of the tumour [2].

In summary, the role of telomere biology during childhood cancer development is complex and not yet fully understood. There is no simple connection between telomerase activity, telomere length and either disease stage or diagnosis of a malignancy. Telomere lengths have been found to differ at different ages and even among individuals of the same ageand telomerase activity and length vary in various organs [107, 108]. These inter- and intra-individual differences complicate the interpretation of telomere length abnormalities in cancer. Whether embryonal cancer cells reactivate the telomerase or up-regulated telomerase activity causes the cancer is still uncertain. A better understanding of the factors that contribute to telomerase biology and telomere length variability during intrauterine life, together with the uncovering of telomere biology in cells that mediate tumour initiation in childhood cancer and the information needed for these cells to transform themselves into neoplastic cells, will help to elucidate the presumed causal

connections between early development and the later abnormal phenotype.

MEDULLOBLASTOMA

MB results from the transformation of primitive neuroectodermal cells. MB is a malignant and invasive tumour with a relatively poor prognosis and represents more than 20% of all pediatric brain tumours [109]. It is predominately neuronal in nature and typically located in the cerebellum. It has been postulated that MB tumour formation is a phenotypic outcome of dysregulated neurogenesis, with tumours viewed as abnormally differentiated neural tissue. In relation to this, evidence is accumulating that MB tumours are maintained by a small fraction of cells that share properties of tissue stem cells. It is not yet clear whether multipotent stem cells, developmentally restricted progenitors, or other cells give rise to these paediatric malignancies. It is hoped that by defining the cell (or cells) from which they form and the relationship between normal development and oncogenesis, improved therapies can be developed [110].

Data on telomere maintenance or the association between telomerase expression and clinical outcome in MB are scarce while examination of the few reports that do exist yields conflicting results. Studies have shown that large increases in chromosomal material in the 5p15 region, where the TERT gene is located, are detectable in MB, suggesting that the TERT gene could be amplified in CNS embryonal tumours [65, 111, 112]. Fan *et al.* used differential PCR and real-time RT-PCR to determine the relationship between TERT gene copy number, TERT mRNA expressionand clinical outcome in CNS embryonal tumours including MB [113]. The group found that the TERT gene was amplified in 42% of 36 primary MB samples examined. The TERT amplification was found to correlate with the increased expression of TERT mRNA in almost all the tumours, while MB patients with increased TERT expression in their tumours showed a trend towards worse clinical outcomes. The group suggested that changes may have happened at the TERT locus during the evolution of MB, indicating a possible role for telomerase in the pathogenesis of MB [113]. Other groups, including our laboratory [114-117], detected telomerase enzyme activity in cultured MB cells *in vitro*. Our group investigated the mRNA expression level of TERT in 50 primary MB samples and compared it with 7 normal brain samples. 76% of the primary MB samples had upregulated TERT mRNA expression [114]. While a positive correlation between TERT mRNA expression and telomerase activity was detected in MB cell lines, no correlation was found between telomerase activity and telomere length. Treatment of MB cell lines with the telomerase inhibitor epigallocatechin gallate (EGCG) displayed strong dose dependent proliferation inhibitory effects against telomere repeat amplification protocol (TRAP)-positive MB cell lines with IC_{50} between 100 and 300μM [114]. Our results suggest that inhibition of telomerase function could represent a novel experimental therapeutic strategy in childhood MB. Together these data suggest changes at the TERT locus in the evolution of primitive neuroepithelial tumours of the CNS and a possible role for telomerase in the pathogenesis of MB.

In contrast, however, by screening a heterogeneous group of brain tumours for telomerase activity, MB was found to be the only telomerase negative in the series of brain tumours tested [118, 119]. Hence these results may provide a stimulus for future research aimed at uncovering the real role, if any, that telomere maintenance might play in the oncogenesis of MB.

NEUROBLASTOMA

NB is the most common extra-cranial solid tumour of childhood and accounts for at least 15 % of cancer-related deaths in children [120]. It originates in cells of the neural crest and so it can be found anywhere along the paravertebral sympathetic chain or in the adrenal gland [121]. The varied clinical behaviour of this disease, ranging from spontaneous regression in some cases to progression while under aggressive therapy in others, has been associated with various biologic differences (such as *MYCN* amplification) and provides insight into the possibilities for more specific treatments [122].

