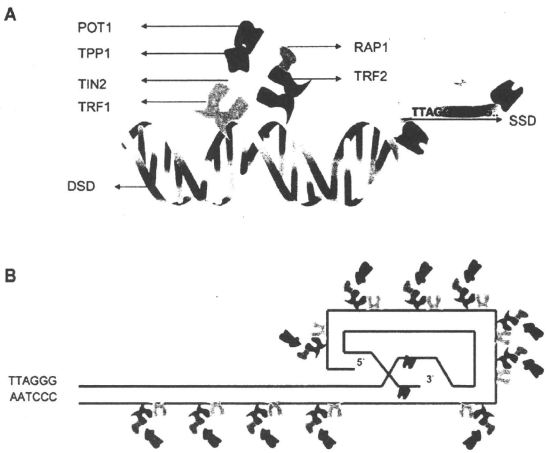

Fig. (1). Shelterin complexes.
(A) TRF1 and TRF 2 binds double-stranded telomeric DNA while POT1 protein bind the single-stranded DNA, resulting in a higher-order complex (t-loop) **(B)** Schematic of shelterin on telomeric DNA: The single-stranded G-rich overhang is protected by invasion within the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

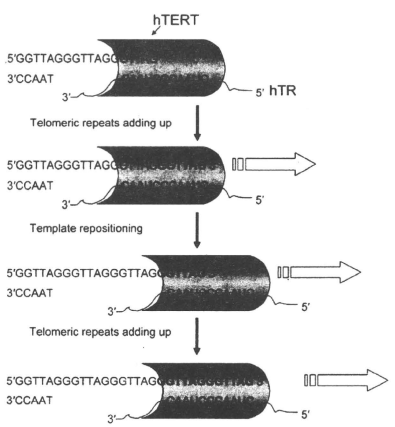

Fig. (2). Telomere elongation by telomerase.
TERT adds TTAGGG repeats to the telomereic ends utilizing the telomerase internal template hTR to specify the sequence.

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

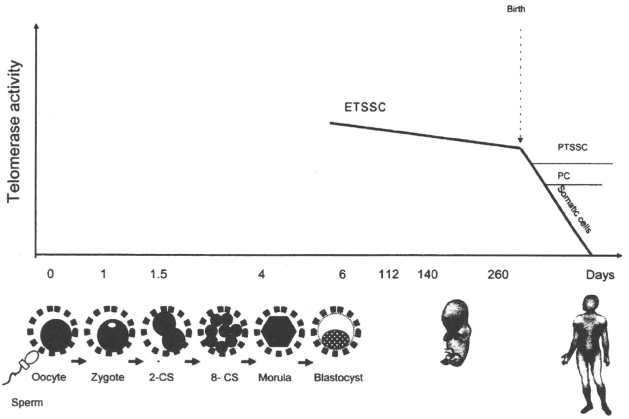

Fig. (3). Diagram depicting the telomerase activity during human embryonal development.
Telomerase activity is detected in all early developmental stages from oocytes through to blastocyst stage embryos. Telomerase activity is found to be relatively low in matured oocytes, increases after fertilization 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-

mour. In a case-cohort study of 78 patients with favourable histology Wilms' tumour, the research group compared tumour telomerase levels in patients with and without eventual recurrence, measuring TERT activity, TERT mRNA and expression of the RNA component. In their study 81% had detectable telomerase activity, 97% had detectable TERT transcriptand 100% had detectable hTR. Of the variables assessed, only TERT mRNA expression correlated with tumour recurrence marker [127]. Recently the same group studied a larger set of 244 Wilms' tumours and found that high telomerase RNA expression level is an adverse prognostic factor for favourable-histology Wilms' tumour [152]. Although these reports may reflect the level of telomerase expression in Wilms' tumour, the link between high TERT expression and unfavourable prognosis (whether it is causative or correlative) has not been addressed experimentally. An alternative focus for research is whether telomere dysfunction, rather than high telomerase level, correlates with high-risk tumours. In a recent study abnormal telomere shortening was found in cultured cells and in tissue sections from highly aggressive Wilms' tumours. Dysfunctional telomeres were associated with specific cell division abnormalities, including anaphase bridges and multipolar mitoses. Telomere-dependent mitotic instability was found to be present in a subgroup of Wilms' tumours, predominantly consisting of high-risk tumours [95].

