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TELOMERE MAINTENANCE MECHANISMS IN TUMOUR OF MESENCHYMAL ORIGIN: EVALUATION OF PROGNOSTIC SIGNIFICANCE AND CHARACTERIZATION OF RELEVANT MOLECULAR PATHWAYS

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Contents

Part I

Part II

Published papers

Published manuscript not included in the present thesis

1. **ALT-associated promyelocytic leukaemia body (APB) detection as a reproducible tool to assess alternative lengthening of telomere stability in liposarcomas**. Venturini L, Erdas R, Costa A, Gronchi A, Pilotti S, Zaffaroni N, Daidone M.G. J Pathol. 2008 Mar;214(4):410-4.
2. **Multiple mechanisms of telomere maintenance exist and differentially affect clinical outcome in diffuse malignant peritoneal mesothelioma.** Villa R, Daidone MG, Motta R, Venturini L, De Marco C, Vannelli A, Kusamura S, Baratti D, Deraco M, Costa A, Reddel RR, Zaffaroni N. Clin Cancer Res. 2008 Jul 1;14(13):4134-40.

PART I

Abstract

A limitless proliferative potential is one of the hallmarks of tumour cells and can be achieved through the activation of telomere maintenance mechanisms (TMM), which rely on telomerase reactivation (TA) or, alternatively, on recombination-based processes known as alternative lengthening of telomeres (ALT). Since a substantial fraction of tumours of mesenchymal origin utilizes ALT mechanisms, they represent an interesting model to study the molecular pathways involved in the activation of TMM.

With the present work, we extended our knowledge about the prevalence and the prognostic significance of the two known TMMs in different soft-tissue sarcoma histotypes (Malignant peripheral nerve sheath tumors –MPNST-, leiomyosarcoma, liposarcoma) and mixed origin tumours (Wilms' tumour), showing the relatively low frequency of telomerase activity in soft tissue sarcomas if compared to tumors of epithelial origin, and the important role of ALT in these malignancies. Controversial results have been obtained about the clinical relevance of TMMs, whose prognostic role seems to be dependent on tumor histotypes. Specifically, ALT is a strong determinant of an unfavorable outcome in liposarcoma and leiomyosarcoma patients, whereas it failed to significantly affect the outcome of patients with MPNST (for whom TA proved to be an important predictor of poor survival).

This scenario could be due, at least in part, to the lack of standardized methods to properly classify tumours with respect to their TMM status; in this context, we comparatively analysed the prognostic relevance of ALT in a series of liposarcoma as a function of the characteristic used to classify the tumour, with the final aim to identify the most suitable ALT marker.

Moreover, we also proposed to identify microRNAs expressed as a function of the different TMM operating in the tumour, which could be either involved in the regulation of such mechanisms or represent surrogate markers of TA-positive or ALT-positive phenotypes.

The knowledge of the specific factors/pathways by which the two known TMMs are differentially regulated in distinct tumour histotypes of mesenchymal origin might be important for a better understanding of the pathogenesis of these malignancies and for the identification of new therapeutic targets.

State of the Art

The process of carcinogenesis involves a number of changes in cellular phenotype, which are largely based on acquired genetic alterations in cells that are not terminally differentiated. The ability of cancer cells to grow and their failure to respond to the usual controls on such proliferation are obvious features, but they can also evade cell death and most have no limits on their ability to replicate beyond the limits imposed by telomere length in normal cells.

By 2000, it was widely accepted that cancer cells require unlimited replicative potential in order to generate macroscopic tumours. This capability stands in marked contrast to the behaviour of the cells in most normal cell lineages in the body, which are able to pass through only a limited number of successive cell growth-and-division cycles. This limitation has been associated with two distinct barriers to proliferation: senescence, which is an irreversible nonproliferative state, and crisis, which leads to cell death. Accordingly, when cells are propagated in culture, repeated cycles of cell division lead first to senescence and then to crisis, a phase in which the majority of cells in the population die. Rarely, some cells can emerge from a population in crisis and exhibit unlimited replicative potential (immortalization). Multiple lines of evidence indicate that telomeres protecting the ends of chromosomes are centrally involved in the capability for unlimited proliferation (Blasco, 2005; Shay and Wright, 2000).

The telomere

Telomeres are essential components of linear chromosomes, but their existence was postulated several years before the discover of the DNA double helix. In the late 1930s, Hermann Muller and Barbara McClintock independently noted that the ends of chromosomes (termed “telomeres” or “natural ends”) were not as sticky as broken chromosomal ends. Decades later, after the discovery of the structure of DNA, Alexey Olovnikov implicated telomeres in the “Hayflick limit” of cellular duplication; further, he realized that in DNA semi-conservative replication, the replica would always be shorter than the template, eventually producing extremely short chromosomes and senescent cells; this theory hypothesized that telomeres would buffer genetic loss with each mitotic divisions and that telomere shortening could explain many aging-related disorders. Telomeres are DNA-protein structures that distinguish natural chromosome ends from double strand breaks (DSBs). Because telomeres protect chromosome ends from processing events that result in degradation and/or end-to-end fusions, they are essential for chromosome integrity.

Telomeric DNA consists of a tandem array of short repeated sequences, in which the strand running 5’–3’ from the centromere towards the chromosome end is usually guanine-rich, specifically, in human species the tandem repeated sequence is TTAGGG. The amount of duplex telomeric DNA per chromosome end varies enormously among different species and in mammals reaches more than 10kb. Extension of the guanine-rich strand forms a conserved feature of telomere structure, so called G-tail, of 30 single stranded overhang which plays a crucial role in telomere function (Cech T.R., 2004, Chan S.W.-L. 2003, deLange T.

2005, Dionne I. 1996, Wellinger R.J. 1997).

As part of their end-protection function, telomeric DNA is constitutively bound by a core complex of proteins called shelterin. Shelterin components include both duplex (TRF proteins in mammals, Rap1 in S. cerevisiae, Taz1 in S. pombe) and single strand (Pot1 in mammals and S. pombe, Cdc13 in S. cerevisiae) binding proteins as well as proteins that associate via protein–protein interactions (deLange T. 2005, Rhodes D. 2002, Palm, W., 2009, Vega, L. 2003).

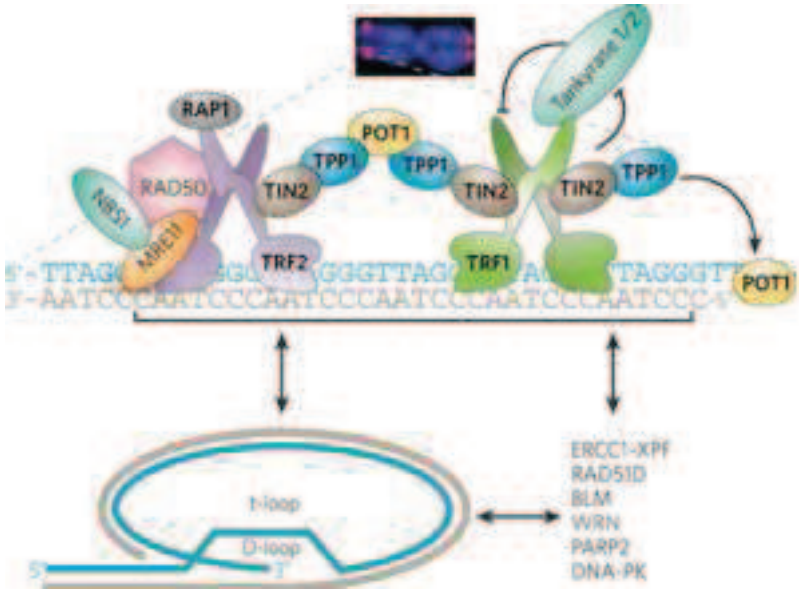


Figure 1. telomere structure. Telomeric DNA and telomere-binding proteins.

The overall composition of the shelterin complex varies between species in respect to protein content and higher order structure, but the general design is conserved (Linger B.R., 2009). The role of the shelterin complex in telomere function and regulation includes distinguishing natural ends from DNA breaks and recruiting telomerase to DNA ends (Palm, W., 2009). In addition to the shelterin complex, higher-order DNA

structures are thought to contribute to telomere functions. A well documented example of such structures are T-loops forming when the single stranded telomeric G- overhang invades the double stranded telomeric region of the same chromosome (Makarov V.L., 1997). By sequestering chromosome ends, T-loops are proposed to protect telomeres against checkpoint recognition, DNA repair, and telomerase-mediated extension (Griffith J.D., 1999, Poulet, 2009, van Steensel B., 1998). Since T- loops are structurally similar to Holliday junctions, they may also be important for telomere recombination (Dunham M.A., 2000, Stansel R.M., 2002). It is not yet known if T-loops exist at each telomere, how they are regulated in the cell cycle, or how they are displaced to allow both semi-conservative and telomerase-mediated telomere replication. G-quadruplex structures are another secondary DNA structure that can affect telomere function. G-quadruplexes involve the association of four guanines into a cyclic Hoogsteen hydrogen bonding arrangement in which each guanine shares a hydrogen bond with its neighbor (N1–O6 and N2–N7). The G-rich single stranded telomeric overhang can form intra- and inter- molecular G-quadruplex structures in vitro.

Telomere maintenance mechanisms

Since linear chromosomes shorten with every round of DNA replication and cell division, leading cells to replicative senescence or apoptosis (Gilson E, 2007), telomere maintenance is crucial for cells to escape a crisis and to become immortalized. For this reason, the activation of a telomere maintenance mechanism (TMM) is essential for the transformation of most human cancer cells. In a high percentage of human tumours (more than 85%), proliferation-dependent telomere

shortening is counterbalanced by the synthesis of telomeric sequences by telomerase (Folini M, 2009). However, there are human tumours, about 15%, that lack telomerase and maintain telomere length by a pathway known as the alternative lengthening of telomeres (ALT) mechanism (Cesare AJ, 2010).

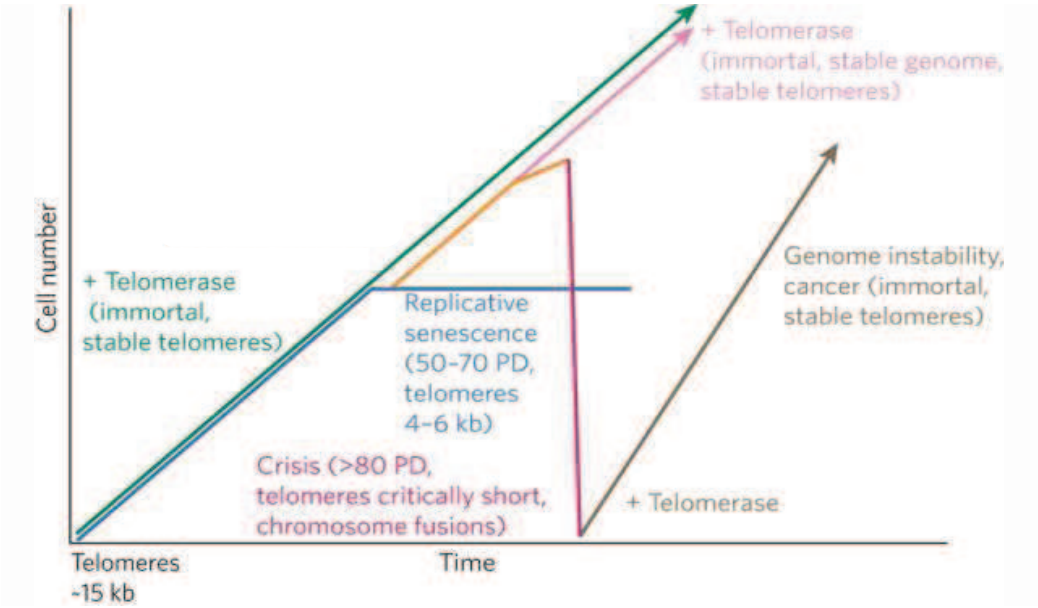


Figure 2: Primary cells divide exponentially, and telomeres shorten until they reach a critical length and irreversible cell-cycle arrest then occurs (senescence). Activation of TMM allows cells to divide indefinitely until end protection is completely lost, resulting in telomeric crisis, cell death and massive genomic instability. Activation of telomerase or other TMMs after the accumulation of mutations allows clones that carry multiple mutations to escape cell death (that is, to become immortal). Such cells are predisposed to oncogenic transformation. PD, population doublings

Telomerase

The minimal telomerase enzyme consists of a protein subunit (TERT) and an integral RNA component (TR), which contains the template used by TERT to add multiple identical repeats of DNA to the ends of chromosomes, a process known as repeat addition processivity (Blackburn EH, 2000, Shippen-Lentz D. 1990, Miller MC 2000, Lai CK 2002, Lamond AI 1989). The initiation of telomere replication requires that the enzyme telomerase is recruited to the telomeres, usually by a single-stranded telomere binding protein such as, in humans, the components of the shelterin complex, TPIP1 and POT1 (Xin H, 2007). Telomerase loading to the telomeres leads to partial pairing of the template RNA with the DNA overhang and positioning of the 3′-end of the telomeric DNA at the active site of the enzyme for catalysis. Following replication of one telomeric repeat, the RNA–DNA hybrid transiently dissociates to allow for pairing of the DNA overhang with the distal region of the RNA-templating region and initiation of replication of another telomeric repeat (Lee MS 1993) (Figure 3).

Although TR and TERT are sufficient to obtain telomerase activity in vitro, additional protein components such as dyskerin and telomerase Cajal body protein 1 are required for the correct assembly and trafficking of the enzyme in human cells (Folini M, 2009, Mitchell M, 2010, Venteicher AS, 2009). In addition to its role in maintaining chromosome ends through the regulation of telomere length, telomerase has been recently implicated in providing growth-promoting properties to tumour cells, independently of its catalytic activity (Bollmann FM., 2008).

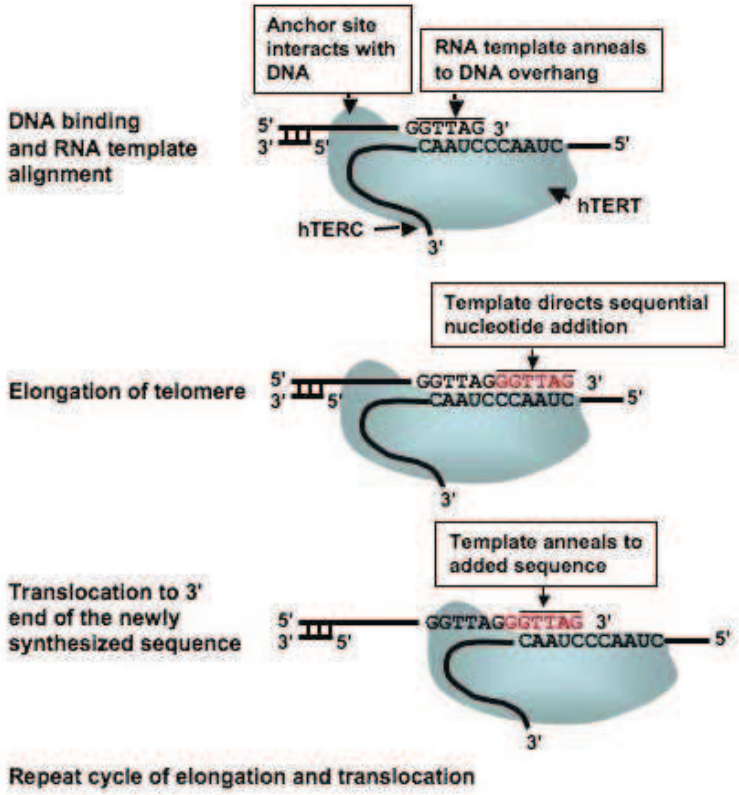


Figure 3: telomere extension by telomerase.

There are currently a number of methods used to detect telomerase activity. The telomeric repeat amplification protocol assay (TRAP), a method based on a polymerase chain reaction (PCR) (Figure 4), has become the standard method for studying the diagnostic relevance of the enzyme (Kim NW, 1994) (for further details see Part II, Published Papers, materials and methods). Telomerase activity has also been investigated qualitatively and quantitatively using several modified TRAP protocols. Other methods focused on the detection of the telomerase components, hTERT and hTR, by reverse transcriptase PCR (RT-PCR)

methods. In particular, real-time PCR methods in more recent years allowed the quantitative determination of telomerase subunits.

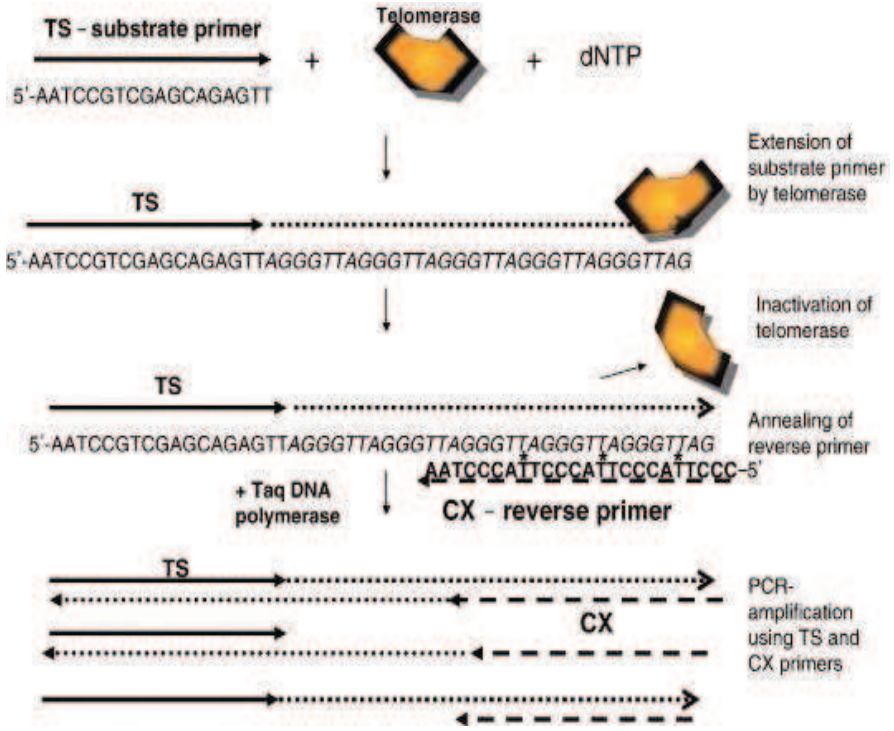


Figure 4 schematic representation of TRAP assay

During the process of carcinogenesis, telomerase is activated at different stages. In some instances, telomerase can be activated gradually throughout the progression of the cancer, whereas in other cases, this enzyme might already be expressed at the *in situ* or precancerous stage. These differences might affect the clinical utility of telomerase as a diagnostic marker or prognostic index. Early studies of telomerase activity in cancerous tissue were marked by the use of different approaches; different objectives were stipulated and several methods, both qualitative and quantitative, were used. Initially, most

studies aimed at analyzing telomerase activity in cancerous lesion and the surrounding macroscopically healthy tissue. Later, the main objective of these studies was to verify whether telomerase activity could offer a reliable and non-invasive diagnostic or prognostic tool.

One problem for the TRAP assay is that it is a solution-phase technique, thus, information about the cell type that expresses the telomerase is lost. Cancer cells, as well as some cells from healthy tissue and benign lesions (including germ cells, lymphocytes, stem or its progenitor cells, and certain epithelial cells) exhibit telomerase activity; therefore, it is not possible to know whether telomerase activity is coming from the tumour cells in the sample. To overcome this limitation, an *in situ* TRAP assay, which uses fluorescent dyes and microscopy to visualize telomerase activity in the nuclei of cells of interest, is needed to determine whether telomerase expression is derived from normal telomerase-positive cells or from malignant cells. Another solution is to use quantitative TRAP or real-time PCR methods to define threshold values that can help distinguish cancer from normal tissue.

Telomerase is activated in about 85% of human cancers and its expression is not usually detectable in somatic cells; therefore, its detection holds promise as a diagnostic tool for many types of tumour. In addition, telomerase is also activated in some premalignant tissue; thus, it is reasonable for physicians to expect that testing for telomerase activity is a useful method for the screening of high-risk patients. Hence, it is crucial to determine which cancers possess early robust telomerase activity and which manifest little to no telomerase activity, even in the most advanced stages of malignancy. Expression of the TR subunit has been found in all cells, irrespectively from the presence of telomerase

expression and activity, although it could be often amplified in cancer cells. By counterpart, TERT expression correlates with telomerase activity because its presence is essential for enzymatic activity. Thus, detection of the TERT mRNA is considered to be a more reliable marker of the presence of cancer cells in clinical samples, although its presence is necessary but not sufficient to obtain an active form of the enzyme (Villa R, 2001; Villa R, 2008; Zaffaroni N, 2005).

Due to its selective reactivation in most human tumours, the possibility to interfere with telomerase expression and functions has been considered a useful strategy for the development of effective anticancer therapies since its discovery (Folini M, 2007; Shay JW, 2006).
Data obtained from preclinical studies concerning the effects of telomerase inhibition have provided compelling arguments to indicate that the enzyme is a well-validated cancer target (Folini M, 2011). Data obtained from preclinical studies on the effects of telomerase inhibition have provided compelling arguments to indicate that the enzyme is a well-validated cancer target and have led the enzyme's inhibitor GRN163L to enter the first clinical trials — as a single agent or in combination with conventional anticancer drugs — for different malignancies (Harley C.B., 2008) (For more details see Part II, published manuscript: "Telomeres as targets for anticancer therapies").