Although the relative level of telomerase activity carries prognostic information in a variety of tumours, this correlation seems to be particularly emphasized in NB. Telomerase activity has been shown independently in several laboratories to be a robust prognostic indicator in NB [123-127]. Earlier investigations had demonstrated that telomerase activity may discriminate between prognostically different subsets of NB [67, 128, 129]. Similarly, in a study of a large cohort telomerase activity was detected in 39/133 (29%) tumours including 25/41 (61%) Stage 4, 8/23 (35%) Stage 3, 0/13 (0%) Stage 2, 2/32 (6%) Stage 1 and 4/24 (17%) Stage 4S NB. In this study telomerase activity emerged as an independent predictor of clinical outcome with greater prognostic impact than the *MYCN* status and even the clinical stage [130]. Hiyama *et al.* reported that telomerase was expressed in 94% of NB, but not in benign ganglioneuromas or adjacent adrenal tissues: 75% of tumours with high telomerase activity had a poor prognosis, 97% of tumours with low telomerase activity had a good prognosis and 100% of tumours with no detectable telomerase activity regressed [67, 123]. The level of RNA subunit of telomerase (hTR) has also been found to be associated with the clinical stage of NB at diagnosis [128, 131]. High expression of hTR was associated with advanced disease and with unfavourable prognosis, while most patients with weak or absent hTR expression were found to belong to early tumour stages [123, 128, 131]. NB patients classified as 4S stage, known to have a good prognosis and usually demonstrating spontaneous regression, were found to exhibit short telomeres and to express no detectable telomerase activity at diagnosis, in contrast to patients with progressive disease [123, 126]. Hence it has been hypothesized that the aggressive tumours express telomerase (and therefore have stabilized telomeres), whereas the regressing tumours may have absent or low levels of telomerase activity (allowing telomeres to continue shortening).

The clinical application of telomerase activity measurements is nevertheless hampered by the fact that it requires well-preserved fresh or snap-frozen tumour tissue that is rarely available in a routine setting. It has therefore been proposed to assess TERT expression as a substitute for activity measurements in archival material. In a retrospective study on 124 NB, Krams *et al.* have shown that both spliced and full-length hTERT transcripts were significantly associated with *MYCN* amplification. While hTERT in general showed no correlation with other prognostic factors such as staging, or age at diagnosis, the presence of full-length transcripts was significantly associated with higher stages and full-length hTERT transcripts were highly predictive of poor outcome. In a multivariate analysis, full-length hTERT transcripts emerged as the sole independent predictor of event-free survival. The authors then concluded that the strong statistical correlation of full-length TERT transcripts with clinical outcome in NB suggests that the reverse transcriptase-polymerase chain reaction analysis of TERT transcripts may be equivalent to telomerase activity measurements. Because this assay is well suited for archival material, it could become a useful adjunct in evaluating the prognosis of individual NB cases [132].

EWING'S SARCOMA

Ewing's sarcoma is the second most common solid bone malignancy of children and young adults. Ewing's sarcoma is composed of small, round cells showing limited neuroectodermal differentiation and it is associated in 85% of cases with the t(11;22)(q24:q12) chromosomal translocation [133].

Studies have shown that telomere maintenance mechanisms in childhood Ewing's sarcoma is different than in other types of bone and soft-tissue sarcomas that have been reported to exhibit high

prevalence of ALT rather than telomerase [106, 134-136]. Telomerase activity has been demonstrated in 70% of primary Ewing's sarcoma samples and in 9 of 10 cell lines with evidence of ALT in only one cell line. The low prevalence of ALT in Ewing's sarcoma contrasts sharply with the data on telomere maintenance in osteosarcomas, which showed ALT in 38 of 60 cases [134]. Further support of the high prevalence of telomerase activity in Ewing's sarcomas was demonstrated by Ohali *et al.* [137]. It has been suggested that the predominance of telomerase activation in the absence of ALT may characterize sarcomas with specific chromosomal translocations (such as Ewing's sarcoma), whereas a high prevalence of ALT appears typical of sarcomas with non-specific complex karyotypes (such as osteosarcoma). These results suggest important differences in telomere biology between different sarcomas. Recognizing this heterogeneity might contribute to understanding the pathogenesis of these sarcomas and to devising appropriate therapeutic and diagnostic approaches. These differences between Ewing's sarcomas and osteosarcomas are also pertinent to the application of telomerase-based diagnostic assays in these sarcomas.

In a comparative analysis of telomere maintenance in primary tumours and metastatic lesions from osteosarcoma and Ewing's sarcoma patients, telomerase activity was detected in 85% of the bone tumour metastases (100% Ewing's sarcomas and 75% osteosarcomas) but only in 12% of the primary tumours (13% Ewing's sarcomas and 11% osteosarcomas). Bone tumour tissues with telomerase activity had mean telomere lengths of 3 kb shorter than those with no detectable telomerase activity [138]. This observation was supported by another study by Ulaner *et al.* [134]. The results of both studies suggest that in the development of Ewing's sarcomas, telomerase may be reactivated earlier as a result of telomere shortening rather than that a tumour might arise from a telomerase-expressing stem cell, although this has not been confirmed experimentally [43, 89, 139-141]. These results concur with the finding that longer event-free survival periods were found in patients who lacked telomerase activity compared with those who had detectable telomerase activity levels in their tumour tissues. This is consistent with two other independent reports: one which described TERT transcript as being highly expressed in 78% of the samples from patients with Ewing's sarcoma that were analyzed immunohistochemically [142] and one which found that the high TERT mRNA expression correlated with worst outcome [137]. Collectively these findings suggest a role for telomerase activation in the malignant progression and acquisition of invasive capability of bone tumours [138]. It is known that telomere alterations change the cellular response to chemotherapy [143] and radiation [144, 145]. Thus, the knowledge that Ewing's sarcomas rely on telomerase activity for telomere maintenance may also be clinically valuable, both for conventional therapies and for targeted approaches such as telomerase inhibitors.