HEPATOBLASTOMA (HB)

Hepatic tumours comprise approximately 5% of the total neoplasms occurring in the foetus and neonate [153]. HB is one of the common paediatric tumours and more than 70% of the tumours are diagnosed in children less than 2 years old [153, 154]. This tumour, which is derived from hepatic precursor cells, is morphologically similar to immature hepatocytes and the prognosis of the patients is variable [155, 156]. Data on telomere maintenance in childhood HB neoplasms, including TERT gene dosage and mRNA levels, is sparse. Hiyama *et al.* have detected TERT mRNA expression in 96% of 61 primary HB in patients listed in the Japanese Study Group of Pediatric Liver Tumour between 1991 and 2002 [157]. The group showed that an increased level of TERT mRNA expression or telomerase activity is a prognostic indicator of poor outcome in children with HB, independent of disease stage and histological classification. Since telomerase activity has been detected in HB, the use of telomerase inhibitors may provide an attractive approach to therapy. To that end, a few studies using different approaches have succeeded in inhibiting TERT using siRNA and the hTR template antagonist oligonucleotide (GRN163L), tamoxifen, or interleukin-4(hIL-4) gene transfer and have demonstrated a reduction in cell proliferation, an increase in cell differentiation and apoptosis [158-161]. Although it would need a larger series to clarify the correlation between clinical variables and the levels of TERT mRNA or the inhibition of telomerase activity, these findings have drawn attention to the fact that high telomerase activity may stratify patients who are likely to have cancer recurrence requiring postoperative aggressive chemo-adjuvant therapy or, in the future, telomerase-targeting therapy.

THERAPEUTIC STRATEGIES TARGETING TELOMERE MAINTENANCE

The interest in telomere maintenance mechanisms in a cancer therapeutics context has emerged following the observations that: (i) Immortality of cancer cells is intimately related to the maintenance of the ends of human chromosomes [25, 63, 162-164]; (ii) Telomerase activity is detectable in over 85% of human tumour samples *in vivo* and some studies have suggested that cancer stem or stem-like cells are also telomerase-positive [15-18]. The reactivation of telomerase in cancer cells stabilizes telomere length, thereby counteracting the cell division-related telomere erosion and providing unlimited proliferative capacity to malignant cells; (iii) Telomerase repression and tight regulation in humans function as tumour suppressor mechanisms [19]; and (iv) Telomerase is usually not expressed in normal tissues of somatic origin and is expressed only transiently or at low levels in proliferative tissues including hematopoietic progenitor cells, intestinal crypt cells, endometrial cells and basal layer cells of skin and cervical keratinocytes.

The early findings that HeLa cells express telomerase [165] and that immortalization of cells *in vitro* occurs concomitantly with the activation of telomerase activity [64] have established a close link between the limitless replicative potential of cancer cells and the maintenance of the telomeres sequences. The key finding that related the telomeres to cancer emerged later when it was shown that ectopic expression of the TERT subunit in cultured human retinal pigment epithelial cells or foreskin fibroblasts extended their life span and conferred an indefinite proliferative potential to the cells [166], also elongating the short telomeres by 2.5 kbp [167]. This concept was coupled with the remarkable reports by Hahn showing that cloning a mutant TERT gene into a cancer cell *in vitro* causes the cell to lose the ability to form tumours in mice, leads to shortening of telomeres and forces the cell into replicative senescence [162, 168]. These results argue strongly that telomere maintenance is the key to cell immortality and establish telomere length as the clock that keeps track of cell division. Soon afterwards cumulative reports continued to demonstrate and provide evidence for the genetic validation of telomere maintenance as an anticancer target [169-171]. Moreover, the concept of a close relationship between cancer cell survival and telomere/telomerase biology presented novel molecular targets for cancer therapy and accordingly a number of different approaches have been developed to inhibit telomere preservation in human cancer cells. The most advanced of these is inhibition of telomerase.