Alternative lengthening of telomeres

Some cell lines and tumour do not use telomerase to maintain telomeres, but a telomerase-independent mechanism, referred to as Alternative Lengthening of Telomeres (ALT) (Bryan TM, 1995). Although the definition of ALT encompasses any non-telomerase TMM (Bryan TM 1997), so far there is no clear evidence that there is more than one ALT mechanism in human cells. In contrast to RNA-templated DNA synthesis by telomerase, synthesis of new telomeric DNA by ALT involves the use of a DNA template (Dunham MA, 2000). It has been suggested that the homologous-recombination machinery is responsible for the amplification of the telomeric sequences in ALT cells, because a selection marker introduced into telomeres of ALT and non-ALT cells spreads throughout telomeres only in ALT cells (Dunham, M. A, 2000) ALT cells show an increased rate of sister chromatid exchange (Bailey SM, 2004; Bechter OE, 2004) suggesting that the homologous-recombination pathway is involved.

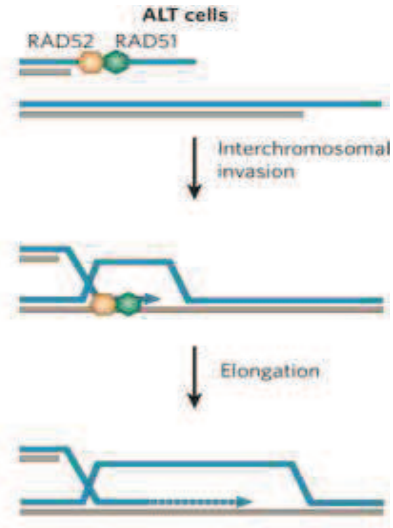


Figure 5 Telomere dynamic in ALT cells.

The maintenance of telomere length in a telomerase-independent way was first described in yeast, where genetic analyses demonstrated it was dependent on homologous recombination (HR) (Lundblad V, 1993). The existence of an ALT mechanism in mammalian cells was deduced from the observation that telomere lengths were maintained for hundreds of population doublings (PD) in telomerase-negative immortalized human cell lines (Bryan TM 1995, Rogan EM, 1995).

With few exceptions, human ALT positive cells show a characteristic pattern of telomere length heterogeneity, with telomeres ranging from very short to greater than 50 kb long and the presence of ALT-associated promyelocitic leukaemia (PML) nuclear bodies (APBs), containing telomeric DNA $(TTAGGG)_n$ and telomere-specific binding proteins (Yeager TR, 1999).

APBs are a subset of PML bodies that are present only in ALT cells, and are not found in mortal cells or telomerase-positive cells (Yeager TR, 1999). Constitutive components of APBs are telomeric DNA, PML protein (specifically, PML3 isoform (Yu, J., 2010) and Sp100 protein, in addition to telomere-associated proteins such as the Shelterin Complex. Telomeric chromatin needs to be SUMOylated by the SMC 5/6 complex (Potts PR, 2007) and a functional MRN complex needs to be involved in DNA recombination (Jiang WQ, 2009). APBs also contain other proteins involved in DNA replication, recombination and repair including RAD51, RAD52, RPA (Yeager TR, 1999), WRN (Johnson FB, 2001), BRCA1, MRE11, RAD50, NBS1 (Zhu XD, 2000; Wu G, 2000).

Although definitive evidence is still lacking, it has long been thought that APBs might have a pivotal role in the ALT mechanism process (), and

consistent with this suggestion, inhibition of ALT in some somatic cell hybrids formed by fusion of ALT and telomerase-positive cell lines resulted in a substantial decrease in APBs (Perrem K, 2001). APBs can be also found in nearly 5% of exponentially dividing “normal” ALT cells, suggesting that APBs can be actually functionally heterogeneous, with only a subset being directly involved in ALT mechanisms.

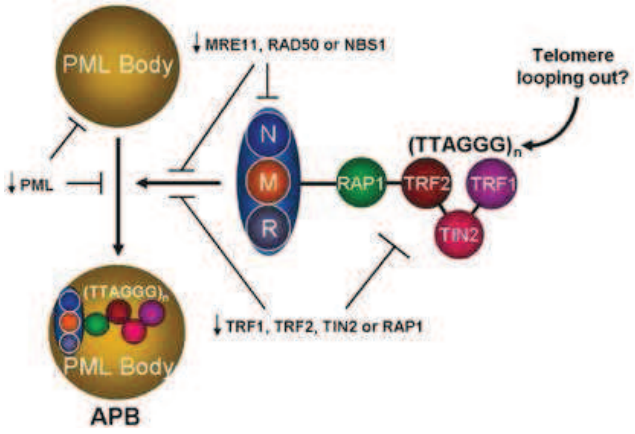


Figure 6: A hypothetical model for APB formation in ALT cells. Telomeric DNA binds to the MRN complex via RAP1, and then translocates to PML bodies to form APBs.

Another specific feature of ALT mechanisms is C-circles (self-priming circular telomeric DNA), which may represent molecular intermediates of the ALT mechanisms and whose specificity for assessing ALT activity in biological samples has recently been demonstrated (Henson J, 2010).
Showing that a cell line is able to maintain the length of its telomeres in the absence of telomerase activity for several population doublings is still the only definitive test for the presence of ALT activity, but of course is not a suitable assay to detect ALT activity in human tumours because it would take a long time and several metacrhounous lesions should be

available. Furthermore, this assay is not suitable for detecting ALT when both ALT and telomerase may be present.

Without the availability of a specific assay for ALT activity, its presence in tumours has been deduced from peculiar phenotypic features of ALT-positive cell lines which are currently used as surrogate markers of ALT.

As already described above, the first hallmark found in immortalized human ALT-positive cell was a characteristic pattern of telomere lengths that can be observed by terminal restriction fragment (TRF) Southern blot analysis (Figure 7). The modal length in ALT cells is approximately twice that in comparable telomerase-positive or normal cells (Bryan TM, 1995, Gollahon LS, 1998).

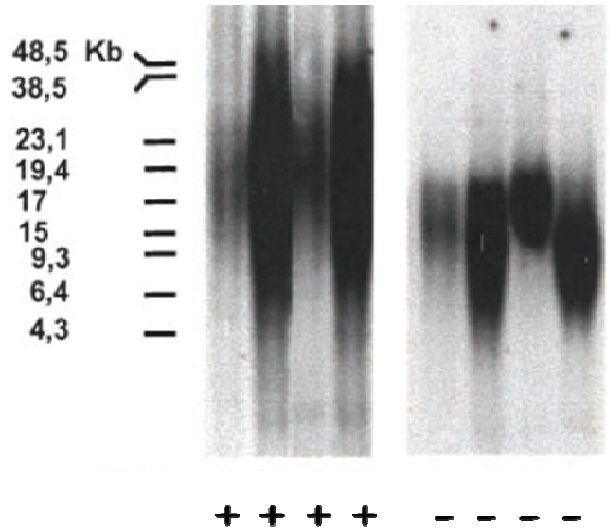


Figure 7: Representative examples of tumour specimens defined as ALT-positive (left) or ALT-negative (right) on the basis of telomere length.

The telomere length heterogeneity can also be visualized in metaphase spreads by telomere fluorescence in situ hybridization (FISH) (Perrem K, 2001). Telomere lengths in ALT cells are not normally distributed, in

contrast to telomerase-positive cells, most of which regulate their telomere lengths about a mean of 5–10kb (Henson JD, 2002, Jeyapalan JN, 2008).

The long heterogeneous telomere length pattern remains the best-established marker for ALT in human cells, including tumours archived under conditions where it is possible to extract high-quality genomic DNA (Bryan TM, 1997; Henson JD, 2005). In tumour specimens the distinction between ALT+ and ALT- is sometimes less obvious due to tumour heterogeneity and to the admixture of non-tumour cells in the specimen (Henson JD, 2005; Venturini L, 2010). For these reasons, tumour length heterogeneity needs to be interpreted cautiously by an expert operator.

Also the assessment of APB presence by a combined technique of immunofluorescence for PML protein and telomere FISH (Yeager TR, 1999) has become a well-established test for ALT (Figure 8). APB assay has been especially useful for determining the ALT status of tumours as it can be used on paraffin embedded specimens (Venturini L, 2008), and it is not affected by intra-tumoural heterogeneity in TMM because it assesses individual cells (Henson JD, 2005, Venturini L., 2010).

The lack of standardized methods for ALT determination has major implications for the classification of ALT+ tumours. In this context, in liposarcoma, the instability at the minisatellite MS32 — usually associated with ALT activation — and the presence of complex telomere mutations have been reported to aid the identification of tumours that have recombination-like activity at telomeres in the absence of APBs

(Jeyapalan J.N., 2008), suggesting that the incidence of ALT may be underestimated using the available methods.

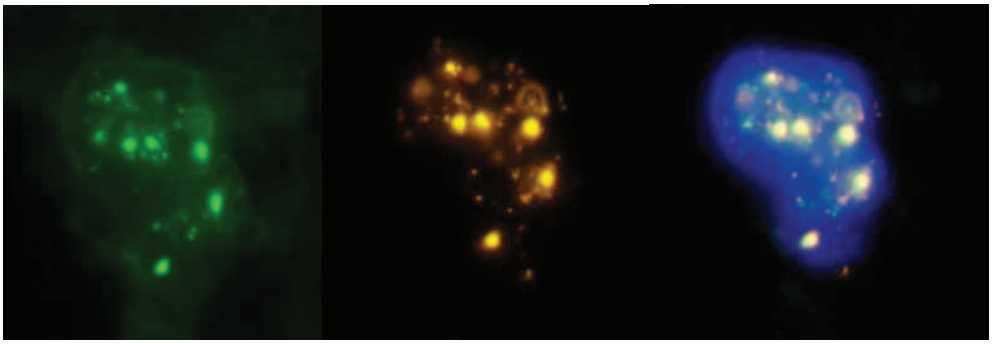


Figure 8: APB assay performed on a tumour frozen section by PML immunofluorescence (green stain) and telomere FISH (red stain). Co localization of the signals determines the presence of an APB.

As these TMMs are either undetectable or have low levels of activity in normal somatic cells (R.R. Reddel, 2000, Shay JW 1997), they provide important targets for detection and treatment of cancer cells. ALT is being found in an increasing number of cancer types, including some that have a very poor prognosis with currently available treatments.
However, inhibitors that specifically target the ALT pathway have not yet been described, probably as a consequence of the fragmentary information about the molecular events driving such a mechanism. Based on results of a recent survey aimed to comparatively evaluate the expression levels of telomere-related proteins in normal and tumour tissues, none of these proteins stands out as a novel cancer-related target (Cookson JC, 2009).

Tumours of mesenchymal origin.

Tumours of mesenchymal origin cover a broad range of uncommon but diverse malignancies such as sarcomas, that arise from bone, cartilage or connective tissues (e.g. muscle, fat, peripheral nerves and fibrous or related tissues). Increasingly, human mesenchymal malignancies are being classified by the abnormalities that drive their pathogenesis. Although many of these aberrations are highly prevalent within particular sarcoma subtypes, very few of them are currently targeted therapeutically. Indeed, most subtypes of sarcoma and other mesenchymal neoplasms are still treated with traditional therapeutic modalities, and in many cases sarcomas are resistant to adjuvant therapies thus given the need for new therapies in such malignancies. In particular, in our studies we focused on:

Liposarcoma: the largest single group of soft-tissue tumours, accounting for 20% of all mesenchymal malignancies. This group is composed of three categories: well-differentiated and dedifferentiated liposarcoma, myxoid and round cell liposarcoma, pleomorphic liposarcoma. While clustered together, these histological subtypes are widely diverse in their clinical, pathological, and molecular characteristics. In general, surgery still remains the mainstay of therapy and the only approach offering the potential of cure Risk of recurrence is high for patients with dedifferentiated histology or retroperitoneal location (Hoffman A, 2010).

Leiomyosarcoma: a malignant neoplasm arising from smooth muscle cells. It's a relatively rare form of cancer, comprising 5-10% of soft tissue sarcomas. These malignancies are generally not very responsive to

chemotherapy or radiation therapy and the best patients' outcome occurs when the tumour can be surgically removed with wide margins. Uterine leiomyosarcomas is a very common type arising from the smooth muscle layer of the uterus.(van Vliet, 2009)

Malignant peripheral nerve sheath tumours (MPNST): a group of rare soft tissue neoplasms accounting for 3-10% of all soft tissue sarcomas that most commonly involve major nerve trunks. MPNSTs are highly aggressive malignancies, complete surgical excision is the primary curative modality. Approximately half of MPNSTs occur in the setting of neurofibromatosis type 1 (NF1), a hereditary tumour syndrome, while the remainder of MPNSTs develop sporadically (Anghileri M, 2006).

Peritoneal mesothelioma: is a neoplasm arising from mesothelial cells lining the peritoneal cavity. Treatment approaches have evolved toward aggressive cytoreductive surgery and perioperative intraperitoneal chemotherapy, but the prognosis still remain very poor with major studies reporting median survivals consistently under a year (Turner K, 2012).

Wilms' tumour (WT): WT or nephroblastoma is a paediatric malignancy of the kidney, and one of the most common solid cancers of childhood. WT is typically composed of a combination of epithelial, mesenchymal, and/or blastemal cells. Nephrectomy represents the established curative modality (Beckwith, 1983).

Telomere maintenance mechanisms in clinical tumours.

While telomerase presence has been screened in a wide variety of cancerous and non-cancerous tissue types, the prevalence of the ALT phenotype has not yet been clearly determined, since the currently available assays for ALT are complicated for screening a large number of clinical samples. In fact, whereas telomerase positive (TA+) tumours can be identified unequivocally by detecting the enzyme's activity, the identification of ALTpositive (ALT+) cancers has been thus far based on telomere restriction fragment analysis and APB detection, two methods that display a suboptimal rate of concordance. On the basis of available data, ALT mechanisms seem to be frequently present in tumours of mesenchymal and neuroepithelial origin (Cesare AJ, 2010; Henson JD, 2010), with respect to carcinomas (Henson JD, 2010). Such evidence suggests that cell type-specific mechanisms can favor the activation of the ALT versus telomerase, and vice versa, during tumourigenesis and may reflect a tighter regulation of telomerase in normal mesenchymal and neuroepithelial than in epithelial cells (Henson JD, 2010).

At present, scanty information is available regarding the molecular pathways leading to the activation of a specific TMM in tumours. The mechanisms by which telomerase activity is regulated in human tumours have been extensively investigated. Evidence suggests that the altered expression of specific oncogenes and tumour suppressor genes is related to reactivation of the enzyme (Deng Y, 2008; Shay JW 2006). In addition, multiple steps for regulation of the enzyme have been unveiled. In particular, telomerase expression is strictly controlled at the transcriptional and post-transcriptional/translational level, and an

association between the overexpression of hTERT and the deregulated expression of a specific microRNA (miR-138) has been recently demonstrated in human anaplastic thyroid carcinoma cell lines (Mitomo S, 2008). As regards the ALT phenotype, although it is thought to be driven by a recombination--based mechanism, factors that can act as the main engine of the ALT pathway have not yet been identified. However, in the attempt to unveil candidate ALT genes, it has been shown that the telomere associated proteins (i.e., TRF1, TRF2, TIN2, RAP1) and the MRE11/RAD50/NBS1 complex (Jiang WQ, 2007, Zhong ZH, 2007) are essential for APB formation, indicating that they are probably required to sustain the ALT mechanism. It has also been reported that epigenetic modifications could influence which TMM is activated in different cancer cells. Specific epigenetic alterations within the chromatin environment of the hTR and hTERT promoters have been reported to correlate with the expression of hTR/hTERT in normal, TA+ and ALT+ cells (Atkinson SP, 2005).

An interesting aspect related to TMM and tumour biology is whether telomerase and ALT confer different properties to tumour cells in vivo. It has been demonstrated that hTERT may affect cellular functions other than telomere length (Bollmann FM, 2008), and although telomerase and ALT appear to be equivalent in their ability to support immortalization, their contribution to tumour growth and survival may differ.

Several studies have been carried out to investigate the prognostic implications of TMMs in clinical tumours. Reported data indicate that the association between prognosis and telomerase expression is not so straightforward in human tumours (Hiyama E, 2003). Whereas some

studies reported that increased telomerase activity/expression is related to a poor prognosis in different tumour types (Hiyama E, 2003), such an association was not confirmed by other studies, probably as a consequence of different assay conditions, data analysis and patient selection, thus making it difficult to draw a general conclusion on the role of telomerase in cancer prognosis. However, the prognostic significance of ALT mechanisms seems to be strictly dependent on tumour type. Specifically, it has been demonstrated that patients with ALT+, high-grade glioblastoma multiforme (GBM) have significantly longer survival than those with ALT-negative tumours (Hakin-Smith V, 2003). In contrast, a poor prognosis characterizes patients with ALT+ (and TA+) osteosarcomas compared with patients with tumours lacking any known TMM (Ulaner GA, 2003). In addition, it has been reported that ALT is a strong determinant of an unfavorable outcome in liposarcoma patients (Costa A, 2006), whereas it failed to significantly affect the clinical outcome of patients with diffuse malignant peritoneal mesothelioma (Villa R, 2008). Differences in TMM-specific genetic alterations might explain the differences observed in the prognosis of patients with different tumour types.

Aims of the project.

A limitless proliferative potential is one of the hallmarks of tumour cells and can be achieved through the activation of telomere maintenance mechanisms (TMM), which rely on telomerase reactivation or, alternatively, on recombination-based processes known as alternative lengthening of telomeres (ALT). To date, the molecular basis that lead to the activation of TMM during tumourigenesis are poorly understood but several lines of evidence indicate that complex signaling networks might be involved. Since telomerase activity has been detected in most tumours of epithelial origin and ALT is more frequently expressed in tumours of mesenchymal and neuroepithelial origin, it could be hypothesized that cell type-specific mechanisms — which are still unknown — may favor the activation of the different TMM during tumourigenesis.

Another interesting aspect related to TMM and tumour biology is whether telomerase and ALT confer different properties to tumour cells in vivo, in terms of biological aggressiveness. In this context, controversial results have been obtained about the clinical relevance of TMM, whose prognostic role seems to be dependent on tumour histotype. This scenario could be, at least in part, the consequence of the lack of standardized methods to properly classify tumours with respect to their TMM status.

Since a substantial fraction of tumours of mesenchymal origin utilizes ALT, they represent a very interesting model to study the molecular pathways involved in the activation of TMM. In particular, the knowledge of the specific factors/pathways by which the two known TMMs are

differentially regulated in different tumour histotypes of mesenchymal origin might be important for a better understanding of the pathogenesis of these malignancies and for the identification of new therapeutic targets.

By taking advantage of the in-house availability of a large collection of tumour specimens with an associated clinical and patho-biological database, our studies were aimed at:

- ***i)*** Extending the knowledge about the prevalence and the prognostic significance of the two known TMMs (TA and ALT) in different soft-tissue sarcoma histotypes (MPNST, leiomyosarcoma) and mixed origin tumours (Wilms' tumour);
- ***ii)*** Comparing the available methods to assess ALT mechanisms in human solid tumours and define the most suitable biomarker;
- ***iii)*** Analyzing the molecular pathways differentially expressed as a function of the different TMM operating in the tumour, using microRNA expression profiles in subsets of TA-positive and ALT-positive tumours of mesenchymal origin (including MPNST, and malignant peritoneal mesotheliomas);
- ***iv)*** Verifying the relevance of putative TMM-related miRNAs by functional analysis in a human mesenchymal tumour cell line. Specifically, miRNA involvement in cancer-related pathways, such as proliferation and TMMs activation was assessed.

Main results

In summary, with our studies, we are able to provide information on:

i) **the prevalence and prognostic relevance of TMMs in MPNST. In particular, at least one TMM is present in 61% of the MPNST with TA and ALT being present in a comparable fraction of tumours. Survival analyses suggested TA as an indicator of poor disease-specific survival.** (main results available in this session, whole unpublished manuscript available in Part II, page 127)

ii) **the prevalence and prognostic relevance of TMMs in leiomyosarcoma, in which ALT proved to be the more frequently expressed mechanism. Also in this tumour type, TMM presence (in particular ALT) proved to negatively affect patients' prognosis.** (manuscript in preparation, main results available in this session, page 35)

iii) **the prognostic relevance of ALT in a series of liposarcoma patients as a function of the characteristic (heterogeneous telomeres versus APB presence) used to classify the tumour, identifying APB presence as the most suitable marker for ALT** (main results reported in this session, whole published manuscript "Venturini L et al., BMC Cancer 2010, 10:254" available in Part II, page 59)

iv) **the presence, for the first time, of ALT mechanisms in Wilms' tumour** (main results reported in this session, whole published manuscript "Venturini L et al., GCC 2011 Oct; 50(10):823-9" available in Part II, page 108)

v) **miRNAs potentially involved in TMM regulation in tumours of mesenchymal origin.** (preliminary results reported in this session, page 42)

i) Telomere maintenance mechanisms in malignant peripheral nerve sheath tumours.