RHABDOMYOSARCOMA (RMS)

RMS is one of the most common extracranial solid tumours in children. Embryonal and alveolar subtypes of RMS represent completely different genetic abnormalities. Telomere maintenance has not been widely described in paediatric RMS and the few published studies regarding the association between survival and telomere maintenance are rather controversial. Using a fluorescence *in situ* hybridization / immunofluorescence method, Montgomery *et al.* assessed telomere lengths in archival tissues from nine sarcomas with characteristic translocations, including alveolar RMS and nine without, including pleomorphic RMS. They found that in all the cases with specific translocations, which generally have few karyotypic abnormalities, telomere lengths were similar to, or reduced, compared with surrounding non-neoplastic tissues whereas telomeres in cases lacking specific translocations, which generally contain complex karyotypes, were found to be dramatically lengthened and heterogeneous. In addition to markedly elongated telomeres, 70 % of the complex cases exhibited large brightly stained regions corresponding to a specific type of promyelocytic leukaemia nuclear body found in immortalized cells that maintain telomeres in a telomerase-independent manner (ALT) [106]. These findings provide additional molecular-genetic evidence supporting the dichotomous grouping of sarcomas into those with characteristic signature translocations without extensive additional karyotypic abnormalities and those without such signature translocations that typically display very complex karyotypes and point to telomere dysfunction as a plausible contributor to the chromosomal aberrations found in complex sarcomas. Ohali *et al.* investigated 31 patients (16 embryonal and 15 alveolar) in relation to the telomere maintenance mechanism and its association with survival in RMS, which is characterized by two major subtypes: one that is harbouring a specific translocation (alveolar) and one that has a non-specific karyotype (embryonal). The group found that the average TRF length of the embryonal tumours was significantly higher than that of the alveolar tumours. While alveolar RMS tumours exhibited no ALT phenotype and the majority demonstrated telomerase activity, both telomerase and ALT may play a role in the telomere maintenance mechanism in the embryonal tumours and neither telomerase activity nor ALT correlated with outcome [146]. These findings have important implications for understanding the role of telomere maintenance mechanism in the development of RMS and for the future design of adapted treatment strategies. On the other hand, to investigate telomerase activity in soft tissue tumours and its possible correlation with disease outcomes, Yoo *et al.* analyzed 24 fresh soft tissue sarcomas for telomerase activity and for telomerase RNA (hTR) and found that telomerase activity was only detectable in 17 % and only in association with tumours expressing hTR. Half of the patients with grade 1 and 2 tumours were found to be expressing hTR, suggesting that telomerase RNA may be useful as a marker for identifying tumour aggressiveness earlier than the conventional histopathology grading scale allows. Although the sample number studied is not large enough to permit a a firm conclusion to be drawn, the low frequency of telomerase activity suggests that telomerase may not play an important role in tumourigenesis in these tumours [147].

Identifying the different regulatory patterns of telomere maintenance mechanisms has important clinical implications in addition to the increased understanding of their role during the development of sarcomas, as telomerase might be a therapeutic target. The above findings highlight the fact that a significant fraction of these tumours would be refractory to such treatment. To date, no strategies have been developed which are aimed at the treatment of tumours that use ALT.

WILMS' TUMOUR

Wilms' tumour (WT), or nephroblastoma, is an embryonal malignancy of the kidney, considered to be the second most common intra-abdominal cancer of childhood and the fifth most common paediatric malignancy overall. It represents approximately 6% of all paediatric cancers and accounts for more than 95% of all tumours of the kidney in the paediatric age group [148]. The molecular signalling pathways determining the origin and behaviour of WT are complex and several genes in several loci may participate. The molecular genetics of WT have been the subject of extensive research and at least 4 WT genes (WT1, WT2, WT3, WTX) have been implicated [149, 150]. This is of particular interest as the WT1 gene was identified as a repressor of the TERT promoter in embryonal kidney cells, using an expression cloning approach [151]. However, evidence to date does not address whether WT1 telomerase interaction is involved in renal malignancy.

The compelling evidence demonstrating that high telomerase activity is an unfavourable prognostic feature for several types of childhood cancer has persuaded Dome *et al.* to investigate whether telomerase level predicts outcomes for patients with Wilms' tu-