Different strategies targeting various telomerase regulatory levels have been reported and can be classified as either targeting production of the telomerase complex using antisense, siRNA, dominant negative telomerase complex approaches or targeting telomerase activity with specific inhibitors (Fig. **4A**), such as: oligonucleotide inhibitors that target the template portion of hTR and small-molecule inhibitors including reverse transcriptase inhibitors (Fig. **4B**), nucleotide analogs and other small molecules (Table **2**). One of the recent and more promising areas of research is targeting the telomere with G-quadruplex stabilizers (Fig. **4C**) [19, 172-176]. The main advantage of targeting telomerase is its wide expression in tumour tissues and its specificity for cancer cells, including putative cancer stem cells. In fact no other tumour-associated gene is as widely expressed in cancer. Importantly, the purely transient expression of telomerase in normal tissues and the longer telomeres in normal stem cells compared with those in tumour cells, reduce the probability of toxicity in normal cells, suggesting that telomerase targeting therapy could have a broad therapeutic window. However, the possibility that cancer therapy targeting telomerase might cause undesired toxicity in telomerase-expressing normal proliferating cells remains to be elucidated by the administration of telomerase inhibitors in long-term clinical trials. It is worth noting that recurrent tumours are characterized by immortal cells that have reactivated telomerase [177-179], hence telomerase inhibitors may also be useful when traditional anti-tumour therapies, which are generally more effective against early stage cancer, have failed. Therefore, anti-telomerase strategies promise to be a novel anticancer approach that might also prove effective against disseminated advanced tumours.

Another promising approach for intervention in telomere maintenance is the disruption of the telomere capping structure at the end of the telomeric sequences. Telomere-capping function requires the integrity of shelterin protein complex (that remodels linear telomeric DNA into T-loops), sufficient telomere length and telomerase expression to maintain the equilibrium of telomere length in the case of critical telomere shortening. Loss of the telomere repeats, mutations of the telomere-associated proteins, or telomerase inhibition may lead to the destruction of telomere structure, which

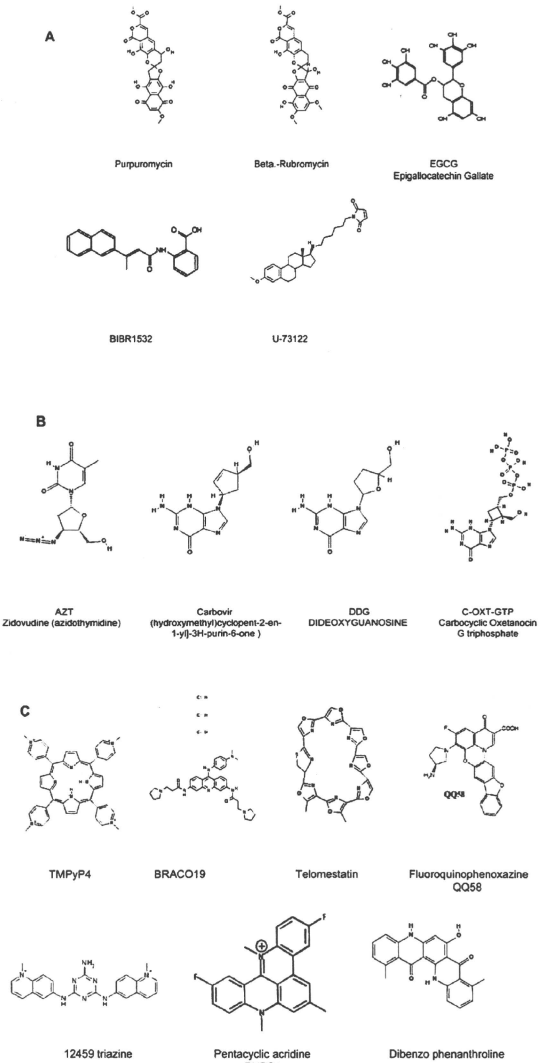

Fig. (4). Telomerase inhibitors.
(A) Small molecules telomerase inhibitors **(B)** Reverse transcriptase inhibitors. **(C)** Small molecules G quadruplex inhibitors