Whole unpublished manuscript available in Part II, page 127, *Venturini et al.: Telomere maintenance mechanisms in malignant peripheral nerve sheath tumours: expression and prognostic relevance.*

The study aimed at investigating the prevalence of the two known telomere maintenance mechanisms (TMMs), telomerase activity (TA) and alternative lengthening of telomeres (ALT), and assessing their prognostic relevance in malignant peripheral nerve sheath tumours (MPNST). In 57 specimens obtained from 49 patients with MPNST (35 sporadic and 14 neurofibromatosis type 1-related), TA was determined using the telomeric repeat amplification protocol and ALT was detected by assaying both ALT-associated promyelocytic leukaemia bodies (APB) and terminal restriction fragment (TRF) length distribution. TA or ALT (as detected by the presence of APB) alone was found in 24.6% or 26.3% of the lesions, respectively, whereas 6 cases (10.5%) were TA+/ALT+. A concordance between APB and TRF results in defining the ALT status was observed in 44 of 57 cases (77.2%; $P<0.0001$). TA was found to be more frequently expressed in samples from patients with neurofibromatosis type 1-related MPNST than in those with sporadic disease (60% vs. 29.4%, $P=0.087$). In the overall series, TA proved to be prognostic for 5-year disease-specific death (Hazard Ratio, 3.78; 95% CI, 1.60-8.95; $P=0.002$). These findings held true even when adjusted for the concomitant presence of neurofibromatosis type 1 syndrome (Hazard Ratio, 4.22; 95% CI, 1.804-9.874; $P=0.001$) and

margin status after surgery (Hazard Ratio, 5.78; 95% CI, 2.19-15.26; p<0.001). Conversely, ALT failed to significantly affect clinical outcome of MPNST patients either using APB expression (Hazard Ratio,1.25; 95% CI 0.54-2.89; *P*=0.605) or TRF distribution (Hazard Ratio 0.57; 95% CI 0.17-1.96; *P*=0.375) as the detection approach. Our results indicate for the first time that both known TMMs, TA and ALT, are present in MPNST and differentially affect patient prognosis.

ii) Telomere maintenance mechanisms in leiomyosarcoma (unpublished results).

A total of 137 leiomyosarcoma frozen specimens from 116 adult patients, were available for TA and ALT analysis (Table 1).

All the patients have been followed and treated at the Istituto Nazionale Tumouri of Milan from 1990 to 2008 and underwent different surgical procedures according to disease presentation. The median age was 56 years (range 26-78) and the median follow-up for the entire group, as of February 2011, was 81 months. The TMM phenotype has been investigated on the overall case series by assessing the presence of APBs for assaying tumours for ALT, as well as by verifying the presence of telomerase activity by TRAP assay.

Total patients	116 (100%)
Gender	
M	44 (37.9%)
F	72 (62.1%)
Tumor type at presentation	
primary	58 (50.0%)
recurrence	49 (42.2%)
metastasis	9 (7.8%)
grade	
1	9 (7.8%)
2	31 (26.7%)
3	53 (45.7%)
missing	23 (19.8%)

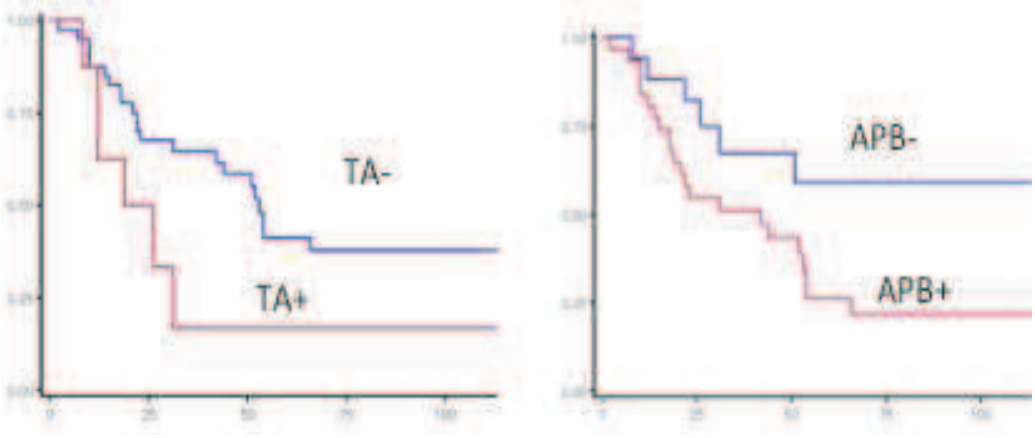
Tab.1: Leiomyosarcoma patients characteristics

Twenty-five of 137 (18.2%) specimens were classified as TA+ based on positive TRAP results and more than half of cases (72/137, 52.6%) were classified as ALT+; among these, only 3 lesions were defined as ALT+/TA+, due to the concomitant expression of APBs and TA, and about a third (31.4%) of the lesions did not express any known TMM (Tab.2).

total 137 lesion	TA+/ALT+	TA+/ALT-	TA-/ALT+	TA-/ALT-
	3 (2.2%)	22 (16.0%)	69 (50.4%)	43 (31.4%)

Tab. 2: TMM prevalence in leiomyosarcoma.

In this study we also analysed the relevance of the two different known TMM on disease specific survival of the patients, who underwent surgery followed by different treatment modalities including radiotherapy and/or chemotherapy, according to the Institute guidelines. The presence of at least one TMM significantly affect patient prognosis (after 6 years of follow-up: TMM+ vs. TMM-, hazard ratio, 5.99; 95% CI 1.42-25.35; P=0.015). Specifically, TA proved to be prognostic for 3-year disease-specific survival but such a prognostic value was not maintained at 6 years of follow-up (At 6 years: TA+ vs. TA-, HR, 2.15; 95% CI, 0.86-5.38 *P* = 0.102). By counterpart, after 3 years, ALT-positive patients showed a lower although not statistically significant probability of being alive, and such a trend reached statistical significance at longer follow-up period (At 6 years: ALT+ vs. ALT-, HR, 2.55; 95% CI, 1.03-6.30; *P*= 0.043) (Figure 9).



TA+ vs TA-, HR, 2.15; 95% CI, 0.86-5.38 *P* = 0.102 ALT+ vs ALT-, HR, 2.55; 95% CI, 1.03-6.30; *P*= 0.043

Figure 9 : prognostic relevance of TMM in leiomyosarcoma patients. Disease-specific survival at 6 years of follow-up.

iii) Prognostic relevance of ALT-associated markers in liposarcoma: a comparative analysis

Whole published article available on Part II, page 59: *Venturini et al. Prognostic relevance of ALT-associated markers in liposarcoma: a comparative analysis. BMC Cancer 2010, 10:254*

Characteristics of ALT-positive tumour cells include an extreme heterogeneity of telomere length, as well as the presence of APBs, and different assays have been developed and alternatively used to screen human tumour specimens for the occurrence of ALT.

In a previous paper from our group we reported how the presence of ALT in liposarcoma negatively affected patients' prognosis (Costa A, 2006). In this study we comparatively analysed the prognostic relevance of ALT in a monoinstitutional series of 85 liposarcoma patients as a function of the characteristic (heterogeneous telomeres vs. APB presence) used to classify the tumour, with the final aim to identify the most suitable ALT marker. We determined ALT status on each liposarcoma specimen by using both APB detection and telomere length analysis. Overall, 27 (31.8%) lesions were defined as ALT+ based on the presence of APB, whereas 24 (27.5%) samples were classified as ALT+ on the basis of TRF length distribution. A concordance between APB and TRF results in defining a specimen as ALT+ or ALT- was found in 66 of 85 cases (77.6%; kappa=0.469; 95%CI, 0.265-0.672; $P<0.0001$). Specifically, 16 lesions (18.8%) were defined as ALT+ and 50 (58.8%) were scored as ALT- with both detection methods. As regards the remaining lesions, 11 were defined as ALT+ on the basis of

APB expression but did not show a TRF length distribution consistent with an ALT phenotype, and 8 were scored as ALT+ on the basis of TRF analysis but showed a very low percentage of APB-expressing tumour cells (from 0.01 to 0.2%). The prognostic significance of TMM was analysed on the overall series of 85 patients. When a tumour was defined as ALT+ according to at least one method (APB or TRF), ALT proved to be prognostic for 10-year disease-specific survival (ALT+ versus ALT-, 45.5% vs. 71.1%; HR, 2.38; 95% CI, 1.15-4.90; *P*=0.019), and such a prognostic value was maintained and strengthened at 15 years of follow-up (ALT+ versus ALT-, 25.3% versus 71.1%; HR, 2.76; 95%CI, 1.36-5.06; *P*=0.005). These results held true also when APB expression was used as the only parameter to classify tumours for ALT. Specifically, the APB presence proved to be an indicator of increased mortality at both 10 years (HR, 2.14; 95% CI, 1.04-4.41; *P*=0.040) and 15 years (HR, 2.54; 95%CI, 1.27-5.11; *P*=0.009) of follow-up. Conversely, at 10 years of follow-up, patients with a tumour defined as ALT-positive on the basis of TRF length distribution showed a lower although not statistically significant probability of being alive (HR, 1.77; 95% CI, 0.84-3.73; *P*=0.130). Such a trend reached statistical significance at 15 years (HR, 2.14; 95% CI, 1.06-4.34; *P*=0.035). The incomplete overlapping of the TRF and APB results may be due to the different liposarcoma histological subtypes and this heterogeneity may result in a slight difference on the time-dependence of each assay to provide significant prognostic information. Overall, APB may be more appropriate than TRF pattern to assay ALT in tumours because they can be detected in both frozen and formalin-fixed, paraffin-embedded tumour samples as well as in needle biopsies or cytology specimens.

iv) Telomere maintenance mechanisms in Wilms' tumour

Whole published article available on Part II, page 108: *Venturini et al. Telomere maintenance in Wilms tumour: first evidence for the presence of alternative lengthening of telomeres mechanism. Genes Chromosomes and Cancer. 2011 Oct;50(10):823-9*.

To extend our knowledge about the prevalence of ALT mechanisms in human tumours, we took advantage of a collaboration with the Paediatric Oncology Unit of the Istituto Nazionale dei Tumouri. In particular, we investigated the presence of ALT in Wilms Tumour (WT), a paediatric malignancy of the kidney and one of the most common solid cancers of childhood composed of a combination of epithelial, mesenchymal and blastemal cells. We investigated ALT in 34 WT samples from 30 patients by using both APB detection and the assessment of terminal restriction fragment (TRF) length distribution. In parallel, telomerase activity was determined by TRAP assay. Based on APB expression, ALT was detectable in 5 samples as the sole TMM and in 6 samples in association with telomerase. Seventeen samples only expressed TA and in 6 cases no known TMM was appreciable. Expression of the catalytic component of telomerase h*TERT* was found in all TA+ specimens and in 5/11 of TA- cases, confirming that the presence of TERT mRNA is necessary but not sufficient to obtain an active form of the enzyme. The presence of TA was more frequently observed in tumours from untreated patients compared to samples obtained from patients who underwent preoperative chemotherapy (P=0.042), while the presence of APB proved to be unrelated to previous treatment. Results of TRF length distribution were available in

32 cases, and a concordance between APB and TRF data in defining the ALT phenotype was found in a valuable percentage of cases (81%). The study provided the first evidence of the presence of ALT in approximately one-third of WT and indicated that in a small but defined fraction of cases (about 15%) ALT could be the only TMM supporting the development of WT.

v) Identification of miRNAs differentially expressed as a function of the TMM operating in the tumour.

We performed miRNA expression profiling of peritoneal mesothelioma and MPNST samples by using the Illumina human V2 miRNA chip, which contains 1,146 assays, covering more than 97% of the miRNAs described in the miRBase database v12.0 (Sanger Institute). For this step we extracted high-quality RNA using Trizol reagent® that was then converted to cDNA by standard methods. Our aim was to identify specific miRNAs differentially expressed as a function of the TMM operating in the tumour. Specifically, we performed miRNA expression profiles of 29 MPNST and 50 peritoneal mesothelioma specimens that have been already screened for TMM during the past years. The 50 peritoneal mesotheliomas expressed TMMs as follows: 22 cases TA+/ALT-, 9 cases TA-/ALT+, 16 cases TA-/ALT- and 3 cases TA+/ALT+ (Supplementary table 1).

The first classification allowed us to divide samples on the basis of telomerase expression, irrespectively of the ALT status, in order to determine which miRNAs could be potentially involved in TA regulation in peritoneal mesothelioma. We found a signature of 84 miRNAs differentially expressed in TA+ vs. TA- samples ($p<0.05$) (Supplementary Table 2). We then divided mesothelioma specimens on the basis of ALT expression, irrespectively of TA status, defining a list of 61 miRNAs differentially expressed between ALT+ vs. ALT- tumours (Supplementary Table 3). Similarly, we analysed data from miRNA expression profiles of MPNST samples. The tumours were classified on the basis of TMM as follows: 10 cases TA+/ALT-, 5 cases TA-/ALT+, 10 cases TA-/ALT-, 4 cases TA+/ALT+ (Supplementary table 4).

The comparison between telomerase posive and telomerase negative cases generated a signature of 32 miRNA significantly differentially expressed ($p<0.01$) (Supplementary Table 5). We then compared ALT-posive and ALT-negative cases, generating a signature of 15 miRNA differentially expressed between the two classes ($p<0.05$) (Supplementary Table 6).

MiRNA expression profiles will be carried out also from a subset of the leiomyosarcoma samples we screened for TMM and these results will be analysed and compared to those obtained in mesotheliomas and MPNSTs. Through the screening of tumour specimens of different histotypes, we want to assess the expression of specific miRNA signatures in ALT+ and TA+ tumours independently of the histologic origin.

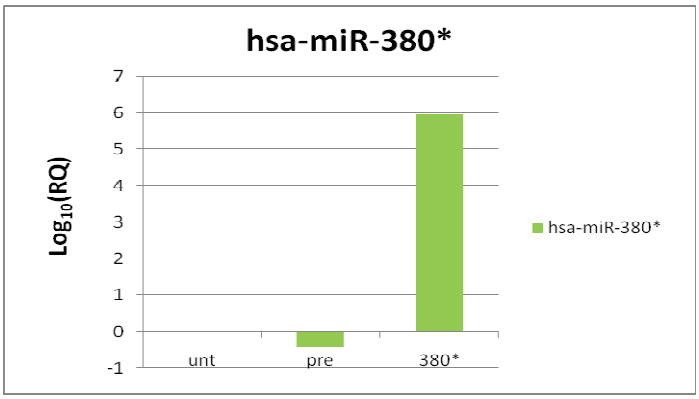
Gene expression analyses will be carried out on the tumour types considered and already characterized for TMM (peritoneal mesothelioma, MPNST and leiomyosarcoma) with an Agilent-based gene expression platform. To this purpose, we will use total RNA that we already extracted from these tumour specimens using Trizol Reagent (Invitrogen). Data generated from miRNA and gene expression profiles will be integrated to identify possible miRNA/target gene interactions involved in the regulation of TMM. This analysis will help us to find a putative connection between TMM-associated miRNAs and the targets they regulate. In addition, the identification of TMM-associated genes and miRNAs, as surrogate markers of ALT+ and TA+ phenotypes, will help to correctly classify tumours according to TMM status, allowing to overcome the misclassification bias introduced by the current available detection methods.

Ongoing results

To get further insight the regulation of TMM, we selected some of the miRNAs potentially involved in telomerase activity regulation and validated their expression by quantitative RT-PCR using TaqMan® MicroRNA. We then choosed miR380* (Supplementary Figure 1, Part III) for functional validation since it proved to be significantly down-regulated in telomerase positive mesothelioma specimens and in a telomerase positive peritoneal mesothelioma cell line already available and well characterized in our laboratory (STO cell line, Zaffaroni N, 2007).

To unravel if miR380* could play a specific role in telomerase regulation and in cellular pathways relevant in the tumourigenic process, we restored miR-380* levels in STO cell line. Ectopic expression of miR-380* was pursued by transfection of its synthetic precursor (Pre-miR$_{TM}$ Precursor Molecules, Ambion). Transfection efficiency was evaluated by using 6-carboxyfluorescein-labeled microRNA (data not shown).

A 20nM concentration proved to be effective in restoring miR-380* expression in STO cells, as assessed by end-point RT-PCR 3 days after transfection, while miR-380* levels remained undetectable in cells exposed to the lipidic vehicle alone (not shown) or transfected with a negative control oligomer (preNeg) (Figure 10).



	Ct (miR-380*)	Ct (endogenous control)	ΔCt	P (Sudent's t-test)	
UNT	34.8±1.2	24.5±0.03	10.4±1.23		
pre-Neg	36.4±0.6	24.6±0.02	11.7±0.54	0.14	
Pre-miR-380*	17.6±0.2	26.9±1.05	-9.33±1.1	0.0012	$3.7\times^{10-5}$

Figure 10: expression of miR-380* (72hs post transfection). Expression of miR380* is significantly higher in transfected cells with respect to negative control-transfected cells (PRE NEG). Measurement was performed by qRT-PCR. RQ=relative quantity, UNT=untransfected cells, PRE NEG=cells transfected with negative precursor, 380*=cells transfected with pre-miR-380*. RNU48 was used as PCR endogenous control.

Ectopic miR-380* expression in STO cells induced perturbations of cell growth. Specifically, we observed an appreciable cell growth impairment in miR380*-transfected cells, which exhibited significantly longer population doubling time than those transfected with control microRNA (Figure 11). These results suggest that miR-203 could inhibit the proliferation of STO cell.

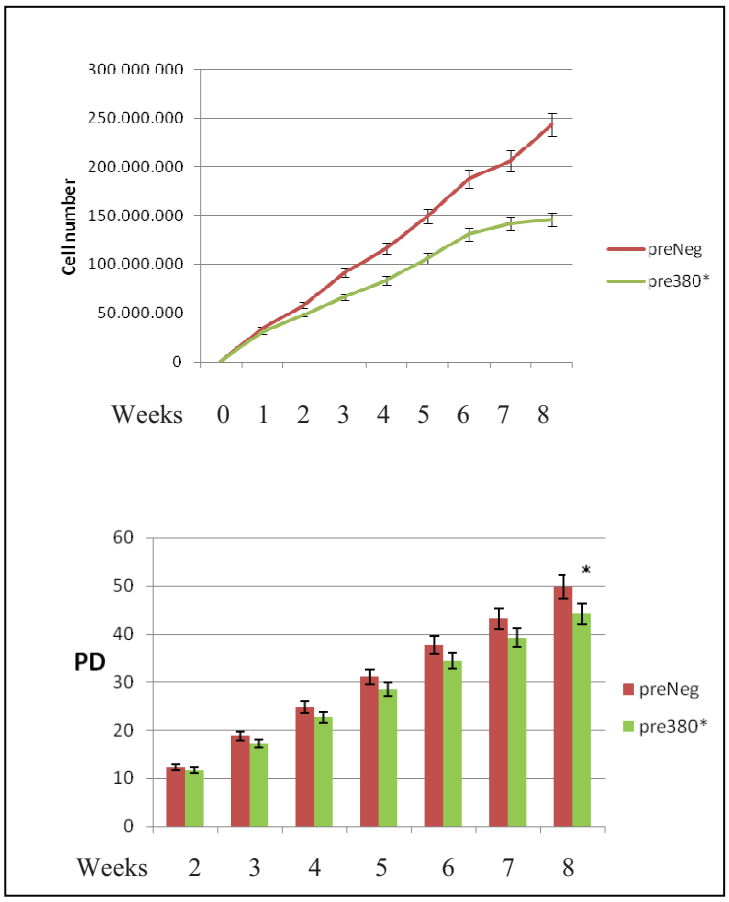


Figure 11. vital counting in each week after transfection using Trypan Blue Solution 0,4%.. P<0.05. *Population Dubling time* PD=Log_{10}(harvested cells/seeded cells)/$Log_{10}2$. Obtaining the number of replication in each week. In the following weeks PD are added to the previous one. P<0.05.

The ectopic expression of miR380* induced appreciable morphological changes in transfected cells starting from the first week after transfection and becoming more and more evident in the following weeks. Specifically, we observed changes usually associated with the acquisition of an epithelial phenotype, with a decreasing fraction of cells with elongated shape and the appearance of large, flattened, polygonal-shaped cells (Figure 12).

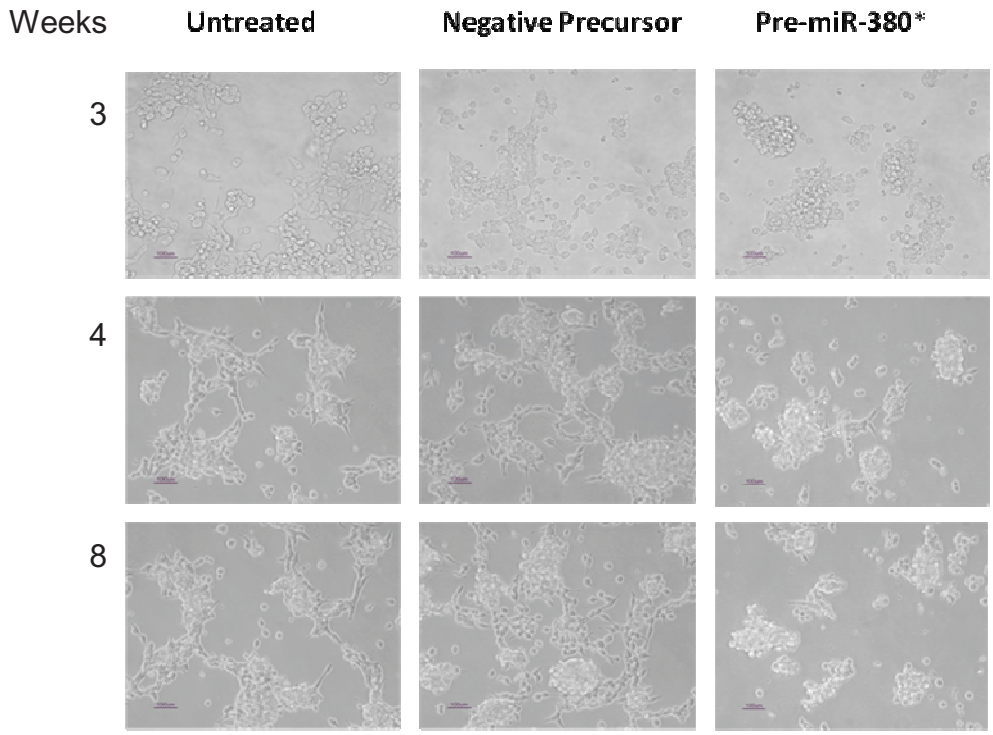


Figure 12. Morphology changes of pre-miR-380*-transfected cells. Pre-miR-380*-transfected cells progressively lose their original elongated shape and became more rounded and flattened.