Table 2. Strategies for Targeting Telomere Maintenance

Target	Mode of Action	Inhibitors	References
Telomerase	**Agents blocking telomerase biosynthesis** 1) Oligonucleotide inhibitors : A) Antisense: Prevent translation / promote mRNA degradation • Against hTR • Against hTERT mRNA	Chemically modified oligonucleotides including: phosphorothioates, RNA oligomers nucleotides with alkyl modifications at the 2′ position of the ribose, 2′,5′-oligoadenylate antisense oligomers, N3′—P5′ (GRN163*) phosphoramidates and peptide nucleic acids (PNAs)	[10, 219-225]
	B) Small interfering RNAs, (siRNA): Targeting protein destabilize • Against hTR • Against hTERT mRNA	Chemically synthesized derivatives of natural toxic compounds	[226-228]
	2) Small molecules inhibitors: Targeting the active site of hTERT.	Purpuromycin, BIBR1532, U-73122, Epigallocatechin Gallate, FJ5002	[114, 229-233]
	TERT mutants: Inhibition of the telomerase catalytic protein subunit	Dominant negative TERT constructs mutants that are catalytically inactive.	[234-236]
	Agents blocking reverse transcriptase Blocks chain elongation using the reverse transcriptase enzyme.	Azidothymidine (AZT), carbovir, Dideoxyguanosine (DDG)	[237-240]
	Indirect telomerase inhibitors: Compounds affects telomerase via an indirect mechanism.	Heat shock protein 90 (HSP90), protein kinase C, histone deacetylase, COX-2 and tyrosine kinase inhibitors, tamoxifen as well as nonsteroidal anti-inflammatory drugs and anti-oxidants	[224,241]
	Immunotherapy Using hTERT as a tumour antigen.	GRNVAC1* (Autologous dendritic cells transduced with TERT) GV1001* (TERT peptide p611-626) p540-548* (TERT peptide p540-548) Vx01* (TERT cryptic peptide p572Y-580 and native TERT p572(R)-580) TLI* (TERT fragment-transduced B-lymphocyte immunization)	Reviewed in [9]
	Suicide gene therapy Using telomerase promoter-driven expression of a toxic gene (for example, oncolytic viral replication or toxic prodrug conversion		[242-244]
Telomere maintenance distruption	**G-quadraplex-stabilizing molecules** Inhibiting telomerase and/or telomere maintenance.	2,6 diamidoanthraquinone, TMPyP4 Cationic porphyrin, BRACO19 Trisubstituted acridine, Telomestatin, Dibenzo phenanthroline, Ethidium derivative, 12459 triazine, Pentacyclic acridine RHPS4, Fluoroquinophenoxazine QQ58,	[4, 245]
	Interference with telosome proteins Telomere dysfunctional telomeres	• TRF1 overexpression • Inhibition of TRF2 • Pin2 mutant	[224, 246-250]

* Telomerase inhibitors in clincal trias.

is recognized as DNA damage [11, 180-184]. Identifying shelterin proteins and understanding how they are involved in telomere maintenance has enriched the list of possible targets for therapeutic intervention. Genetic and biochemical studies suggest that targeting components of shelterin, such as TRF2 [185] or POT1 [14, 186, 187], or exposing the telomere 3' overhang [188] can activate a DNA damage checkpoint response and in so doing induce telomere-initiated senescence or apoptosis in cancer cells [189]. Optimal telomerase activity requires the unfolding of the single-stranded substrate that gives access to the telomerase RNA to allow priming and elongation of the telomere length. Therefore, it has been hypothesized that molecules that selectively bind to and stabilize the telomere sequence in unfolded structures such as quadruplexes may interfere with telomere conformation and telomere elongation via telomerase [190].