Our data from miRNA expression profiles of peritoneal mesothelioma specimens showed miR-380* as significantly down-regulated in telomerase positive tumours. We then supposed that the reduced cell growth could be due, at least in part, to the occurrence of any telomere dysfunction related to the ectopic expression of miR-380*. For instance we measured telomere length of the transfected cell populations by Southern Blot analysis of Telomere restriction Fragments (TRF) (Villa R, 2000) (for further details see materials and methods in Part II, published papers). After 8 weeks of transfection, the expression of miR-380* and the cell growth impairment were accompanied by a small but significant

telomere shortening (Figure 13). Specifically, the mean TRF was 5,12 Kb in pre-Neg-transfected cells and 4,71 Kb in pre-miR-380*-transfected cells.

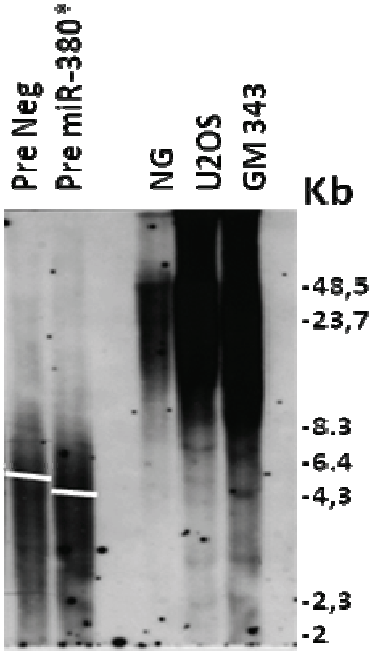
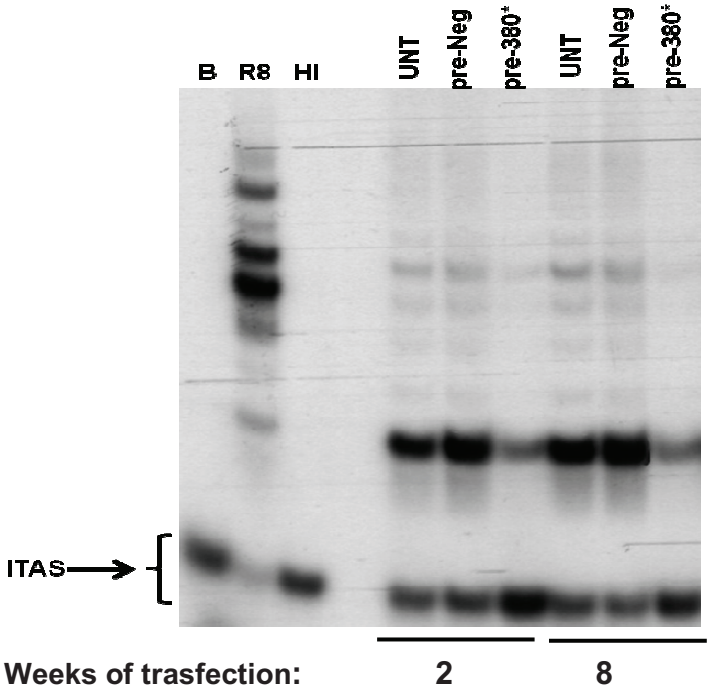


Figure 13. Telomere Restriction Fragment lenght analysis by Southern blot. Unt: untreated cells, Pre Neg: cells transfected with negative precursor, Pre-miR-380*: cells trasfected with miR-380* precursor, U2OS: osteosarcoma cell line and GM 847: human fibroblasts included as control.

We then argued that the observed telomere shortening in miR-380*-expressing cells could be due to a direct or indirect role of miR380* in telomerase activity regulation. TRAP assay showed a strong inhibition of telomerase activity in miR380*-transfected cells after 8 weeks of treatment when compared to the cells transfected with negative precursor and to untreated cells. Specifically, a quantitative analysis showed a reduction of about 90% of telomerase activity in pre-miR-380*-treated cells when compared to pre-neg treated samples. (Figure 14).



	Week 2	Week 8
UNT	1.0±0.3	1.2±0.07
pre-NEG	1.0±0.12	1.05±0.01
pre-380*	0.1±0.004	0.06±0.001

Figure 14. TRAP assay for telomerase activity. 0.1 ug of protein was used for each sample in this assay. The signal intensity for each lane is normalized with the corresponding ITAS signal. TSR8=positive control, HI=heat inactivated negative control, ITAS=internal amplification standard.

To further verify whether mir-380* modulation can affect telomere dynamic, we will evaluate also eventual changes in terms of APB expression changes in the APB-negative STO cell line treated with miR-380* synthetic precursor.

Putative target genes for miR380* TA-associated miRNA will be found using the available miRNA target prediction databases, such as miRanda, TargetScan and PicTar, allowing us to identify potential molecular targets related to telomerase regulation.

Conclusions and future perspectives

With respect to human tumours of epithelial origin, our data show the relatively low frequency of telomerase activity in soft tissue sarcomas, such as MPNST and leiomyosarcoma as discussed in the present thesis, and in liposarcoma as reported in previous works of our group, implicating that telomerase reactivation is an important, although not crucial event involved in the progression of such malignancies.

By counterpart, the evidence that in liposarcoma and MPNST the frequency of tumours that activate ALT is comparable with that of tumours that express telomerase, and in leiomyosarcoma approximately half of the cases use ALT as TMM, opens important questions about the mechanisms that may determine which TMM becomes activated in individual tumours and the requirement for telomere maintenance during the development of these malignancies.

From our data we can address an important issue for the clinical use of telomerase inhibitors related to the choice of the best patient population. The reported evidence that many solid tumours express the ALT phenotype instead of telomerase and, as a consequence, are not likely to be affected by telomerase inhibitors, suggests that TMM status should be determined in individual patients before starting any hypothetical anti-telomerase treatment.

Moreover, since ALT and telomerase may coexist in the same tumour, it is also conceivable that treatment of TA-positive tumours with telomerase inhibitors could exert a potent selection pressure, leading to the emergence of subpopulations of ALT-positive tumour cells refractory to telomerase inhibitors.

On the other hand, we found that a significant percentage of tumours (about 40% of MPNSTs, a third of leiomyosarcoma and about 15% of WT) possessed no apparent TMM despite being informative for the different assays, suggesting that the presence of a constitutively active TMM is not a stringent requirement for these malignancies, or, alternatively, that these tumours use a mechanism that has not yet been identified. Moreover, such a lack of any known TMM, is in accord with experimental data suggesting that TMM acquisition is not always required for malignant transformation of normal human cells. However, the possibility that the lack of TMM expression observed in a high percentage of the tested MPNSTs may be related, at least in part, to the sensitivity of the assays used cannot be excluded.

In this way, the identification of TMM-associated miRNAs, as surrogate markers of ALT+ and TA+ phenotypes, will help to correctly classify tumours according to TMM status, eventually allowing to overcome the classification biases introduced by the current available detection methods and, possibly, provide new potential indicators of prognosis.

In addition, the validation of candidate TMM-associated miRNAs will be instrumental to discover new cancer-related targets, paving the way for the development of innovative anticancer therapeutics, which could be tailored to patients as a function of the specific TMM, also taking into account, the lack of reliable therapeutic options other than complete surgery.

References

Anghileri M, Miceli R, Fiore M, Mariani L, Ferrari A, Mussi C, Lozza L, Collini P, Olmi P, Casali PG, Pilotti S, Gronchi A. Malignant peripheral nerve sheath tumours: prognostic factors and survival in a series of patients treated at a single institution. Cancer. 2006 Sep 1;107(5):1065-74.

Atkinson SP, Hoare SF, Glasspool RM, et al. Lack of telomerase gene expression in alternative lengthening of telomere cells is associated with chromatin remodeling of the hTR and hTERT gene promoters. Cancer Res 2005;65:7585-90.

Bailey, S. M., Brenneman, M. A. & Goodwin, E. H. Frequent recombination in telomeric DNA may extend the proliferative life of telomerase-negative cells. Nucleic Acids Res. 32, 3743–3751 (2004).

Bechter, O. E., Shay, J. W. & Wright, W. E. The frequency of homologous recombination in human ALT cells. Cell Cycle 3, 547–549 (2004).

Beckwith JB. 1983. Wilms' tumour and other renal tumours of childhood: A selective review from the National Wilms' Tumour Study Pathology Center. Hum Pathol 14:481–492.

Blackburn EH., The end of the (DNA) line. *Nat Struct Biol*, **7** 10 (2000), pp. 847–850.

Bollmann FM. The many faces of telomerase: emerging extratelomeric effects. Bioessays 2008;30:728-32.

Borde, V. and Cobb, J. (2009) Double functions for the Mre11 complex during DNA double-strand break repair and replication. Int. J. Biochem. Cell Biol. 41, 1249–1253.

Bryan TM and R.R. Reddel, Telomere dynamics and telomerase activity in *in vitro* immortalised human cells. *Eur. J. Cancer*, **33** (1997), pp. 767–773.

Bryan TM, A. Englezou, L. Dalla-Pozza, M.A. Dunham and R.R. Reddel, Evidence for an alternative mechanism for maintaining telomere length in human tumours and tumour-derived cell lines. *Nat. Med.*, **3** (1997), pp. 1271–1274.

Bryan TM., A. Englezou, J. Gupta, S. Bacchetti and R.R. Reddel, Telomere elongation in immortal human cells without detectable telomerase activity. *EMBO J.*, **14** (1995), pp. 4240–4248.

Cech, T.R. (2004) Beginning to understand the end of the chromosome. Cell 116, 273–279.

Cesare AJ, Reddel RR. Alternative lengthening of telomeres: models, mechanisms and implications. Nat Rev Genet 2010;11:319-30

Chan, S.W.-L. and Blackburn, E.H. (2003) Telomerase and ATM/Tel1p protect telomeres from nonhomologous end joining. Mol. Cell 11, 1379–1387.

Cookson JC, Laughton CA. The levels of telomere-binding proteins in human tumours and therapeutic implications. Eur J Cancer 2009;45:536-50

Costa A, Daidone MG, Daprai L, et al. Telomere maintenance mechanisms in liposarcomas: association with histologic subtypes and disease progression.Cancer Res; 66:8918 – 24, 2006.

deLange,T.(2005) Shelterin: the protein complex that shapes and safeguards human telomeres. Genes Dev. 19, 2100–2110.

Deng Y, Chang SS, Chang S. Telomere dysfunction and tumour suppression: the senescence connection. Nat Rev Cancer 2008;8:450-8

Dionne, I. and Wellinger,R.J. (1996) Cell cycle-regulated generation of single-stranded G-rich DNA in the absence of telomerase. Proc. Natl. Acad. Sci. USA 93, 13902–13907.

Dunham MA, A.A. Neumann, C.L. Fasching and R.R. Reddel, Telomere maintenance by recombination in human cells. *Nat. Genet.*, **26** (2000), 447–450.

Dunham, M.A., Neumann, A.A., Fasching, C.L. and Reddel, R.R. (2000) Telomere maintenance by recombination in human cells. Nat. Genet. 26, 447–450.

Folini M, Gandellini P, Zaffaroni N. Targeting the telosome: therapeutic implications. Biochim Biophs Acta 2009;1792:309-16.

Gollahon, L.S., Kraus, E., Wu, T.A., Yim, S.O., Strong, L.C., Shay, J.W. and Tainsky, M.A. (1998) Telomerase activity during spontaneous immortalization of Li-Fraumeni syndrome skin fibroblasts. Oncogene 17, 709–717.

Griffith,J.D., Comeau, L.,Rosenfield,S., Stansel,R.M., Bianchi,A., Moss,H. and de Lange, T. (1999) Mammalian telomeres end in a large duplex loop. Cell 97, 503–514.

Hakin-Smith V, Jellinek DA, Levy D, et al. Alternative lengthening of telomeres and survival in patients with glioblastoma multiforme. Lancet 2003;361:836-8.

Henson JD, Hannay, J.A., McCarthy, S.W., Royds, J.A., Yeager, T.R., Robinson, R.A., Wharton, S.B., Jellinek, D.A., Arbuckle, S.M., Yoo, J., Robinson, B.G., Learoyd, D.L., Stalley, P.D., Bonar, S.F., Yu, D., Pollock, R.E. and Reddel, R.R. (2005) A robust assay for alternative lengthening of telomeres (ALT) in tumours demonstrates the significance of ALT in sarcomas and astrocytomas. Clin. Cancer Res. 11, 217–225.

Henson, J.D., Neumann, A.A., Yeager, T.R. and Reddel, R.R. (2002) Alternative lengthening of telomeres in mammalian cells. Oncogene 21, 598–610.

Hiyama E, Hiyama K. Telomerase as tumour marker. Cancer Lett 2003;194:221-33

Hoffman A, Lazar AJ, Pollock RE, Lev D. New frontiers in the treatment of liposarcoma, a therapeutically resistant malignant cohort. Drug Resist Updat. 2011 Feb;14(1):52-66. Epub 2010 Dec 18.

Jeyapalan, J.N., Mendez-Bermudez, A., Zaffaroni, N., Dubrova, Y.E. and Royle, N.J. (2008) Evidence for alternative lengthening of telomeres in liposarcomas in the absence of ALT-associated PML bodies. Int. J. Cancer 122, 2414–2421.

Jiang, W.Q., Zhong, Z.H., Henson, J.D. and Reddel, R.R. (2007) Identification of candidate alternative lengthening of telomeres genes by methionine restriction and RNA interference. Oncogene 26, 4635–4647.

Johnson FB, Marciniak RA, McVey M, Stewart SA, Hahn WC, Guarente L. The Saccharomyces cerevisiae WRN homolog Sgs1p participates in telomere maintenance in cells lacking telomerase. EMBO J. 2001 Feb 15;20(4):905-13.

Johnson JE, Gettings EJ, Schwalm J, et al. Whole-genome profiling in liposarcomas reveals genetic alterations common to specific telomere maintenance mechanisms. Cancer Res 2007;67:9221-8

Kim NW, Piatyszek MA, Prowse KR, et al. Specific association of human telomerase activity with immortal cells and cancer. Science 1994;66:2011–5.

Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL, Shay JW. 1994.

Lai CK., M.C. Miller and K. Collins, Template boundary definition in *Tetrahymena telomerase*. *Genes Dev*, **16** 4 (2002), pp. 415–420.

Lamond AI, *Tetrahymena telomerase* contains an internal RNA template. *Trends Biochem Sci*, **14** 6 (1989), pp. 202–204.

Lee MS. and E.H. Blackburn, Sequence-specific DNA primer effects on telomerase polymerization activity *Mol Cell Biol*, **13** 10 (1993), pp. 6586–6599.

Linger, B.R. and Price, C.M. (2009) Conservation of telomere protein complexes: shuffling through evolution. Crit. Rev. Biochem. Mol. Biol. 44, 434–446.

Lundblad V and E.H. Blackburn, An alternative pathway for yeast telomere maintenance rescues *est1*$^{-}$ senescence. *Cell*, **73** (1993), pp. 347–360.

Makarov,V.L., Hirose,Y. and Langmore,J.P. (1997) Long Gtails at both ends of human chromosomes suggest a C strand degradation mechanism for telomere shortening. Cell 88, 657–666.

Miller MC, J.K. Liu and K. Collins, Template definition by *Tetrahymena telomerase* reverse transcriptase. *EMBO J*, **19** 16 (2000), pp. 4412–4422.

Mitchell M, Gillis A, Futahashi M, et al. Structural basis for telomerase catalytic subunit TERT binding to RNA template and telomeric DNA. Nat Struct Mol Biol 2010;17:513-18.

Mitomo S, Maesawa C, Ogasawara S, et al. Downregulation of miR-138 is associated with overexpression of human telomerase reverse transcriptase protein in human anaplastic thyroid carcinoma cell lines. Cancer Sci 2008;99:280-6

Palm, W., Hockemeyer, D., Kibe, T. and de Lange, T. (2009) Functional dissection of human and mouse POT1 proteins. Mol. Cell. Biol. 29, 471–482.

Palm, W., Hockemeyer, D., Kibe, T. and de Lange, T. (2009) Functional dissection of human and mouse POT1 proteins. Mol. Cell. Biol. 29, 471–482.

Perrem K, Colgin LM, Neumann AA, Yeager TR, Reddel RR. Coexistence of alternative lengthening of telomeres and telomerase in hTERT-transfected GM847 cells. *Mol Cell Biol.* 2001;21.

Potts P.R. and Yu, H. (2007) The SMC5/6 complex maintains telomere length in ALT cancer cells through SUMOylation of telomere-binding proteins. Nat. Struct. Mol. Biol. 14, 581–590.

Poulet, A. et al. (2009) TRF2 promotes, remodels and protects telomeric Holliday junctions. EMBO J. 28, 641–651.

Reddel RR, The role of senescence and immortalization in carcinogenesis. *Carcinogenesis*, **21** (2000), pp. 477–484.

Rhodes, D., Fairall, L., Simonsson, T., Court, R. and Chapman, L. (2002) Telomere architecture. EMBO Rep. 3, 1139–1145.

Rogan EM, T.M. Bryan, B. Hukku, K. Maclean, A.C. Chang, E.L. Moy, A. Englezou, S.G. Warneford, L. Dalla-Pozza and R.R. Reddel, Alterations in p53 and $p16^{INK4}$ expression and telomere length during spontaneous immortalization of Li-Fraumeni syndrome fibroblasts. *Mol. Cell. Biol.*, **15** (1995), pp. 4745–4753.

Shay JW and S. Bacchetti, A survey of telomerase activity in human cancer. *Eur. J. Cancer*, **33** (1997), pp. 787–791.

Shay JW, Wright WE. Telomerase therapeutics for cancer: challenges and new directions. Nat Rev Drug Discov 2006;5:577-84

Shippen-Lentz D. and E.H. Blackburn, Functional evidence for an RNA template in telomerase. *Science*, **247** 4942 (1990), pp. 546–552.

Stansel, R.M., Subramanian, D. and Griffith, J.D. (2002) p53 binds telomeric single strand overhangs and t-loop junctions in vitro. J. Biol. Chem. 277, 11625–11628.

Turner K, Varghese S, Alexander HR. Current concepts in the evaluation and treatment of patients with diffuse malignant peritoneal mesothelioma. J Natl Compr Canc Netw. 2012 Jan 1;10(1):49-57

Ulaner GA, Huang HY, Otero J, et al. Absence of a telomere maintenance mechanism as a favorable prognostic factor in patients with osteosarcoma. Cancer Res 2003;63:1759-63

van Steensel, B., Smogorzewska, A. and de lange, T. (1998) TRF2 protects human telomeres from end-to-end fusions. Cell 92, 401–413.

van Vliet M, Kliffen M, Krestin GP, van Dijke CF. Soft tissue sarcomas at a glance: clinical, histological, and MR imaging features of malignant extremity soft tissue tumours. Eur Radiol. 2009 Jun;19(6):1499-511. Epub 2009 Mar 6. Review.

Vega, L., Mateyak, M. and Zakian, V. (2003) Getting to the end: telomerase access in yeast and humans. Nat. Rev. Mol. Cell Biol. 4, 948–959.

Venteicher AS, Abreu EB, Meng Z, et al. A human telomerase holoenzyme protein required for Cajal body localization and telomere synthesis. Science 2009;323:644-8.

Venturini L., Erdas R, Costa A, Gronchi A, Pilotti S, Zaffaroni N, Daidone M. ALT-associated promyelocytic leukaemia body (APB) detection as a reproducible tool to assess alternative lengthening of telomere stability in liposarcomas. J Pathol. 214(4):410-4, 2008.

Verdun, R.E. and Karlseder, J. (2007) Replication and protection of telomeres. Nature 447, 924–931.

Villa R, Daidone MG, Motta R, Venturini L, De Marco C, Vannelli A, Kusamura S, Baratti D, Deraco M, Costa A, Reddel RR, Zaffaroni N. Multiple mechanisms of telomere maintenance exist and differentially affect clinical outcome in diffuse malignant peritoneal mesothelioma. Clin Cancer Res. 1; 14(13):4134-40, 2008.

Villa R, Folini M, Perego P, et al. Telomerase activity and telomere length in human ovarian cancer and melanoma cell lines: correlation with sensitivity to DNA damaging agents. Int J Oncol. 2000 May;16(5):995-1002.

Wellinger, R.J. and Sen, D. (1997) The DNA structures at the ends of eukaryotic chromosomes. Eur. J. Cancer 33, 735–749.

Wu G, Lee WH, Chen PL. NBS1 and TRF1 colocalize at promyelocytic leukemia bodies during late S/G2 phases in immortalized telomerase-negative cells. Implication of NBS1 in alternative lengthening of telomeres. J Biol Chem. 2000 Sep 29;275(39): 30618-22.

Xin H., D. Liu, M. Wan, A. Safari, H. Kim, W. Sun, M.S. O'Connor and Z. Songyang, TPP1 is a homologue of ciliate TEBP-beta and interacts with POT1 to recruit telomerase. *Nature*, **445** 7127 (2007), pp. 559–562.