Quadruplex nucleic acids are defined as higher order four-stranded structures formed by DNA sequences containing at least one contiguous tract of guanine nucleotides. The occurrence of such sequences in human telomeres and within genomic sequences has been recognised for many years. Recently the diverse structures of G-quadruplexes have been the focus of attention as novel anticancer targets since their formation inhibits the telomerase complex from maintaining telomere length in cancer cells [191-193]. The concept of telomeric quadruplex DNA as a therapeutic target was established with the finding that a group of disubstituted amidoanthraquinone small molecules, containing a planar aromatic chromophore, could inhibit telomerase activity [194, 195]. The hypothesis underlying this observation was that a ligand molecule can induce the single-stranded telomeric DNA substrate to fold into a quadruplex structure, which is known to be incompatible with telomerase-catalysed telomere elongation [190]. More recent studies have provided compelling evidence that the ligand effectively competes with hPOT1 and telomerase for the single-stranded overhang [196-199]. The formation of a quadruplex-ligand complex at telomere ends appears to be equivalent to the exposure of damaged DNA, since it elicits a rapid DNA damage response that is lethal to the affected cells [11, 200], reviewed in [191]. Notably, however, it

was observed that G-quadruplex ligands also induce a short-term response of growth arrest and apoptosis before any detectable telomere shortening, a finding which cannot be explained solely by telomerase inhibition but which to a certain extent indicates that the direct target of these ligands is the telomere dysfunction rather than telomerase inhibition [170, 201-203]. Studies which reported that G-quadruplex ligands block the proliferation of ALT cell lines, together with the finding that neither overexpression nor the introduction of dominant negative domain of TERT in a telomerase positive cell line modify the antiproliferative effect of the G-quadruplex ligand, provided further evidence that the antiproliferative effect of G-quadruplex ligands is independent of telomerase inhibition [204-206]. Chromosome end-to-end fusion with typical images of telophase bridges is yet another evidence that supports the proposal that G-quadruplex ligands primarily act by disrupting the telomere structure [207]. Such telomeric dysfunction has been observed in diverse cancer cell lines treated with different quadruplex ligands [203, 208-210]. No specific inhibitor has so far been specifically designed to inhibit the ALT pathway. Telomere interacting agents targeting telomere sequence or telomere structure however could, in principle, act both on telomerase positive and ALT cells [211-213].

CONCLUSION

In summary, the high proliferation 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 acquired capabilities of cancerous cells. Within the context of our current knowledge concerning telomere biology in embryonal tumours, telomerase activity has been detected in most advanced embryonal tumours of childhood. The emerging view of a close relationship between embryonic development, telomere/telomerase biology and embryonal cancer encourages new approaches in the development of innovative therapeutic interventions for childhood malignancy. In pre-clinical studies, telomerase inhibitors have shown promise as effective agents for a wide variety of malignancies and for some embryonal tumours such as NB and MB, but their usefulness in clinical practice has yet to be proven and more research is definitely required to define how telomere biology can be utilised to clinical advantage in malignancies of childhood. At the current state of knowledge it is still not possible to decide the best therapeutic target, between telomerase and telomeres. Although telomerase activation is not the sole mechanism responsible for maintaining telomeric sequences, the enzyme appears to be crucial in the maintenance of telomeres in most embryonal cancers and it has been found to be associated with unfavourable outcomes. However, results vary according to tumour type. Small molecules that target G-quadruplexes in telomeric DNA disrupt telomere maintenance in cancer cells and hence become attractive potential anticancer agents. Genetic-based validation studies have provided a compelling argument which suggests that the telomere maintenance pathway is a well validated target at the preclinical level; hence interference with telomere maintenance may provide an attractive approach to therapy for these deadly diseases and may prove most effective in reducing the risk of relapse by targeting cancer stem cells. Although telomerase and telomere maintenance mechanisms may not yet be the universal targets for anti-cancer therapy, we certainly believe that they will be important targets in future research aimed towards a successful strategy for curing childhood cancer.

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CONFLICT OF INTEREST STATEMENTS

No conflicts of interest exist among the authors related to this project.