Yeager TR, Neumann AA, Englezou A, Huschtscha LI, Noble JR, Reddel RR. Telomerase-negative immortalized human cells contain a novel type of promyelocytic leukemia (PML) body. Cancer Res. 1999 Sep 1;59(17):4175-9.

Yu, J., Lan, J., Wang, C., Wu, Q., Zhu, Y., Lai, X., Sun, J., Jin, C. and Huang, H. (2010) PML3 interacts with TRF1 and is essential for ALT-associated PML bodies assembly in U2OS cells. Cancer Lett. 291, 177–186.

Zaffaroni N, Costa A, Pennati M, De Marco C, Affini E, Madeo M, Erdas R, Cabras A, Kusamura S, Baratti D, Deraco M, Daidone MG. Survivin is highly expressed and promotes cell survival in malignant peritoneal mesothelioma. Cell Oncol. 2007;29(6):453-66.

Zhong ZH, Jiang WQ, Cesare AJ, et al. Disruption of telomere maintenance by depletion of the MRE11/RAD50/NBS1 complex in cells that use alternative lengthening of telomeres. J Biol Chem 2007;282:29314-22

Zhu XD, Küster B, Mann M, Petrini JH, de Lange T. Cell-cycle-regulated association of RAD50/MRE11/NBS1 with TRF2 and human telomeres. Nat Genet. 2000 Jul;25(3):347-52.

PART II

Prognostic relevance of ALT-associated markers in liposarcoma: a comparative analysis.

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Abstract

Background: Most cancers maintain telomeres by activating telomerase but a significant minority, mainly of mesenchymal origin, utilize an alternative lengthening of telomeres (ALT) mechanism.

Methods: In this study we comparatively analyzed the prognostic relevance of ALT in a monoinstitutional series of 85 liposarcoma patients as a function of the marker (ALT-associated promyelocytic leukemia bodies (APB) versus heterogeneous telomeres) used to classify the tumor.

Results: Independently of the detection approach, ALT proved to be a prognostic discriminant of increased mortality, although the prognostic relevance of the two markers appeared at different follow-up intervals (at 10 years for APB and 15 years for telomeres).

Conclusions: Overall, we confirmed ALT as an indicator of poor clinical outcome in this disease and provide the first evidence that the sensitivity of the ALT predictive power depends, at least in part, on the method used.

Background

A hallmark of cancer cells is their limitless proliferative potential, which is sustained by the activation of a telomere maintenance mechanism (TMM) [1]. In a high percentage of human tumors (> 85%), proliferation dependent telomere shortening is counterbalanced by the synthesis of telomeric DNA, which is catalyzed by telomerase [2]. However, in few cancers that lack telomerase, an alternative lengthening of telomeres (ALT) mechanism is used [3]. There may be more than one ALT mechanism, but in at least some ALT-positive human cancer cells telomere length is maintained by recombination mediated replication of telomeric DNA [4]. Characteristics of ALT-positive tumor cells include an extreme heterogeneity of telomere length, with telomeres ranging from very short to extremely long within the same cell, as well as the presence of subnuclear structures termed ALT-associated promyelocytic leukemia (PML) bodies (APB), which contain telomeric DNA, telomere binding proteins and proteins involved in DNA recombination and replication [5]. Assays to detect telomere length and APB have been developed and alternatively used to screen human tumor specimens for the occurrence of ALT. Available results indicate that ALT is more common in tumors of mesenchymal and neuroepithelial origin, including osteosarcomas [6], soft tissue sarcomas [7] and glioblastoma multiforme [8], and that the presence of ALT has prognostic significance that depends on tumor type. Specifically, in liposarcoma ALT proved to be a strong prognostic discriminant of increased mortality [9], whereas in glioblastoma the presence of ALT was associated to a better patient survival [8], suggesting that the prognostic relevance of ALT presumably reflects the distinct set of genetic changes that are associated to the activation of ALT in a given tumor type. In the present study, we

comparatively analyzed the prognostic relevance of ALT in a monoinstitutional series of liposarcoma patients as a function of the characteristic (heterogenoeus telomeres versus APB presence) used to classify the tumor, with the final aim to identify the most suitable marker.

Methods

Study population

Samples from 85 liposarcomas, all from adult patients (36 women and 49 men; median age, 52 years; range, 18-91) treated with a curative intent at the *Istituto Nazionale Tumori* of Milan from December 1986 to November 2003 were available for TMM analysis (Additional file 1, Table S1). The specimens, which represent a subset of a larger case series already characterized for TMM (Costa *et al*, 2006), were consecutive with respect to the availability of frozen tissue and adequate clinicopathologic and follow-up information. Twenty-two patients presented with primary tumors and 63 with recurrent disease (59 localregional recurrences and 4 metastases), and they underwent different surgical procedures according to disease presentation. The median follow-up for the entire group, as of December 2008, was 118 months. During the follow-up, 36 patients died for cancer-related causes (30 within 10 years, another 2 from 10 to 15 years, and 4 after 15 years). Postoperative treatment was given when there was a high risk of recurrence: 18 patients were submitted to radiotherapy, 8 to chemotherapy, and 5 to radio-chemotherapy according to the treatment protocols of the multidisciplinary Soft Tissue Sarcoma Group of the Institute. This study was approved by the Institutional Review Board of the Institute, and all patients provided written informed consent to donate to the Institute the leftover tissue after diagnostic procedures.

Detection of APB, telomere length and telomerase activity (TA)

Tumor tissue was sampled by a pathologist at the time of surgery and flash frozen. A fragment of about 100 mg was cut from each lesion and further subdivided for APB detection, DNA extraction (for telomere length assessment) and protein extraction (for TA assay). APB were assayed by combined PML immunofluorescence and telomere fluorescence *in situ* hybridization [10]. PML was detected with anti-PML mouse antibody (Dako Cytomation; Glostrup, Denmark) plus anti-mouse FITC-labeled goat antibody (Sigma; St. Louis, MO). Telomere fluorescence in situ hybrization (FISH) was performed by denaturing slides together with 5'labeled Cy3-(5'CCCTAA3')3 PNA probe (Applied Biosystems, Framingham, MA) for 3 min at 80℃ and h ybridizing for 3 hs at room temperature. Slides were washed and counterstained with 4'6-Diamino-2-phenylindole (DAPI). Images were captured on a Nikon Eclipse E600 fluorescence microscope using ACT-1 (Nikon, Tokyo, Japan) image analysis software and processed using Adobe Photoshop Image Reader 7.0 software. APB status was determined according to previously defined criteria: the presence of an APB was defined by the localization of a telomeric DNA focus within a nuclear PML body, sections were scored as APB+ if they contained APB in ≥0.5% of tumor cells and a tumor was considered ALT+ when at least one section was APB+. A set of criteria was used to determine the APB status of tumor section. An APB was considered to be present only when the telomeric DNA fluorescence within a PML body was more intense than that of telomeres, and a cell was not considered to contain APB if more than 25% of the co-localized foci occurred outside the nucleus. To avoid false negatives, at least 2,000 tumor nuclei were examined, and the assay was repeated in the presence of negative results.

Telomere length was assessed by pulsed-field gel electrophoresis as previously described [11]. ALT status was determined by calculating whether the mean, variance, and semi-interquartile range of the terminal restriction fragment (TRF) length distribution were greater than 16 kb, 1,000 kb2, and 4 kb, respectively. Tumors were classified as ALT+ when two of three or three of three of these criteria were met for unimodal or bimodal TRF length distributions, respectively. Statistical analysis of TRF length distributions was done with Telometric software [12]. TA was measured by the telomeric repeat amplification protocol (TRAP) [13], with the TRAPeze kit (Intergen, Oxford, UK) as outlined in Costa *et al*. [9].

Data analysis

The agreement between APB and TRF data was assessed by kappa statistics. The clinical end point of the study was cancer-related survival, and the time of its occurrence was computed from the date of first diagnosis to the time of death, or censored at the date of the last recorded follow-up for living patients. Survival curves were estimated by means of the Kaplan-Meier product limit method [14], and the Cox proportional hazards model [15] was used to calculate the hazard ratios (HR) and their confidence interval (CI). SAS software (SAS Institutes, Inc., Cary, NC) was used to perform statistical calculations, and a two-sided *P* value ≤0.05 was considered statistically significant.

Results and discussion

ALT status was determined on each liposarcoma specimen by using both APB detection and telomere length analysis (Fig. 1). Overall, 27 (31.8%) lesions were defined as ALT+ based on the presence of APB,

whereas 24 (27.5%) samples were classified as ALT+ on the basis of TRF length distribution.

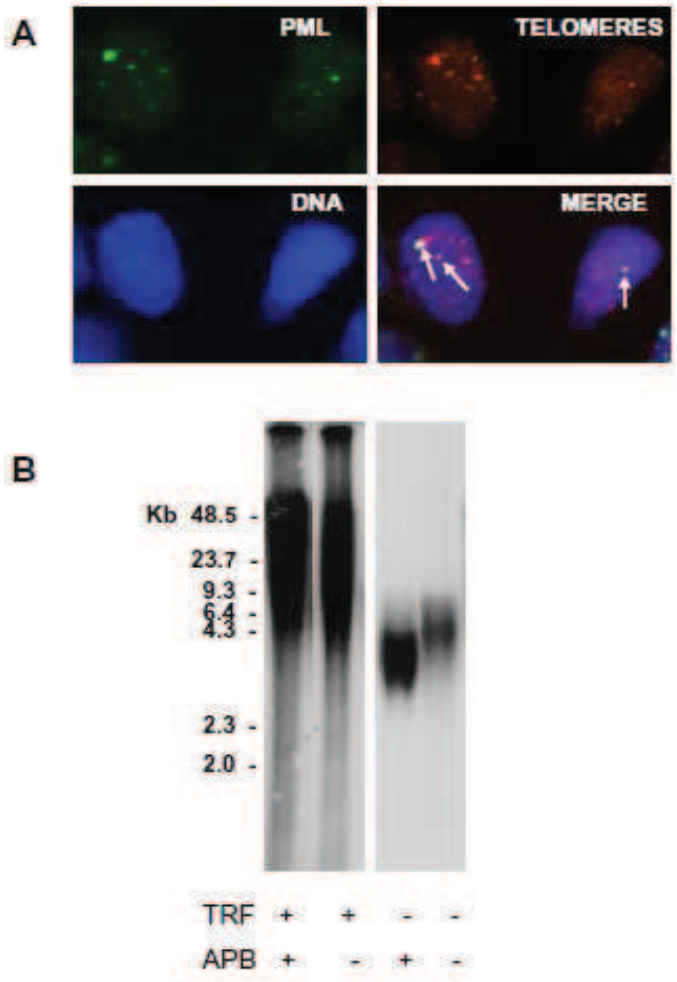
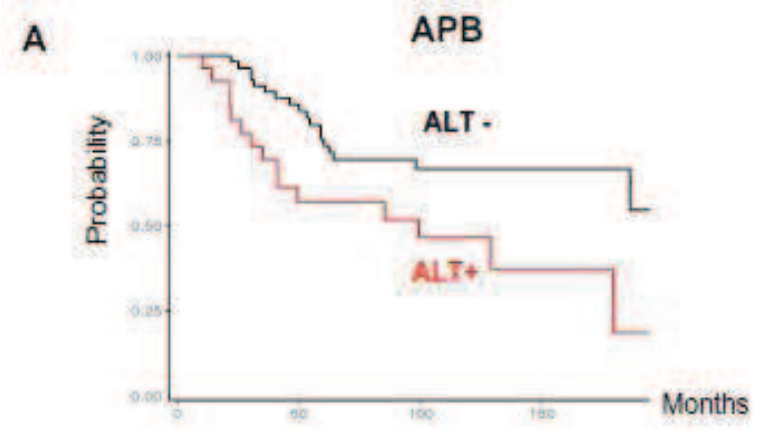


***Figure 1 ALT assays in liposarcomas**. **A)** APB assay: combined PML immunofluorescence and telomere fluorescence in situ hybridization (FISH) in a frozen section of an ABP-positive liposarcoma. Indirect immunofluorescence was used for the PML protein (FITC label, green stain). Telomere FISH was done using a Cy3-conjugated telomeric peptide nucleic acid probe (red stain). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (blue stain). The foci of telomeric DNA that co-localize with PML represent APB. **B)** TRF southern blot analysis. Telomere length distribution of a representative series of liposarcomas. The lengths of telomeres in ALT-positive cells typically range from < 3 to > 50 kb. ALT-negative cells typically have a more homogeneous distribution of telomere length and a shorter average length than ALTpositive cells.*

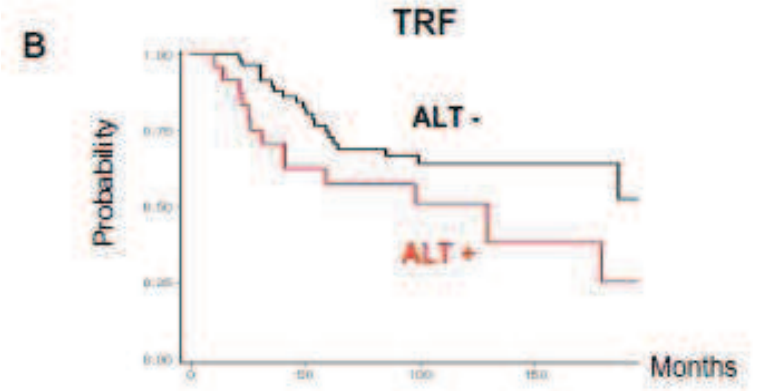
A concordance between APB and TRF results in defining a specimen as ALT+ or ALTwas found in 66 of 85 cases (77.6%; kappa = 0.469; 95% CI, 0.265-0.672; *P* < 0.0001). Specifically, 16 lesions (18.8%) were defined as ALT+ and 50 (58.8%) were scored as ALT- with both detection methods. As regards the remaining lesions, 11 were defined as ALT+ on the basis of APB expression but did not show a TRF length distribution consistent with an ALT phenotype, and 8 were classified as ALT+ on the basis of TRF analysis but showed a very low percentage of APB-expressing tumor cells (from 0.01 to 0.2%).

The incomplete overlapping of the results obtained with the two methods is not surprising. In fact, while the APB assay allows the analysis of individual tumor cells, the TRF pattern could be misleading due to the admixture of normal and tumor cells present in the specimen. However, it has been recently shown by Jeyapalan *et al*. [16] that some telomerase-negative liposarcomas without APB express recombination-like activity at the telomere, suggesting that the incidence of ALT, as defined solely on the basis of APB expression, could be underestimated. Thirty of 85 (35.3%) liposarcoma specimens were classified as TA+ based on positive TRAP results. Among these, 6 and 8 lesions were defined as ALT+/TA+ based on the expression of APB or on TRF length distribution, respectively, thus confirming the possibility that the two TMM coexist in the same lesion as previously reported also for other tumor types [3,17,18]. The prognostic significance of TMM was analyzed on the overall series of 85 patients. TA alone did not prove to be associated with disease-specific mortality (120 months: TA+ versus TA-, 62.0% versus 60.0%; HR, 0.91; 95% CI, 0.43-1.95; *P* = 0.814) (180 months: 62.0% versus 48.5%; HR, 0.80; 95% CI, 0.38-1.70; *P* = 0.566), whereas significant results were obtained for ALT. Specifically, when a

tumor was defined as ALT+ according to at least one method (APB or TRF), ALT proved to be prognostic for 10-year disease-specific survival (ALT+ versus ALT-, 45.5% versus 71.1%; HR, 2.38; 95% CI, 1.15-4.90; *P* = 0.019), and such a prognostic value was maintained and strengthened at 15 years of follow-up (ALT+ versus ALT-, 25.3% versus 71.1%; HR, 2.76; 95% CI, 1.36-5.06; *P* =0.005). These results held true also when APB expression was used as the only parameter to classify tumors for ALT. Specifically, the APB presence proved to be an indicator of increased mortality at both 10 years (HR, 2.14; 95% CI, 1.04-4.41; *P* = 0.040) and 15 years (HR, 2.54; 95% CI, 1.27-5.11; *P* = 0.009) of follow-up (Fig. 2A). Conversely, at 10 years of follow-up, patients with a tumor defined as ALT-positive on the basis of TRF length distribution showed a lower although not statistically significant probability of being alive (HR, 1.77; 95% CI, 0.84-3.73; *P* = 0.130). Such a trend reached statistical significance at 15 years (HR, 2.14; 95% CI, 1.06-4.34; *P* = 0.035) (Fig. 2B). These results held true even after adjustment for TA. In fact, the prognostic significance of APB expression was evident both at 10 (HR, 2.16; 95% CI, 1.03-4.51; *P* = 0.041) and at 15 years (HR, 2.53; 95% CI, 1.24-5.15; *P*= 0.011) of follow up, whereas TRF length distribution provided significant information only at 15 years of follow-up (HR, 2.11; 95% CI, 1.04-4.23; *P* = 0.038).



Patients at risk at:				
month	0	60	120	180
ALT-	58	36	20	12
ALT+	27	12	6	1



Patients at risk at:				
month	0	60	120	180
ALT-	61	37	20	12
ALT+	24	11	6	1

Figure 2 *Probability of disease-specific survival as a function of ALT, detected by APB presence (A) or TRF length distribution (B), according to the criteria reported in Methods, in liposarcoma patients.*

Conclusions

In agreement with previously published results based on APB detection [9], we confirmed ALT as a prognostic discriminant of increased mortality in liposarcomas and provide the first evidence that sensitivity of the ALT predictive power depends, at least in part, on the marker (APB expression versus TRF length distribution) used. Notwithstanding the good agreement observed between the two assays in defining the ALT phenotype, they do not precisely identify the same subset of patients, conversely to that observed in glioblastoma multiforme, where a complete agreement in the results of the two assays was observed [10]. The incomplete overlapping of the TRF and APB results may be due to the different liposarcoma histological subtypes and this heterogeneity may result in a slight difference on the time-dependence of each assay to provide significant prognostic information. Overall, APB may be more appropriate than TRF pattern to assay ALT in tumors because they can be detected in both frozen and formalin-fixed, paraffin-embedded tumor samples - as we recently reported in this tumor type [19] - as well as in needle biopsies or cytology specimens. However, additional studies aimed at comparing the prognostic significance of results obtained with APB and TRF assays in other tumor types are warranted to provide reliable indications on the most appropriate ALT-related marker to be used for prognostic purposes.

Abbreviations

ALT: Alternative lengthening of telomeres; TMM: Telomere maintenance mechanisms; APB: ALT-associated Promyelocitic leukaemia bodies; TRF: Terminal restriction fragment; TA: Telomerase activity; HR: Hazard ratio.

Table S1: patients and tumors characteristics.

Case	Age (years)	Gender	Sample	Histological subtype	Site	Post-treatment	Status	TA	APB	TRF
1	91	M	R	DE	extremities	no	DO	+	-	-
2	35	M	R	MY	extremities	no	AWD	+	-	-
3	64	F	R	PL	extremities	no	DOD	-	+	+
4	55	F	R	PL	extremities	no	DOD	-	+	+
5	38	M	P	RC	extremities	RT	DOD	-	+	+
6	47	F	R	RC	extremities	no	DOD	+	-	-
7	41	M	R	MY	extremities	RT	NED	+	-	-
8	43	F	M	MY	extremities	CTRT	DOD	+	-	-
9	58	F	R	DE	retroperitoneum	no	DOD	-	-	-
10	76	F	P	DE	retroperitoneum	no	DOD	-	-	-
11	49	F	R	DE	retroperitoneum	RT	DOD	-	-	-
12	42	F	R	RC	retroperitoneum	CT	DOD	+	-	+
13	33	F	R	MY	retroperitoneum	RT	DOD	+	-	-
14	73	M	R	DE	retroperitoneum	CT	DOD	-	+	+
15	49	M	M	RC	extremities	no	AWD	+	-	-
16	62	F	R	RC	extremities	no	DOD	+	-	-
17	41	M	M	MY	extremities	no	DOD	+	-	+
18	39	M	R	DE	retroperitoneum	CT	DOD	+	-	-
19	43	M	M	RC	extremities	no	AWD	+	-	+
20	36	M	R	WD	retroperitoneum	no	DOD	-	-	-
21	51	M	R	DE	trunk	no	NED	-	+	+
22	27	M	R	MY	extremities	no	DOD	-	-	+
23	52	M	P	WD	extremities	no	NED	-	-	-
24	46	F	R	WD	extremities	no	NED	-	-	-
25	62	M	P	WD	extremities	no	NED	-	-	-
26	50	M	P	WD	retroperitoneum	no	AWD	-	-	-
27	61	M	R	WD	retroperitoneum	CT	DOD	-	-	-
28	41	M	R	DE	retroperitoneum	no	AWD	-	-	-
29	81	M	R	PL	extremities	no	DOD	-	+	-
30	57	M	R	DE	retroperitoneum	CT+RT	DOD	-	-	-
31	65	M	R	WD	extremities	RT	NED	-	-	-
32	64	F	R	MY	extremities	no	DO	-	-	-
33	57	M	R	PL	retroperitoneum	no	DOD	-	+	-
34	68	M	R	WD	retroperitoneum	no	AWD	-	+	+
35	37	F	R	WD	trunk	no	NED	-	-	-
36	68	M	P	WD	retroperitoneum	no	NED	-	-	-
37	60	F	R	DE	extremities	RT	NED	-	-	-
38	47	F	R	WD	retroperitoneum	no	AWD	-	-	-
39	53	F	R	DE	retroperitoneum	no	DOD	+	+	+
40	40	F	R	MY	extremities	RT	NED	-	-	-
41	23	F	R	WD	extremities	no	NED	-	-	-
42	53	M	R	DE	retroperitoneum	CT	DOD	-	-	-
43	65	F	R	WD	extremities	no	NED	-	+	+
44	68	M	R	WD	retroperitoneum	no	NED	-	-	-
45	59	M	R	WD	retroperitoneum	no	DOD	+	-	-
46	47	M	P	RC	extremities	RT	NED	+	-	-
47	72	F	R	DE	extremities	no	DOD	-	+	+
48	59	F	P	DE	retroperitoneum	CT	DOD	-	+	-
49	43	M	R	RC	extremities	CT+RT	AWD	+	-	-
50	80	M	R	DE	extremities	RT	DOD	-	+	+
51	62	F	R	DE	retroperitoneum	no	DOD	+	+	-
52	79	M	P	WD	extremities	no	NED	-	-	-
53	50	F	R	DE	retroperitoneum	CT	DOD	+	-	-
54	63	M	R	WD	retroperitoneum	no	DO	-	-	-
55	53	F	R	MY	extremities	no	NED	+	-	+
56	55	M	R	RC	extremities	no	NED	+	-	-
57	56	F	R	DE	extremities	no	DOD	-	+	+
58	42	F	P	RC	extremities	RT	NED	-	-	-
59	56	M	R	DE	extremities	no	DOD	-	+	+
60	52	M	R	PM	trunk	no	NED	-	+	-
61	53	M	R	MY	extremities	no	NED	-	+	+
62	57	F	R	WD	extremities	no	NED	-	-	-
63	53	M	R	DE	retroperitoneum	no	DOD	-	+	+
64	34	F	P	MY	extremities	RT	NED	-	-	-
65	42	M	R	WD	extremities	no	NED	-	-	-
66	55	M	P	MY	extremities	no	NED	-	-	-
67	62	M	R	WD	extremities	no	NED	-	-	-
68	71	M	P	MY	trunk	RT	DO	-	+	+
69	61	F	R	RC	extremities	RT	DOD	+	-	-
70	54	F	P	RC	extremities	no	NED	+	+	-
71	45	F	R	DE	retroperitoneum	no	DO	-	-	-
72	51	F	P	MY	extremities	no	DOD	+	-	+
73	45	M	P	RC	extremities	no	NED	+	-	+
74	44	F	R	RC	extremities	CT+RT	DOD	-	+	-
75	47	M	R	RC	extremities	RT	NED	+	+	-
76	31	M	P	RC	extremities	RT	NED	+	-	-
77	62	M	R	RC	extremities	no	AWD	+	+	-
78	49	M	P	RC	trunk	CT+RT	NED	+	+	+
79	30	F	P	RC	extremities	RT	NED	-	-	+
80	33	M	R	MY	extremities	no	DOD	-	+	-
81	32	F	P	MY	extremities	RT	NED	-	+	-
82	18	M	P	MY	extremities	no	NED	-	-	-
83	41	M	P	RC	extremities	CT	DOD	-	-	-
84	42	F	R	MY	extremities	RT	NED	+	-	-
85	34	M	R	MY	extremities	no	AWD	+	-	-

M= male, F= female, P= primary, R= local recurrence, M= metastasis, WD= well-differentiated, DE= dedifferentiated, MY= myxoid, RC= round-cell, PL= pleomorphic, CT= chemotherapy, RT= radiotherapy, DOD= dead of disease, DO= dead of other causes, AWD= alive with disease, NED= not evidence of disease, TA= telomerase activity, APB= ALT-associated promyelocytic leukaemia bodies, TRF= terminal restriction fragments.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LV carried out the molecular studies (APB detection) and drafted the manuscript. RM carried out the molecular studies (TRF analysis). AG provided tumour material and follow-up data MGD participated in the design of the study, performed the statistical analysis and helped to draft the manuscript. NZ conceived the study, partecipated in its coordination and helped to draft the manuscript. All the authors read and approved the final manuscript.

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References

1. Hanahan D, Weinberg RA: The hallmarks of cancer**.** *Cell* 2000, 100:57-70.
2. Shay JW, Zou Y, Hiyama E, Wright WE, *et al.*: Telomerase and cancer**.** *Hum Mol Genet* 2001, 10:677-85.
3. Bryan TM, Englezou A, Dalla-Pozza L, Dunham MA, Reddel RR, *et al.*: Evidence for an alternative mechanism for maintaining telomere length in human tumours and tumor-derived cell lines**.** *Nat Med* 1997, 3:1271-4.
4. Dunham MA, Neumann AA, Fasching CL, Reddel RR: Telomere maintenance by recombination in human cells**.** *Nat Genet* 2000, 26:447-50.
5. Yeager TR, Neumann AA, Englezou A, Huschtscha LI, Noble JR, Reddel RR: telomerase-negative immortalized human cells contain a novel type of promyelocytic leukemia (PML) body**.** *Cancer Res* 1999, 59:4175-9.
6. Ulaner GA, Huang HY, Otero J, Zhao Z, Ben-Porat L, Satagopan JM, Gorlick R, Meyers P, Healey JH, Huvos AG, Hoffman AR, Ladanyi M: Absence of a telomere maintenance mechanism as a favorable prognostic factor in patients with osteosarcoma**.** *Cancer Res* 2003, 63:1759-63.
7. Montgomery E, Argani P, Hicks JL, Hicks JL, DeMarzo AM, Meeker AK, *et al.*: Telomere lengths of traslocation-associated and non translocation associated sarcomas differ dramatically**.** *Am J Pathol* 2004, 164:1523-9.
8. Hakin-Smith V, Jellinek DA, Levy D, Carroll T, Teo M, Timperley WR, McKay MJ, Reddel RR, Royds JA: Alternative lengthnening of telomeres and survival in patients with glioblastoma multiforme**.** *Lancet* 2003, 361:836-8.
9. Costa A, Daidone MG, Daprai L, Villa R, Cantù S, Pilotti S, Mariani L, Gronchi A, Henson JD, Reddel RR, Zaffaroni N: Telomere maintenance mechanisms in liposarcomas: association with histologic subtypes and disease progression**.** *Cancer Res* 2006, 1;66(17):8918-24.
10. Henson JD, Hannay JA, McCarthy SW, Royds JA, Yeager TR, Robinson RA, Wharton SB, Jellinek DA, Arbuckle SM, Yoo J, Robinson BG, Learoyd DL, Stalley PD, Bonar SF, Yu D, Pollock RE, Reddel RR: A robust assay for alternative lengthening of telomeres in sarcomas and astrocytomas**.** *Clin Cancer Res* 2005, 11:217-25.
11. Villa R, Folini M, Perego P, Supino R, Setti E, Daidone MG, Zunino F, Zaffaroni N: Telomerase activity and telomere length in human ovarian cancer and melanoma cell lines: correlation with sensitivity to DNA damaging agents**.** *Int J Oncol* 2000, 16:995-1002.
12. Grant JD, Broccoli D, Muquit M, Manion FJ, Tisdall J, Ochs MF: Telometric: a tool providing simplified, reproducible measurements of telomeric DNA from constant field agarose gels**.** *Biotechniques* 2001, 31:1314-16.
13. Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL, Shay JW: Specific association of human telomerase activity with immortal cells and cancer**.** *Science* 1994, 66:2011-15.
14. Kaplan EL, Meier P: Nonparametric estimation from incomplete observations**.** *J Am Stat Assoc* 1958, 53:457-81.
15. Cox DR: Regression models and life tables**.** *J Stat Soc B* 1972, 34:187-220.
16. Jeyapalan JN, Mendez-Bermudez A, Zaffaroni N, Dubrova YE, Royle NJ: Evidence for alternative lengthening of telomeres in liposarcomas in the absence of ALT-associated PML bodies**.** *Int J Cancer* 2008, 1;122(11):2414-21.

17. Johnson JE, Varkonyi RJ, Schwalm J, Cragle R, Klein-zanto A, Patchefsky A, Cukierman E, von Mehren M, Broccoli D: Multiple mechanisms of telomere maintenance exist in liposarcomas**.** *Clin Cancer Res* 2005, 11(15):5347-55.
18. Schneider-Stock R, Epplen JT, Walter H, Radig K, Rys J, Epplen C, Hoang-Vu C, Niezabitowski A, Roessner A: Telomeric lengths and telomerase activity in liposarcomas**.** *Mol Carcinog* 1999, 24(2):144-51.
19. Venturini L, Erdas R, Costa A, Gronchi A, Pilotti S, Zaffaroni N, Daidone MG: ALT-associated promyelocytic leukaemia body (APB) detection as a reproducible tool to assess alternative lengthening of telomere stability in liposarcomas**.** *J Pathol* 2008, 214:410-414.

Telomeres as targets for anticancer therapies

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Introduction: The limitless replicative potential of cancer cells relies on telomere integrity (which is guaranteed by a complex interaction between several specialized proteins and telomeric DNA) and the activation of specific mechanisms for telomere length maintenance. Two mechanisms are currently known in human cancer, namely telomerase activity and the alternative lengthening of telomere pathway.

Expert opinion: In this review, we summarize the available data concerning the therapeutic strategies proposed thus far and the current challenges posed for the development of innovative telomere-based therapeutic approaches with broad-spectrum anticancer activity and for their translation into the clinical setting.

Areas covered: Due to their essential role in tumor cell proliferation, telomere maintenance mechanisms have become extremely attractive targets for the development of new anticancer interventions. Although numerous efforts have been made to identify specific approaches to interfere with telomere maintenance mechanisms in human cancers, the only molecule currently tested in clinical trials is the oligonucleotide GRN163L. However, a growing body of evidence suggests that interfering with telomeres, through the direct targeting of telomeric G-quadruplex structures, may be a valuable antitumor therapeutic strategy, independent of the specific telomere maintenance mechanism operating in the tumor.

Keywords: ALT mechanisms, bisantrene, G4-ligands, G-quadruplex, naphthalene diimide, telomerase, telomeres

Article highlights.

- Telomeres are specialized DNA--protein structures located at the end of eukaryotic chromosomes. Their functions are crucial to guarantee genome stability (by protecting chromosomes from degradation, end-to-end chromosomes fusion, and unsuitable recombination) and to limit cell proliferation (mitotic clock).
- A hallmark of cancer cells is their limitless proliferative potential, which is at least in part sustained by the activation of a telomere maintenance mechanism (TMM).
- Two TMMs are currently known in human cancer, namely telomerase activity and the alternative lengthening of telomeres (ALT) pathway. Due to their essential role in tumor cell proliferation, such mechanisms have become extremely attractive targets for the development of new anticancer interventions.
- Due to its selective reactivation in many human tumors, the possibility to interfere with telomerase expression or functions represents a useful strategy for the development of specific anticancer therapies.
- Data obtained from preclinical studies concerning the effects of telomerase inhibition have provided compelling arguments to indicate that the enzyme is a well-validated cancer target. However, the only telomerase inhibitor currently tested in clinical trials is the oligonucleotide GRN163L (Imetelstat).
- Interfering with telomeres, through the direct targeting of telomeric G-quadruplex structures (G4), could be a valuable and innovative antitumor therapeutic approach due to the possibility to interfere with unlimited cell proliferation independently of the specific TMM operating in the tumor (telomerase versus ALT). Several molecules able to stabilize telomeric G4 structures has been identified thus far.
- At the preclinical level, it was shown that telomeric G4 ligands efficiently interfered with the growth of a variety of tumor models. In addition, they have shown synergistic activity in vivo when combined with conventional anticancer drugs. Despite such well-documented antitumor and chemosensitizing effects in preclinical models, none of the available telomeric G4 ligands has entered clinical trials.
- The development of suitable methodological approaches to precisely define G4 structures and to dissect their role in normal versus tumor cells may provide important clues for the synthesis of novel and more specific G4 ligands for the development of broad spectrum, telomere-based anticancer therapeutic strategies.

1. Introduction

1.1 The telomere

Telomeres are specialized DNA--protein structures located at the ends of eukaryotic chromosomes. Their functions are crucial to protect the genome from degradation, end-to-end chromosome fusion and unsuitable recombination [1,2]. Human telomeric DNA consists of tandem repeated (TTAGGG)n sequences, on the average of about 10 kb, with a single-stranded terminus on the 3'-oriented strand (3'-overhang) [1]. In normal somatic cells, telomeres shorten with each round of cell division as a natural consequence of the inability of the DNA polymerase to completely replicate the chromosome ends. Therefore, telomere erosion imposes in normal cells a finite number of cell divisions, thus representing a cell-autonomous mechanism to prevent excessive telomere shortening and, as a consequence, genomic instability and malignant transformation.

Another problem associated with the ends of linear chromosomes is that they must be distinguished from chromosome breaks to prevent them from undergoing improper repair processes. In this context, several proteins are involved in DNA binding, which allows telomeric DNA to fold back on itself, resulting in the formation of a telomere loop (t-loop) [3] in which the singlestrand tail can invade duplex telomeric DNA. In humans, telomeric DNA is associated with the shelterin protein complex [3], which includes telomeric-repeat binding factor 1 (TRF1) and 2 (TRF2), which bind to double-stranded DNA [3], and protection of telomeres 1 (POT1), that binds directly to single-stranded DNA [3]. These proteins directly or indirectly interact with other components of shelterin, such as TRF1-interacting protein 2 (TIN2), POT1- and TIN2-organizing protein, and the

transcriptional repressor/activator protein RAP1 [3]. The contribution of RAP1 in the shelterin complex and telomere stabilization is just now emerging [4], and recent evidence suggests a role in repressing non-homologous end joining through interaction with TRF2 [5].

In addition to the shelterin complex, mammalian telomeres interact with other proteins, including factors involved in DNA replication, repair and recombination [3,6]. The unique architecture, high GC content, and multi-protein association create particularly stable protein--DNA complexes that are a challenge for telomere replication and recombination [7]. As a consequence, diverse mechanisms have evolved to accommodate these problems at the telomeric level, including i) the presence of shelterin protein components that facilitate fork progression, control 3'-overhang formation and cap the telomere after replication [6] and ii) the activation of DNAdamage checkpoints (relying on ataxia-telangectasia-mutated (ATM) or ATM and Rad3-related signaling pathways) that help to overcome replication constraints by activating repair mechanisms upon telomere replication and uncapping [3,6].

In addition, members of four helicase families -- RecQ, Pif1, FANCJ, and DNA2 -- are known to have roles in telomere biology [7-9]. Specifically, DNA helicases of the RecQ family act on atypical DNA structures, such as intermediates of homologous recombination, stalled sites of replication forks and the G-quartet structure of telomere DNA (discussed in detail below). Furthermore, mutations or perturbation of telomere activities of RecQ-like helicases (i.e., BLM, Bloom's syndrome; WRN, Werner's syndrome; RECQL4, Rothmund-Thomson's syndrome), as well as FANCJ (Fanconi's anemia) helicase can lead to telomere dysfunction and genome instability and cause inherited human diseases

characterized by increased cancer susceptibility and premature aging [7,8]. In addition, BLM and WRN interact with the telomere specific proteins POT1, TRF1 and TRF2 and their helicase activity is modulated by these interactions [8,9]. Recent findings also indicate that the heterotrimer CST (Cdc13-Stn1-Ten1), found in budding yeast, is present in mammal cells, where it seems to contribute to telomere capping [10]. In addition, Snm1B/Apollo, a member of the SNM1/PSO2 family of nucleases, has been reported to be implicated in telomere protection and replication via its interaction with TRF2 [11]. Therefore, proper telomere protection might result from the cooperation between shelterin and CST, and a new model is now emerging in which TRF2 and Apollo facilitate the replication of telomeres by relieving topological constraints from telomeric DNA [10,11]. Altogether, these telomere-associated proteins cooperate to determine telomere replication, protection and stability, thus contributing to the integrity and dynamics of chromosome ends.

1.2 Telomere maintenance mechanisms in cancer

A hallmark of cancer cells is their limitless proliferative potential, which is at least in part sustained by the activation of a telomere maintenance mechanism (TMM). Telomere maintenance is essential for the transformation of most human cancer cells, and the activation of a TMM is crucial to escape a crisis and allow cells to become immortalized.

In a high percentage of human tumors (> 85%), proliferation-dependent telomere shortening is counterbalanced by the synthesis of telomeric sequences by telomerase [12]. The enzyme is an RNA-dependent DNA polymerase that adds multiple copies of the TTAGGG motif to the end of the telomeric G-strand. The core enzyme consists of the RNA

component human telomerase RNA (hTR, which provides the template for the synthesis of telomeric DNA) and the reverse transcriptase catalytic subunit human telomerase reverse transcriptase (hTERT) [13]. Although hTR and hTERT are sufficient to obtain telomerase activity in vitro, additional protein components such as dyskerin and telomerase Cajal body protein 1 are required for the correct assembly and trafficking of the enzyme in human cells [12,14]. In addition to its role in maintaining chromosome ends through the regulation of telomere length, telomerase has been recently implicated in providing growth-promoting properties to tumor cells, independently of its catalytic activity [15]. Whereas hTR has been found to be ubiquitously expressed in all human tissues, the catalytic component of telomerase hTERT has been proposed as a cancer-specific target, since it is almost exclusively expressed in tumor compared with normal tissues [12]. However, there are human tumors (~15%) lacking telomerase that maintain telomere length by a pathway known as the alternative lengthening of telomeres (ALT) mechanism [16]. Characteristics of ALT cells include an extremely heterogeneous telomere length distribution, ranging from very short to more than 50 kb, and the presence of subnuclear structures termed ALT--associated promyelocytic leukemia (PML) bodies (APB) [16]. PML bodies are normal nuclear domains of unknown function that contain the PML protein. In ALT cells, a subset of the PML bodies contains telomeric chromatin (telomeric DNA and the telomere binding proteins TRF-1 and TRF-2) [16]. Another specific feature of ALT mechanisms is C-circles (self-priming circular telomeric DNA), which may represent molecular intermediates of the ALT mechanisms and whose specificity for assessing ALT activity in biological samples has recently been demonstrated [17]. The prevalence of the ALT phenotype in human

cancers has not yet been clearly determined, since the currently available assays for ALT are complicated for screening a large number of clinical samples. In fact, whereas telomerase positive (TA+) tumors can be identified unequivocally by detecting the enzyme's activity, the identification of ALTpositive (ALT+) cancers has been thus far based on telomere restriction fragment analysis and APB detection, two methods that display a suboptimal rate of concordance. The lack of standardized methods for ALT determination has major implications for the classification of ALT+ tumors. In this context, instability at the minisatellite MS32, usually associated with ALT activation in human cancer cell lines [18], and the presence of complex telomere mutations have been described in liposarcomas, which have recombination-like activity at telomeres in the absence of APBs [19], suggesting that the incidence of ALT may be underestimated using conventional assays. Concerning newer detection methods, the C-circle assay has proven to be a sensitive and specific test for ALT [17,20], even though its reliability on a large set of clinical specimens has not yet been confirmed.

On the basis of available data, ALT mechanisms seem to be frequently present in tumors of mesenchymal and neuroepithelial origin [16,20], as well as in tumors of mixed origin, such as Wilms' tumors (L Venturini, pers. commun.), with respect to carcinomas [20]. Such evidence suggests that cell type- specific mechanisms can favor the activation of the ALT versus telomerase, and vice versa, during tumorigenesis and may reflect a tighter regulation of telomerase in normal mesenchymal and neuroepithelial than in epithelial cells [20]. At present, scanty information is available regarding the molecular underpinnings leading to the establishment of a specific TMM in tumors. The mechanisms by which telomerase activity is regulated in human tumors have been

extensively investigated. Evidence suggests that the altered expression of specific oncogenes and tumor suppressor genes is related to reactivation of the enzyme [21,22]. In addition, multiple steps for regulation of the enzyme have been unveiled. In particular, telomerase expression is strictly controlled at the transcriptional and post-transcriptional/translational level, and an association between the overexpression of hTERT and the deregulated expression of a specific microRNA (miR-138) has been recently demonstrated in human anaplastic thyroid carcinoma cell lines [23]. As regards the ALT phenotype, although it is thought to be driven by a recombination--based mechanism, factors that can act as the main engine of the ALT pathway have not yet been identified. However, in the attempt to unveil candidate ALT genes, it has been shown that the telomere associated proteins (i.e., TRF1, TRF2, TIN2, RAP1) and the MRE11/RAD50/NBS1 complex [24,25] are essential for APB formation, indicating that they are probably required to sustain the ALT mechanism. A number of studies [8,9] also suggested that the BLM and WRN RecQ-like helicases play important roles in recombination-mediated mechanisms of telomere elongation in ALT cells. It has also been reported that epigenetic modifications could influence which TMM is activated in different cancer cells. Specific epigenetic alterations within the chromatin environment of the hTR and hTERT promoters have been reported to correlate with the expression of hTR/hTERT in normal, TA+ and ALT+ cells [26].

An interesting aspect related to TMM and tumor biology is whether telomerase and ALT confer different properties to tumor cells in vivo. It has been demonstrated that hTERT may affect cellular functions other than telomere length [15], and although telomerase and ALT appear to

be equivalent in their ability to support immortalization, their contribution to tumor growth and survival may differ.