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シンポジウム 骨・軟部腫瘍の病理診断の問題点

Ewing 肉腫の病理診断上の問題点*

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I. はじめに

1921年にEwingにより骨に発生する小円形細胞腫瘍として“diffuse endothelioma of bone”の名称で報告されたことに始まるEwing肉腫(Ewing sarcoma)は，小児や若年者の骨，ときに軟部組織に好発する小円形細胞肉腫である[1),2)]．その後，Ewing肉腫に類似した特徴を有する原始神経外胚葉性腫瘍(primitive neuroectodermal tumor; PNET)，神経上皮腫(neuroepithelioma)，あるいはAskin腫瘍の存在が報告された[3)]．従来からこれらの腫瘍とEwing肉腫との異同が議論されてきたが，両者にt(11; 22)(q24; q12)などの共通の染色体転座，それに由来する*EWS-FLI1*などの共通の融合遺伝子の発現を有することが明らかとなり，同一の腫瘍と考えられるようになった．最近では，Ewing肉腫ファミリー腫瘍(Ewing sarcoma family of tumors: ESFT)という名称でこれらの腫瘍を包括し，単一の疾患として扱われるようになった[4)-7)]．

ここでは，日本ユーイング肉腫研究グループ(JESS)の中央病理診断に供された症例の解析からESFTの病理診断上の問題点を明らかにし，さらにESFTを中心とした病理診断のアルゴリズムを示して，小円形細胞腫瘍における日常病理診断の一助としたい．

Key words: Ewing sarcoma family of tumors, Primitive neuroectodermal tumor, Pathology, Diagnostic criteria

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II. 対象と方法

JESSによる限局性ESFTに対する集学的治療法の第II相臨床試験(JESS04と略記)に登録された53例(登録は2004年12月から2008年5月)のうち，中央病理診断のために検体が送付された50例を対象とした．なお，本稿の著者4人はJESSの中央病理診断委員ならびに前中央病理診断委員(J.H.)である．

検体は，パラフィン切片を用いてヘマトキシリンエオジン(HE)染色とCD99(MIC2)の免疫染色を行い，これに加えて適宜，Periodic acid Schiff反応，desmin, muscle specific actin (HHF35), myogenin, α-smooth muscle actin, cytokeratin (AE1/AE3), S-100 protein, CD45 (leukocyte common antigen), TdT, neuron specific enolase (NSE), neurofilamant protein (NFP), vimentin, epithelial membrane antigen (EMA)の免疫染色を行った．凍結検体が送付された場合には，RT-PCR法により，*EWS-FLI1*および*EWS-ERG*の融合遺伝子の発現を検討した．

組織学的，免疫組織化学的所見から腫瘍の病理組織学的診断を行った．融合遺伝子の発現を検討した症例についてはその結果も合わせて総合的に判断した．診断に関しては，病理診断の確からしさの段階から腫瘍を，カテゴリー1：ESFT確診例，カテゴリー2：ESFT疑い例，カテゴリー3：Small round cell tumor, not otherwise specified (SRCT, NOS)，カテゴリー4：ESFT否定例，の4つのカテゴリーに分け，各カテゴリーに分類される要件について検討した．SRCT, NOSはESFTを否定することはできないが積極的に肯定することもできない小円形細胞腫瘍を意味している．この結果をもとにESFTの診断のため

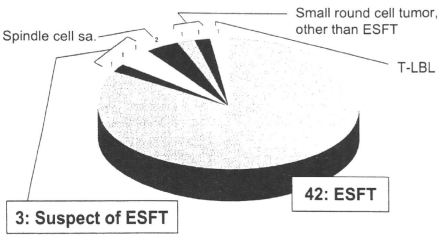

図1 対象50例の中央病理診断内訳

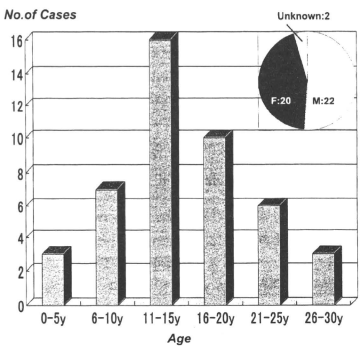

図2 ESFT例およびESFT疑い例の年齢分布と性別

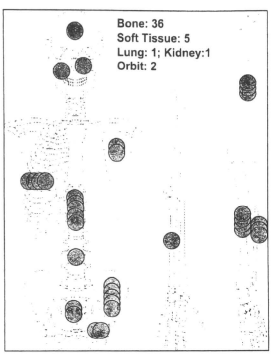

図3 ESFT例およびESFT疑い例の発生部位

のアルゴリズムを検討した.

III. 結　　果

検討した50例のうち，カテゴリー1(ESFT確診例)は42例，カテゴリー2(ESFT疑い例)は3例，カテゴリー3(SRCT, NOS)は2例，カテゴリー4(ESFT否定例)は3例であった(図1)．カテゴリー1および2の45例は，男性23例，女性20例，不明2例であった．登録の対象が30歳未満であるため，年齢は30歳未満に限られるが10歳台前半の症例がもっとも多かった(図2)．発生部位は，腸骨と胸椎が5例ともっとも多く，次いで脛骨および胸壁(肋骨発生を含む)が4例であった(図3)．骨発生例は36例，軟部組織発生例は5例，その他臓器などの発生例は4例であ

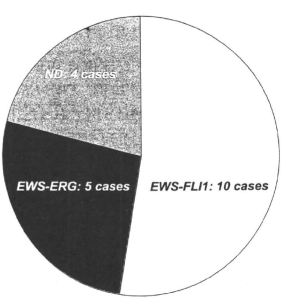

図4 融合遺伝子解析結果

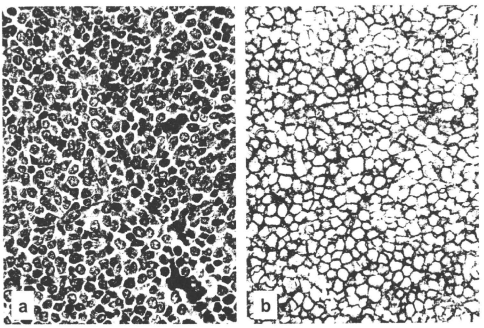

図5　(a)典型的ESFTの組織像(HE染色)．(b)CD99免疫染色像．びまん性に細胞膜に強く陽性である．

表1　Ewing肉腫ファミリー腫瘍の組織学的所見

典型的所見	Diffuse monotonous proliferation Uniform small round tumor cells Uniform round nuclei containing fine chromatin Inconspicuous nucleoli Fibrillary cytoplasm Indistinct cytoplasmic membrane No ground substance Cytoplasmic glycogen [PAS statin(+)]
時に見られる所見	Homer Wright rosettes Large tumor cell
稀に見られる所見	Spindle cell Clear cytoplasm with distinct cell membrane

った．

融合遺伝子が検索できた症例は19例で，*EWS-FLI1*の発現を認めた症例が10例，*EWS-ERG*の融合遺伝子の発現を認めた症例は5例であった(図4)．4例はこれらの融合遺伝子の発現を認めなかった．なお，この4例の内訳はSRCT, NOSが2例，ESFT否定例が2例で，組織学的に積極的にESFTを疑った症例ではなかった．

ESFTの典型的な組織像は，比較的均一な小円形細胞から成る腫瘍細胞の単調なびまん性増殖であり，その核クロマチンは繊細で，核小体は目立たない．細胞境界は不明瞭で，fibrillaryな細胞質を持ち，背景に基質は乏しい．また，細胞質にグリコーゲンを認め，Homer Wright型ロゼットを認めることもある(図5-a，表1)．通常のESFTよりも大型の細胞から成るいわゆるlarge cell Ewing sarcomaと呼ばれる例もある．稀に紡錘形細胞が出現したり，細胞境界明瞭な淡明な細胞からなる病変も見られるとされている．細胞質内のグリコーゲンは検索した16例中8例(50%)，Homer Wright型ロゼットは45例中3例(7%)，large cell Ewing sarcomaは45例中2例(4%)，紡錘形細胞の出現は45例中1例(2%)に認められた．細胞境界明瞭な淡明細胞の出現した症例は検索した45例中にはなかった．

免疫組織化学的結果のまとめを表2に示す．ESFTの診断に有用なマーカーとされているCD99は45例

表2 免疫組織化学的結果のまとめ

Positive markers	Negative markers	Other markers
CD99: 44/45 (98%)	Desmin: 0/30 (0%)	CK-AE1/3: 3/22 (14%)
NSE: 11/18 (61%)	HHF-35: 0/17 (0%)	S-100 protein: 3/31 (10%)
NFP: 3/13 (23%)	Myogenin: 0/5 (0%)	
Vimentin: 4/6 (67%)	CD45: 0/15 (0%)	
	TdT: 0/20 (0%)	
	α-SMA: 0/2 (0%)	
	EMA: 0/2 (0%)	