Several studies have been carried out to investigate the prognostic implications of TMM in clinical tumors. Reported data indicate that the association between prognosis and telomerase expression is not so straightforward in human tumors [27]. Whereas some studies reported that increased telomerase activity/expression is related to a poor prognosis in different tumor types [27], such an association was not confirmed by other studies, probably as a consequence of different assay conditions, data analysis and patient selection, thus making it difficult to draw a general conclusion on the role of telomerase in cancer prognosis. However, the prognostic significance of ALT mechanisms seems to be strictly dependent on tumor type. Specifically, it has been demonstrated that patients with ALT+, high-grade glioblastoma multiforme (GBM) have significantly longer survival than those with ALT-negative tumors [28]. In contrast, a poor prognosis characterizes patients with ALT+ (and TA+) osteosarcomas compared with patients with tumors lacking any known TMM [29]. In addition, it has been reported that ALT is a strong determinant of an unfavorable outcome in liposarcoma patients [30], whereas it failed to significantly affect the clinical outcome of patients with diffuse malignant peritoneal mesothelioma [31]. Differences in TMM-specific genetic alterations might explain the differences observed in the prognosis of patients with different tumor types. In this context, it has been reported that ALT+ liposarcomas show significantly higher levels of genome instability and loss of heterozygosity than their TA+ counterparts [32]. Furthermore, whole-genome profiling has revealed genetic alterations that further distinguish ALT+ from TA+ liposarcoma lesions [32].

2. Targeting telomeres

2.1 Telomerase inhibitors

Telomerase attracted attention after its discoverers E.H. Balckburn, C. Greider and J. Szostak won the 2009 Nobel Prize in Physiology and Medicine. Since its discovery in the early 1980s, telomerase has been considered a potential drug target [33]. Due to its selective reactivation in most human tumors, the possibility to interfere with telomerase expression and/or functions has been considered a useful strategy for the development of effective anticancer therapies [12,22,34]. Several approaches (Figure 1) have been pursued with the aim of interfering i) with the enzyme's catalytic activity. A variety of nucleoside analogs and non-nucleoside compounds have been exploited to inhibit telomerase activity [12]. Among them, BIBR1532, a non-competitive inhibitor that binds to a site in the enzyme which is distinct from those for deoxyribonucleotides and the DNA primer, is one of the most potent small molecule inhibitors of telomerase discovered thus far [35]; ii) with the expression of the enzyme's core subunits hTR and hTERT by antisense-based approaches using chemically modified oligonucleotides (e.g., phosphorothioates, 2'-O-modified RNA oligomers, 2',5'-oligoadenylates, N3'→P5' phosphoramidates and peptide nucleic acids), catalytic RNAs, such as ribozymes, and small interfering RNAs [12]; iii) with signaling pathways responsible for post-transcriptional/translational modifications of telomerase (e.g., small molecules) [12,34]. At present, the most promising telomerase 'drug candidate is GRN163L or imetelstat, a lipid palmitate derivative of the N3'→P5' thiophosphoramidate oligomer GRN163 targeting hTR [36]. GRN163L does not exhibit antisense activity, but rather acts as an antagonist of

telomerase by binding with high affinity and specificity to the template region of hTR [36]. Its effects have been thoroughly investigated in many preclinical studies, both in cell cultures and xenografted tumors. Recently, GRN163L has been shown to efficiently inhibit telomerase activity in prostate-tumor-initiating cells [37] as well as in GBM tumor-initiating cells, where it increases the therapeutic efficacy of radiation and temozolomide, two common therapeutic approaches for GBM patients [38]. It was demonstrated in an orthotopic GBM mouse model [38] that imetelstat efficiently penetrates the blood brain barrier and inhibits telomerase activity. In addition, it was reported to prevent lung metastases in vivo [39], probably as a consequence of its hTR-independent anti-adhesive properties [40]. Thus far, imetelstat has been evaluated in seven Phase I and Phase I/II clinical trials for several cancers, including solid and hematological tumors, either as a single agent or in combination regimens [41]. The primary objectives of such studies are safety, tolerability and determination of the maximum tolerated dose. Preliminary data have shown imetelstat to be well tolerated [42], with a toxicity profile consistent with that reported for other oligonucleotides [36].

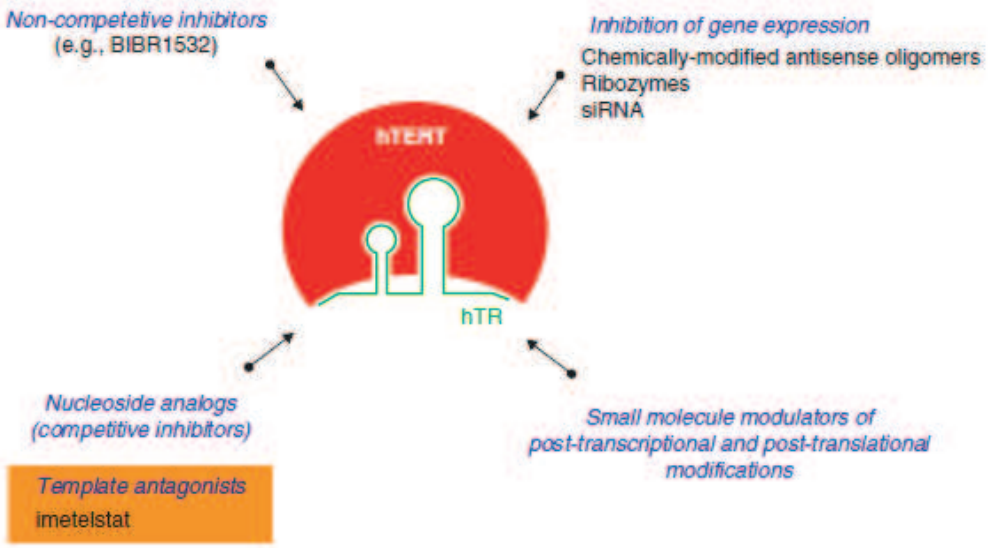

Figure 1. *Telomerase inhibitors. Schematic representation of the most widely exploited strategies to interfere with the expression and function of human telomerase in cancer cells (see text). Colored box: imetelstat (or GRN163L) is the only telomerase inhibitor currently employed in clinical trials. hTERT: Human telomerase reverse transcriptase; hTR: human telomerase RNA.*

Available data indicating that interference with telomerase expression and/or function leads to increased sensitivity of cancer cells to conventional anticancer drugs and radiation [12,22,34] corroborate the notion that telomerase represents a promising anticancer target also for the design of new combination therapies. In this context, combination studies with imetelstat plus paclitaxel and bevacizumab, imetelstat plus paclitaxel and carboplatin, or imetelstat plus bortezomib ± dexamethasone have been activated in breast cancer, NSCLC and multiple myeloma, respectively [41]. Although clinical trials have been activated, there are no available data yet on the efficacy of imetelstat in cancer patients [36].

2.2 Interference with telomere-associated proteins

Studies on shelterin components have contributed knowledge about their specific role in the regulation of telomere structure and dynamics. Specifically, most of the data gathered thus far have shown that the shelterin complex acts as a physical cap to protect the ends of chromosomes from being recognized as natural double-strand breaks, thus distinguishing normal ends from double-strand breaks and avoiding the activation of DNA damage checkpoints [3]. The notion that the deprotection of telomeres results in the activation of a DNA-damage response (DDR) gained support from the evidence that DDR factors, such as tumor protein p53 binding protein 1 (53BP1), histone gH2AX, meiotic recombination 11 (Mre11) and phospho-ataxia telangectasia mutated (ATM), can localize at uncapped telomeres and form cytological structures referred to as telomere dysfunction-induced foci [3]. The main protective role has been assigned to TRF2. The inhibition of TRF2 through the overexpression of a dominant-negative mutant of the protein (TRF2DBDM), able to heterodimerize with the endogenous TRF2 and to block its binding to telomeric DNA [3], resulted in a high frequency of chromosome end-to-end fusions as a consequence of 3¢--overhang loss, the activation of DNA ligase IV-dependent non-homologous end joining, and the ATM/p53-mediated DDR pathway, ultimately leading to cell death in several in vitro models [3]. In addition, mouse models knocked-out for TRF2 showed embryonic lethality, and cells derived from TRF2- and p53-null embryos were characterized by loss of telomere protection and massive increase in end-to-end fusions [43]. Overall, these findings indicate TRF2 to be an early component of the DDR system, and the assessment of its delocalization from telomeres by chromatin immunoprecipitation or combined immunofuorescence has

become a useful approach to check early events related to the induction of telomeric DNA damage. Furthermore, a possible role of TRF2 in mediating drug resistance of cancer cells can be envisaged based on the observation that RNAi-mediated inhibition of TRF2 expression partially reversed the phenotype of multidrug-resistant variant SGC7901 gastric cancer cells [44]. The TRF1 homodimer within the shelterin complex binds double-stranded telomeric repeats, helping to localize shelterin to telomeres [3]. It has been demonstrated that long-term overexpression of the protein in the telomerase positive HT1080 cell line resulted in a gradual and progressive telomere shortening, whereas telomere elongation was induced by expression of a dominant-negative TRF1 mutant that inhibited binding of endogenous TRF1 to telomeres [45]. As TRF1 did not affect the expression of telomerase, it has been suggested that the binding of TRF1 controls telomere length in cis by inhibiting the action of telomerase at the ends of individual telomeres [45]. Thus, it is critical that the levels of TRF1 are properly controlled. Two major pathways regulate TRF1 localization and abundance. TRF1 is poly--ADP--ribosylated by tankyrase 1 leading to TRF1 release from telomeres and subsequent ubiquitination by stem cell factor SCFFbx4, and hence degradation by the proteasome [45,46]. In addition, TIN2 suppresses TRF1 polyubiquitination by sequestering its degradation motif from recognition by SCFFbx4 when both are assembled in the shelterin complex [46]. The dynamic regulation of TRF1 by tankyrase 1, SCFFbx4 and TIN2 appears to play an important role in telomere homeostasis maintenance. As a consequence, interference with any of these mechanisms may represent a useful approach to affect cancer cell growth. In this context, it has been demonstrated that the longterm inhibition of tankyrase 1 poly-ADP-ribosylation activity enhanced

progressive telomere shortening induced by a telomerase inhibitor and hastened cell death in human cancer cells [46]. Furthermore, given the specificity of the TRF1--Fbx4 binding interface, it is possible to envision the development of selective small molecule inhibitors that specifically perturb interaction between SCFFbx4 and TRF1 [46].

Dysfunctional telomeres can also be induced by interference with the expression of other components of shelterin. For instance, the role of hPOT1 in protecting telomeres has been evaluated in human cells exposed to anti-hPOT1 siRNAs. Specifically, hPOT1 knockdown resulted in the induction of apoptosis as a consequence of telomere dysfunction, increased expression of p53 and B-cell leukaemia/ lymphoma 2 associated X protein (Bax), and concomitant decrease of B-cell leukaemia/lymphoma (Bcl-2) levels in breast cancer cells [47]. A marked increase in the levels of telomere-induced foci was also observed in HeLa cells following hPOT1 knockdown [48]. In addition, experimental findings showing that deletion of the pot1 gene in mice resulted in chromosomal instability, and increased telomere recombination further suggested a role of POT1 in carcinogenesis [43].

An alternative approach currently under investigation to target telomeres in cancer cells involves the expression of mutated hTR with an altered template sequence [49-52]. These mutant templates complex with hTERT in tumor cells and direct the addition of mutant telomeric repeats, which are predicted to disrupt binding of shelterin components [52]. Treatment with such mutant templates rapidly induces DNA damage at telomeres and results in cell growth inhibition and apoptosis induction in hTERT-expressing cancer cells, independently of p53 and pRb status [50,51]. Recently, ATM has been reported to be a key mediator of the dysfunctional telomere response mediated by MT-hTer-47A, an hTR

mutant template that directs the addition of TTTGGG repeats instead of wild-type human telomeric repeats [52]. In this context, ATM-depleted melanoma and bladder cancer cells showed increased clonogenic cell survival and a reduced rate of cell death following MT-hTer-47A treatment compared with ATM-competent controls [52].

2.3 Telomeric G-quadruplex ligands

Under physiological ionic conditions, G-rich sequences within nucleic acids are capable of generating a four-stranded helical structure, known as G-quadruplex (G4), which forms when four molecules of guanylic acid are present in a square planar arrangement [53]. The structures exhibit extensive structural polymorphism and may form intramolecular or intermolecular DNA strands [53]. G-quadruplex structures can be classified depending on the orientation of the DNA strands in parallel, antiparallel or a hybrid structure [53]. Interest in these structures has recently increased following the observation of their in vivo existence in oncogene promoters and telomeres [54,55], leading to the notion that G4 may play key roles in biological processes. Indeed, a bioinformatic analysis has identified 375,000 candidate sequences within the human genome that could form G4 structures [53]. The latter are frequently found in proto-oncogenes, such as c-MYC, VEGF, c-kit, hypoxia-inducible factor (HIF)-1a and Bcl-2, rather than in tumor suppressor genes [53]. It has been demonstrated that G4-forming regions within the c-MYC promoter play a critical role in regulating expression of the gene. A single point mutation (which destabilizes the G4) resulted in a threefold increase in c-MYC basal transcriptional activity, whereas a G4-stabilizing cationic porphyrin was able to suppress c-MYC transcriptional activation [53]. It has been recently reported that the hTERT core

promoter also contains a G-rich sequence [56]. Such consecutive G-tracts have the potential to form multiple G4, which in turn may mask specific protein-1 (Sp1) binding sites, thus resulting in inhibition of hTERT promoter activity [56]. Telomeric DNA has received much attention in this regard, as single-stranded G-rich telomeric 3'-overhang can fold into several different intramolecular quadruplex structures that differ for the position of the adjacent loop regions. However, it is becoming clear that single-strandedness is not a prerequisite for G4 formation. Transient destabilization of duplex DNA during transcription, replication, or DNA repair may be sufficient to allow G4 formation at many sites in the genome [53]. At the telomere level, intermolecular G4 could play different roles in telomere--telomere associations, in the alignment of sister chromatids during meiosis (i.e., telomere meiotic bouquet), and in homologous chromosome pairing during meiosis [53]. In addition, telomeric G4 may cap telomeres, thus protecting them from inappropriate elongation by telomerase or from nucleolytic degradation and end-to-end fusion events. In addition, G4 may play an important role in inhibition of the ALT pathway. As the 3'-overhang is essential during the early steps of recombination, sequestration of this single stranded region at the ends of chromosomes into G4 could form an effective barrier against ALT [53]. There is evidence that G4 formation may be relevant also at the RNA level. Specifically, it has been reported that alternative splicing of a number of genes is affected by G4-forming sequences in pre-mRNA [57]. In this context, the G4-inducing drug 12459, initially believed to cause telomerase inhibition, telomere shortening and apoptosis in cancer cells as a consequence of its binding to telomeric DNA, was indeed revealed to mediate the stabilization of G4 in the pre-mRNA of hTERT, causing a shift in the hTERT splicing pattern

toward the production of an inactive form of hTERT [57]. In addition, a G-rich RNA sequence located in the 5'-untraslated region of TRF2 mRNA has been recently reported to be capable of forming a stable G4, substantiating a potential translational mechanism for the downregulation of TRF2 [58]. Another potentially G4-forming RNA is the recently identified G-rich telomeric repeat-containing RNA (TERRA), which is transcribed from promoters located within subtelomeric regions and whose function in human cells still needs to be properly understood [59]. It has been reported that G4 structures formed by TERRA localize at the ends of chromosomes, suggesting a possible association between TERRA and telomeric DNA [59]. In this context, an extensive investigation about the expression levels of TERRA and its possible role in normal versus tumor tissues is strongly warranted. Information derived from such a study will help to define whether or not G4 structures within TERRA may be a valuable target for telomere-based anticancer agents, especially for tumors that do not express telomerase. Elucidation of the crystal and solution structures of telomeric G4 has led to the rational development of effective G4-stabilizing small molecules [60]. The concept that telomeric G4 can represent a therapeutic target was established by the observation that a group of disubstituted amidoanthraquinones containing a planar aromatic chromophore could inhibit telomerase activity [61]. Since then, the number of known G4 ligands has grown rapidly (Table 1).

***Table 1**. Summary of the relevant findings on G4-stabilizing agents obtained in human tumor experimental models.*

G4 ligand*	Experimental models	Biological response [Ref.]
2,6-pyridine-dicarboxamide derivatives	A panel of telomerase-positive cancer cells and ALT-positive SAOS-2 osteosarcoma cells	Inhibition of cell growth and induction of apoptosis; activation of S-phase checkpoint, increase in metaphase duration and cytokinesis defects; telomere instability related to end-to-end fusions and anaphase bridges [73]
4,5-di-substituted acridone	MCF7 and A549 cancer cell lines; IMR90 normal fibroblast cell line	Acute toxicity in cancer cell lines; cell growth inhibition in long-term setting at subtoxic doses; induction of senescence; no inhibition of telomerase activity; no telomere shortening; no effect in normal fibroblasts [77]
Bisantrene derivatives (An1,5)	SKMel-5 melanoma and U2-OS osteogenic sarcoma cell lines	Telomerase activity inhibition; long-term cell growth inhibition in both telomerase- and ALT-positive cell lines; presence of TIFs, induction of senescence & autophagy [70,88]
Cryptolepine derivatives (SYUIQ-5)	HL-60 and K562 leukemic cells; SW620 colon cancer cells	Inhibition of c-myc promoter activity; reduction of hTERT, E2F1 and TRF2 expression levels; inhibition of telomerase activity; telomere shortening; cell growth arrest and induction of senescence or delayed apoptosis [69,86]
	Nasopharyngeal CNE2 and HeLa cell lines	Inhibition of cell viability; induction of a telomere DNA damage response; induction of an autophagic cell death response [87]
HXDV (synthetic derivative of telomestatin)	A panel of normal/cancer telomerase- and ALT-positive cell lines	Inhibition of cell growth independently of telomerase activity; M-phase cell cycle arrest; mitotic defects; induction of apoptosis [76]
Pentacyclic acridines (RHPS4)	Several experimental models	Inhibition of telomerase activity; acute cellular cytotoxicity; delayed cell growth arrest at subtoxic doses; cell cycle perturbations; reduction of telomere length or induction of telomere dysfunctions; delocalization of TRF1,TRF2 and hPOT1; impairment of fork progression and telomere processing; overexpression of WRN and BLM helicases; activation of an ATR-dependent ATM response and induction of TIFs; activation of PARP-1; induction of apoptosis & senescence [67,72,74,75,78,80,81,91] Inhibition of cell growth in the ALT-positive GM847 skin fibroblast cell lines [78] Increased in vitro sensitivity to anticancer drugs; active as single agent in human xenografts of different hystotypes; in vivo synergistic interaction with camptothecins and PARP inhibitors [67,75,81,91]
Porphyrin derivatives (TMPyP4)	HeLa S3 cervical and MiaPaCa-2 pancreatic cancer cell lines	Inhibition of telomerase activity; down-regulation of c-myc and hTERT gene expression [84]

	MX-1 breast and PC-3 prostate tumor xenografts	Significant decrease in tumor growth rate and increase in mice survival [84]
	A panel of myeloma cell lines	Inhibition of telomerse activity and telomere shortening; reduction of cell viability after the first 14 days of treatment [66]
S2T1 -- 6OTD (synthetic derivative of telomestatin)	Medulloblastoma and atypical teratoid/rhabdoid childhood brain cancer cell lines	Strong inhibition of c-myc promoter activity; reduced expression of c-myc and hTERT; dose- and time-dependent antiproliferative effect; c-myc-dependent telomere shortening after long-term treatment with subtoxic doses, cell growth arrest and induction of senescence and apoptosis [69]
Telomestatin (SOT-095)	OM9;22 and K562 leukemic cell lines; normal bone marrow $CD34_+$ cells	Inhibition of telomerase activity; long-term inhibition of cell growth; telomere shortening and dysfunctions; activation of an ATM-dependent DNA damage response; modest effects on normal bone marrow cells [64] Enhanced sensitivity toward imatinib, doxorubicin, mitoxantrone & vincristine in K562 cells
Tetrasubstituted napthalene diimides	A panel of cancer cell lines;	Potent cytotoxic activity in cancer cell lines; inhibition of telomerase activity; no toxicity in normal fibroblasts [82]
	WI38 normal fibroblast cell line KIT-dependent gastrointestinal tumor cell line (GIST882)	Marked cell growth arrest and effective inhibition of telomerase activity; complete suppression of KIT mRNA & protein expression [98]
Triazine derivatives	A panel of cancer and normal fibroblast cell lines	Inhibition of telomerase activity in the nanomolar range; delayed cell growth arrest at subtoxic doses; telomere shortening and induction of senescence [63,65]
Trisubstituted acridine (AS1410)	MCF7 breast cancer and A549 lung cancer cell lines	Growth arrest at subtoxic doses; synergistic activity in combination with cisplatin [93]
Trisubstituted acridine (BRACO-19)	DU145 prostate cancer cell line	Rapid inhibition of cell growth, onset of senescence, increased incidence of chromosomal fusion (partially independent of telomerase activity inhibition) [71]
	UXF1138L uterus carcinoma cell line and Xenografts	Decrease of hTERT expression; cell growth arrest after 15 days; induction of senescence; 96% of tumor growth inhibition; loss of nuclear hTERT protein expression and increase in atypical mitoses in xenografted tissues [90]
	A431 vulval carcinoma xenografts	Synergistic antitumor effect when combined with paclitaxel [92].

*Listed in alphabetical order.

ALT: Alternative lengthening of telomeres; ATM: Ataxia-telangectasia mutated; ATR: Ataxia-telangectasia mutated and Rad3-related; BLM: Bloom's syndrome; hTERT: Human telomerase reverse transcriptase; hPOT: Human protection of telomeres; PARP-1: Poly (ADP-ribose) polymerase 1; TIF: Telomeric-induced focus; TRF: Telomeric-repeat binding factor; WRN: Werner's syndrome.