中44例(98%)に，びまん性に細胞膜に陽性であった(図5-b)．NSEは検索した18例中11例(61%)，neurofilamentは13例中3例(23%)，vimentinは6例中4例(67%)に陽性であった．これらの免疫組織化学的マーカーは，ESFTで比較的高頻度に発現する，あるいはESFTのneuroectodermal differentiationを示すもので，ESFTにとってもpositive markerと捉えることができるが，NSEやvimentinはESFTに全く特異的ではない．CytokeratinやS-100 proteinはESFTでときに発現を認めることがあるマーカーであるが，cytokeratin (AE1/AE3)は検索した22例中3例(14%)に，S-100 proteinは31例中3例(3%)に認められた．ESFTでは通常陽性とならず，陽性であった場合には逆にESFTではなく他の小円形細胞腫瘍を示唆するものは，ESFTにとってのnegative markerと考えられるが，表2に示すようにこれらはすべて陰性であった．これらnegative markerに相当するものとして，desmin, muscle specific actin, myogenin, α-smooth muscle actin, CD45, TdT, EMAなどが挙げられる．

IV. 考　　察

検討した50例のうち，カテゴリー3の症例は2例，カテゴリー4の症例は3例と少ないのは，CD99陽性というESFTの免疫組織化学的特徴と*EWS-FLI1*をはじめとする特異的な融合遺伝子の存在が明らかとなり，PNETなどの類縁疾患を含めてESFTの疾患概念が整理され，小児の他の小円形細胞腫瘍との鑑別点がかなり明らかにされたことによると思われる．カテゴリー3, 4を含めて中央病理診断に標本が送付された50例のうち48例(96%)はCD99陽性であった．また，HE像がいわゆる小円形細胞腫瘍の範疇に入るものは50例のうち48例(96%)であった．したがって，小円形細胞という腫瘍細胞の形態とCD99陽性所見とで，大部分のESFTの病理診断がなされていると推測される．

カテゴリー4に分類した3例のうち，2例は紡錘形細胞肉腫に分類したものであったが，1例はCD99陽性，1例はCD99陰性であった．融合遺伝子は*EWS-FLI1*, *EWS-ERG*ともに発現を認めなかった．滑膜肉腫の可能性を考慮して*SYT-SSX*融合遺伝子も検索したが，この2例は陰性であり，最終的な確定診断には至っていない．残りの1例は前縦隔発生例で，CD99陽性であるが，TdT陽性，CD3陽性でありT細胞性のリンパ芽球性白血病/リンパ腫であった．CD99陽性の小円形細胞腫瘍であってもESFTではない症例があり，実際の診断に際しては注意する必要がある．

カテゴリー3に分類した2例のうち1例は，脱灰のためと思われるHE標本の形態像の保存が悪くCD99も陰性であり，また融合遺伝子検索でも*EWS-FLI1*と*EWS-ERG*ともに発現を認めないものであった．もう1例は，CD99陽性の小円形細胞腫瘍であったが，HE形態像がESFTの典型的所見と異なり，*EWS-FLI1*, *EWS-ERG*ともに陰性であったため，SRCT, NOSと診断した．後に検体送付もとの施設で行われた融合遺伝子検索で*TLS-CHOP*融合遺伝子が検出され，最終的には送付もとの施設で小児(16歳，男性例)では稀な円形細胞型脂肪肉腫と診断された．

カテゴリー2とした3例のうち1例は，HE形態像はESFTに合致するものの壊死が強く認められ，CD99が陰性であった．また，融合遺伝子は検索できていないため，ESFTとの確定診断にいたらずESFTの疑いとしたものであった．別の1例はCD99陽性，遺伝子検索未施行で，HE標本の挫滅が強いため診断をESFT疑いにとどめた例であった．残りの1例は，小円形細胞に加えて一部に紡錘形細胞の増生があり，