Features shared by many of the G4 ligands include a large flat aromatic surface, the presence of cationic charges, and the ability to adopt a terminal stacking mode. Recently, the first example of thermally induced non-metal-based G4 adduct formation, where a reversible binding process is associated with selective alkylation and stabilization of G4, was described by using novel G4 ligand/alkylating hybrid structures, tethering the naphthalene diimide (NDI) moiety to quaternary ammonium salts, as quinone-methide precursors [62]. These new NDI derivatives were shown to efficiently enhance G4 induction and to induce a cytotoxic effect that correlated with their DNA alkylating ability [62]. Due to the inability of telomerase to extend a G4-folded telomeric substrate, G4-interacting agents were first evaluated as telomerase inhibitors. In agreement with the initial paradigm for telomerase inhibition, long-term exposure of human cancer cells to subtoxic doses of specific G4 ligands induced progressive telomere shortening and replicative senescence (Figure 2) [63-69]. Similarly, exposure of telomerase-positive human SK-Mel5 melanoma cells to subtoxic concentrations of the bisantrene regioisomer Ant1,5, identified as a G-quadruplex stabilizer and telomerase inhibitor by screening of a set of anthracene derivatives substituted with 4,5-dihydro-1H-imidazol-2-yl-hydrazonic groups at different positions of the aromatic system, resulted in the inhibition of telomerase activity, delayed antiproliferative effects, and the onset of senescence, thus suggesting a good selectivity window between antitelomerase activity and cell growth impairment [70].

However, there is also evidence that G4 ligands are able to cause a short-term antiproliferative response that cannot be simply explained by telomerase inhibition. Specifically, the observation that the exposure of cancer cells to some G4 ligands caused the induction of a telomeric

DDR and generated telomere dysfunctions, which results in chromosome aberrations independently from telomerase inhibition, led to the proposal that the target of G4 ligands could be the telomere rather than telomerase [71-77].

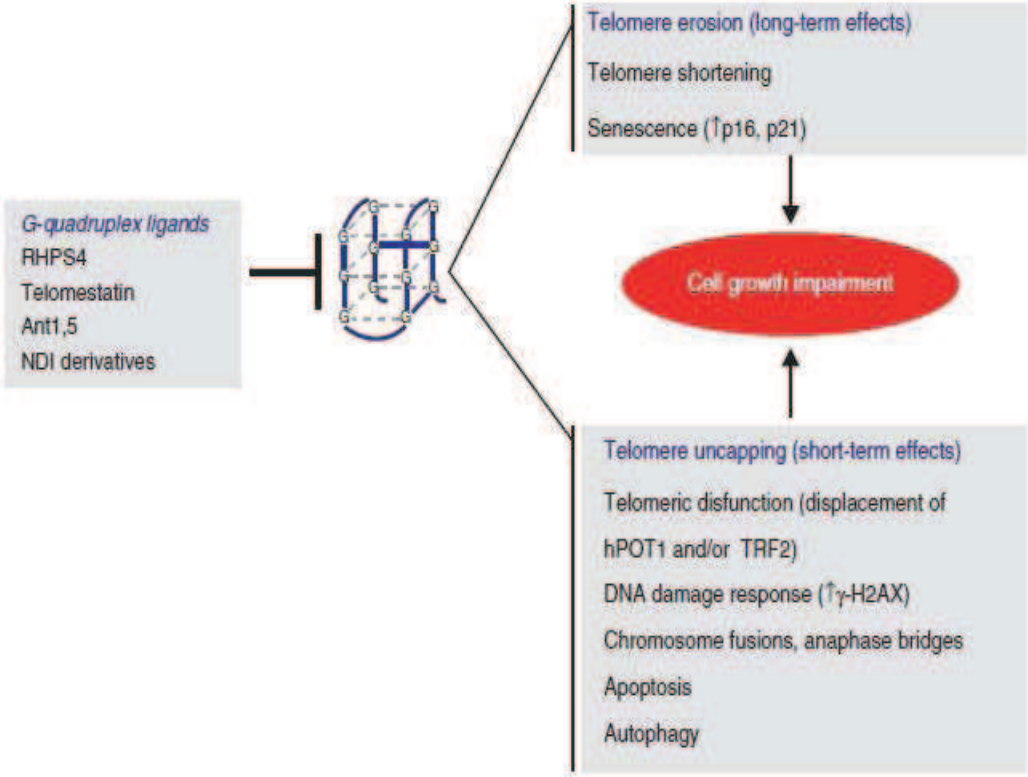

***Figure 2**. Cellular consequences of telomere targeting by G-quadruplex ligands. G-quadruplex stabilizers may induce (1) long--term effects based on telomere erosion resulting in senescence induction and (2) short--term effects through telomere uncapping (human protection of telomeres 1 (hPOT1) and telomeric-repeat binding factor 2 (TRF2) de-localization) and induction of a DNA damage response, resulting in the onset of apoptosis and/or autophagy. Ant1,5: Bisantrene regioisomer; H2A: Histone 2A; NDI: Naphthalene diimide; RHPS4: 3,11-difluoro-6,8,13- trimethyl- 8H-quino[4,3,2-kl] acridinium methosulfate.*

The notion that G4-stabilizing agents can inhibit the growth of ALT cell lines further corroborates the hypothesis that the antiproliferative effects of G4 ligands are largely independent of the presence of active telomerase. In this context, it has been demonstrated that the triazine derivative 115405, the pentacyclic acridine 3,11-difluoro-6,8,13-trimethyl-8H-quino [4,3,2-kl] acridinium methosulfate (RHPS4) and the synthetic derivative of telomestatin HXDV (a 24-membered macrocycle containing six oxazole moieties and two valine residues) were able to inhibit the growth of SV40-immortalized human skin fibroblasts GM847, which maintain telomeres by the ALT pathway [65,76,78]. Similarly, the 2,6-pyridine-dicarboxamide derivatives, the synthetic derivative HXDV and the bisantrene regioisomer Ant1,5 were found to induce antiproliferative effects in telomerase-negative/ALT-positive Saos-2 and U2-OS human osteogenic sarcoma cell lines [70,73,76]. In addition, it has been recently observed that treatment with telomestatin, a natural G4-interacting agent isolated from Streptomyces nanulatus, resulted in depletion of the topoisomerase III/BLM/TRF2 complex and disruption of APBs, with concomitant induction of a DDR at telomeres in the ALT-positive MRC5-V1 lung fibroblast cell line [79]. At present, RHPS4 seems to be the most effective G4 ligand, at least in preclinical models. It shows a high selectivity for G4 structures [80], and long-term exposure of cells to low RHSP4 concentrations causes telomere erosion and an irreversible growth arrest, as a consequence of the drug's ability to inhibit telomerase activity at submicromolar concentrations [67,80]. At higher doses, RHPS4 triggers damage in cells traversing the S phase, as a consequence of its ability to impair fork progression and telomere processing. It also determines alterations of the dynamic association of TRF1, TRF2 and hPOT1 with telomeres [75], the overexpression of

Werner and Bloom helicases, the activation of an ATR-dependent ATM signaling pathway, and the occurrence of chromosome aberrations [75]. It has also been documented that RHPS4 induces the specific activation of poly (ADP-ribose) polymerase 1 (PARP1), resulting in the accumulation of several poly-ADP-ribose residues that co-localize with TRF1 at telomeres [81]. Recently, a series of tetrasubstituted NDI compounds with N--methylpiperazine end groups was evaluated as G4 ligands [82]. They showed high affinity for telomeric G4 over duplex DNA and the ability to stabilize G4 with paralleltype topology, to inhibit the binding of hPOT1 to topoisomerase IIIa to telomeric G4 DNA, and to selectively induce cytoxicity in a panel of cancer cell lines [82]. In addition, three C-11 diamino cryptolepine derivatives, with significant chemical differences between the side chains, have been selected for anticancer drug screening in the National Cancer Institute (NCI) Developmental Therapeutic Program [83]. G4 binding affinity and selectivity studies showed that two compounds with aliphatic amine side chains, NSC748392 and NSC748393, are good but not selective G4 ligands, whereas NSC748394, with an aromatic side chain, showed high selectivity although with decreased affinity. Again, the three compounds showed good in vitro antiproliferative properties at submicromolar concentrations, but only the NSC748393 derivative demonstrated significant activity in the in vivo hollow fiber assay [83]. It has been demonstrated that the cationic porphyrin meso-5,10,15,20-Tetrakis-(N-methyl-4-pyridyl) porphine, Tetratosylate (TMPyP4) is able to prolong survival and decrease tumor growth rates in xenograft tumor models due to its ability to downregulate the expression levels of c-MYC (which harbors G-rich sequences in its promoter) and the resultant transcriptional repression of hTERT [84]. Similarly, the cryptolepine

derivative N'-(10H-Indolo [3,2-b]quinolin-11-yl)-N,N-dimethyl-propane-1,3-diamine (SYUIQ-5) [85] was able to inhibit the c-MYC promoter and telomerase activity, resulting in telomere shortening and apoptosis induction in leukemia cells, after long-term exposure to low drug concentrations [86]. Conversely, at higher concentrations, SYUIQ-5 rapidly inhibited cancer cell growth [87]. Specifically, exposure of nasopharyngeal carcinoma CNE2 cells and HeLa cells to high concentrations of SYUIQ-5 caused a quick antiproliferative effect as a consequence of a potent DNA damage induction, as evidenced by TRF2 delocalization from telomeres, activation of ATM and accumulation of g-H2AX (which co-localizes with TRF1). In addition, an increased expression of LC3-II as well as the evidence of a punctuate pattern of yellow --fluorescent-protein-tagged LC3 [87], two common markers used to detect autophagic activity, have been reported following exposure to SYUIQ-5, suggesting that the biological effects of such a G4 ligand may depend, at least in part, on the induction of autophagy.

In this context, it has been recently reported that SKMel-5 melanoma cells exposed to Ant1,5 display morphological and biochemical features consistent with induction of the autophagic pathway [88].

3. Conclusions

Data obtained from preclinical studies concerning the effects of telomerase inhibition have provided compelling arguments to indicate that the enzyme is a well-validated cancer target [2]. However, the development of therapies to selectively target telomerase in human tumors has been hampered in part by the lack of structural data on the enzyme. In this context, data on the high-resolution structure of human telomerase have been recently reported [13] and could provide a new

opportunity to screen for small molecules to modulate telomerase function in human cancer and to develop novel telomerase-based therapeutic strategies. An important issue that needs to be addressed for the clinical use of telomerase inhibitors is related to the choice of the best patient population. Evidence that many solid tumors express the ALT phenotype instead of telomerase [16] and, as a consequence, are not likely to be affected by telomerase inhibitors suggests that TMM status should be determined in individual patients before starting an anti-telomerase treatment. Moreover, since ALT and telomerase may coexist in the same tumor [16,20], it is also conceivable that treatment of TA+ tumors with telomerase inhibitors could exert a potent selection pressure, leading to the emergence of subpopulations of ALT+ tumor cells refractory to telomerase inhibitors. In this context, inhibitors that specifically target the ALT pathway have not been described, probably as a consequence of the fragmentary information concerning the molecular events driving such a mechanism. Based on results of a recent survey aimed to comparatively evaluate the expression levels of telomere-related proteins in normal and tumor tissues, none of these proteins stands out as a novel cancer-related target [89]. Taken together, these observations highlight the current hurdles to the rational design of novel agents able to target specific factors involved in the maintenance of telomere structure/function. Such novel agents could move in into clinical practice.

At the preclinical level, it has been demonstrated that the use of compounds that selectively target specialized telomere structures, such as G4, can rapidly induce programmed cell death and activate a DDR in a variety of tumor models. Overall, a consistent mechanism of action schema is now emerging for telomeric G4 ligands in tumor cells.

Specifically, they can act through two interconnected pathways (Figure 2). They may either inhibit telomerase activity by locking the single stranded telomere substrate into a quadruplex structure, resulting in long-term effects, or can trigger telomere uncapping, giving rise to short-term effects as a consequence of the induction of telomere dysfunctions. In addition, G4 ligands, such as RHPS4, have been shown to be active in vivo as single agents [75,90,91] and displayed synergistic activity either in vitro or in vivo when combined with conventional anticancer drugs (e.g., platinum compounds, taxanes and topoisomerase I inhibitors), imatinib and PARP1 inhibitors [64,67,81,91-93]. However, despite such well-documented antitumor and chemosensitizing effects in preclinical models, none of the available G4 ligands is currently under clinical development.

4. Expert opinion

Interference with the limitless replicative potential of human cancer cells has emerged as a promising therapeutic strategy from preclinical studies, but telomerase-based cancer therapeutics are moving very slowly to the clinical setting. In fact, the only telomerase inhibitor currently being tested in clinical trials is the template antagonist imetelstat [37]. In addition, lack of knowledge about the molecular determinants of ALT mechanisms makes it difficult to design and develop therapies to selectively interfere with telomere homeostasis in tumors that do not express telomerase. Based on this scenario, the evidence that G4 structures may influence several biological processes other than telomere maintenance makes them an attractive target for broadspectrum anticancer therapies [94]. To this purpose, a precise definition of the structure and the role of G4 in normal and tumor cells is

imperative before G4-stabilizing agents can enter the clinical armamentarium. The development of suitable methodological approaches to detect G4 structures [95] as well as of innovative reagents, including antibodies and aptamers able to discriminate among the different G4 conformations [96,97], will further help to assess the abundance of G4 in vivo and their effects in human diseases and will provide important clues for the synthesis of novel ligands that specifically recognize these structures. An additional issue that still needs to be addressed for G4 ligands with respect to their ability to be exploited as anticancer drugs deals with their therapeutic window. The evidence that G4 ligands selectively impair the growth of cancer cells without affecting the viability of normal cells [64,76,82], together with the ability of these compounds to exert an antitumor activity in different in vivo models and to induce antiproliferative effects also in ALT cells, point to G4 ligands as possible drug candidates for future clinical applications. Furthermore, it cannot be excluded that protein composition at the telomere may differ, quantitatively and/or qualitatively, in normal versus tumor cells and that normal cells may be provided with a higher degree of telomere stability, thus becoming less sensitive to telomere-interacting agents. A therapeutic advantage regarding G4 ligands resides in the evidence that they may have multiple quadruplex targets. In this context, it has been recently reported that an NDI derivative inhibited the growth of gastrointestinal stromal tumor cells as a consequence of its ability to interact with G4 located both at the telomeric level and in the promoter region of the c-kit, an oncogene frequently activated in this tumor type [98]. There is still concern regarding the reliability of the preclinical models currently used to evaluate the therapeutic potential of telomere-based therapeutics. Specifically, more sophisticated human models

(including three-dimensional and organotypic cultures as well as animal models that address the problems of tumor heterogeneity and slow replication of tumor stem cells) should be used to obtain a more realistic proof of the potential of the proposed therapeutic approaches and potentially 'speed' their translation into the clinical setting.

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Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. Gomes NM, Shay JW, Wright WE. Telomere biology in Metazoa. FEBS Lett 2010;584:3741-51

2. Blackburn EH. Telomeres and telomerase: the means to the end (Nobel Lecture). Angew Chem Int Ed Engl 2010;49:7405-21 •• The history of telomere and telomerase discovery narrated by E. Balckburn.

3. de Lange T. How telomeres solve the end-protection problem. Science 2009;326:948-524. Stewart SA, Bertuch AA. The role of telomeres and telomerase in cancer research. Cancer Res 2010;70:7365-71

5. Sarthy J, Bae NS, Scrafford J, et al. Human RAP1 inhibits non-homologous end joining at telomeres. EMBO J 2009;28:3390-9

6. Gilson E, Geli V. How telomeres are replicated. Nat Rev Mol Cell Biol 2007;8:825-38

7. Paeschke K, McDonald KR, Zakian VA. Telomeres: structures in need of unwinding. FEBS Lett 2010;584:3760-72

8. Nabetani A, Ishikawa F. Alternative lengthening of telomeres pathway: recombination-mediated telomere maintenance mechanism in human cells. J Biochem 2011;149:5-14

9. Bhattacharyya S, Sandy A, Groden J. Unwinding protein complexes in ALTernative telomere maintenance. J Cell Biochem 2010;109:7-15

10. Giraud-Panis MJ, Teixeira MT, Geli V, et al. CST meets shelterin to keep telomeres in check. Mol Cell 2010;39:665-76

11. Sarty JF, Baumann P. Apollo-taking the lead in telomere protection. Mol Cell 2010;39:489-91

12. Folini M, Gandellini P, Zaffaroni N. Targeting the telosome: therapeutic implications. Biochim Biophs Acta 2009;1792:309-16

13. Mitchell M, Gillis A, Futahashi M, et al. Structural basis for telomerase catalytic subunit TERT binding to RNA template and telomeric DNA. Nat Struct Mol Biol 2010;17:513-18

14. Venteicher AS, Abreu EB, Meng Z, et al. A human telomerase holoenzyme protein required for Cajal body localization and telomere synthesis. Science 2009;323:644-8

15. Bollmann FM. The many faces of telomerase: emerging extratelomeric effects. Bioessays 2008;30:728-32

16. Cesare AJ, Reddel RR. Alternative lengthening of telomeres: models, mechanisms and implications. Nat Rev Genet 2010;11:319-30 •• An outstanding review on the alternative lengthening of telomere (ALT) mechanisms.

17. Henson JD, Cao Y, Huschtscha LI, et al. DNA C-circles are specific and quantifiable markers of alternative lengthening of-telomeres activity. Nat Biotechnol 2009;27:1181-5 •• The first paper, to our knowledge, showing DNA C-circles as specific markers of (ALT) activity in cancer cell lines and blood samples from tumor patients.

18. Jeyapalan JN, Varley H, Foxon JL, et al. Activation of the ALT pathway for telomere maintenance can affect other sequences in the human genome. Hum Mol Genet 2005;14:1785-94

19. Jeyapalan JN, Mendez-Bermudez A, Zaffaroni N, et al. Evidence for alternative lengthening of telomeres in liposarcomas in the absence of ALT-associated PML bodies. Int J Cancer 2008;122:2414-21

20. Henson JD, Reddel RR. Assaying and investigating alternative lengthening of telomeres activity in human cells and cancers. FEBS Lett 2010;584:3800-11

21. Deng Y, Chang SS, Chang S. Telomere dysfunction and tumour suppression: the senescence connection. Nat Rev Cancer 2008;8:450-8

22. Shay JW, Wright WE. Telomerase therapeutics for cancer: challenges and new directions. Nat Rev Drug Discov 2006;5:577-84

23. Mitomo S, Maesawa C, Ogasawara S, et al. Downregulation of miR-138 is associated with overexpression of human telomerase reverse transcriptase protein in human anaplastic thyroid carcinoma cell lines. Cancer Sci 2008;99:280-6

24. Jiang WQ, Zhong ZH, Henson JD, et al. Identification of candidate alternative lengthening of telomeres genes by methionine restriction and RNA interference. Oncogene 2007;26:4635-47

25. Zhong ZH, Jiang WQ, Cesare AJ, et al. Disruption of telomere maintenance by depletion of the MRE11/RAD50/NBS1 complex in cells that use alternative lengthening of telomeres. J Biol Chem 2007;282:29314-22

26. Atkinson SP, Hoare SF, Glasspool RM, et al. Lack of telomerase gene expression in alternative lengthening of telomere cells is associated with chromatin remodeling of the hTR and hTERT gene promoters. Cancer Res 2005;65:7585-90

27. Hiyama E, Hiyama K. Telomerase as tumor marker. Cancer Lett 2003;194:221-33

28. Hakin-Smith V, Jellinek DA, Levy D, et al. Alternative lengthening of telomeres and survival in patients with glioblastoma multiforme. Lancet 2003;361:836-8

29. Ulaner GA, Huang HY, Otero J, et al. Absence of a telomere maintenance mechanism as a favorable prognostic factor in patients with osteosarcoma. Cancer Res 2003;63:1759-63

30. Costa A, Daidone MG, Daprai L, et al. Telomere maintenance mechanisms in liposarcomas: association with histologic subtypes and disease progression. Cancer Res 2006;66:8918-24

31. Villa R, Daidone MG, Motta R, et al. Multiple mechanisms of telomere maintenance exist and differentially affect clinical outcome in diffuse malignant peritoneal mesothelioma. Clin Cancer Res 2008;14:4134-40

32. Johnson JE, Gettings EJ, Schwalm J, et al. Whole-genome profiling in liposarcomas reveals genetic alterations common to specific telomere maintenance mechanisms. Cancer Res 2007;67:9221-8

33. Brower V. Telomerase-based therapies emerging slowly. J Natl Cancer Inst 2010;102:520-1

34. De Cian A, Lacroix L, Douarre C, et al. Targeting telomeres and telomerase. Biochimie 2008;90:131-5

35. El Daly H, Martens UM. Telomerase inhibition and telomere targeting in hematopoietic cancer cell lines with small non-nucleosidic synthetic compounds (BIBR1532). Methods Mol Biol 2007;405:47-60

36. Roth A, Harley CB, Baerlocher GM. Imetelstat (GRN163L)-telomerase-based cancer therapy. Recent Results Cancer Res 2010;184:221-34 . A comprehensive review on the first telomerase inhibitor entered into clinical trials.

37. Marian CO, Wright WE, Shay JW. The effects of telomerase inhibition on prostate tumor-initiating cells. Int J Cancer 2010;127:321-31

38. Marian CO, Cho SK, McEllin BM, et al. The telomerase antagonist, imetelstat, efficiently targets glioblastoma tumor-initiating cells leading to decreased proliferation and tumor growth. Clin Cancer Res 2010;16:154-63

39. Dikmen ZG, Gellert GC, Jackson S, et al. In vivo inhibition of lung cancer by GRN163L: a novel human telomerase inhibitor, Cancer Res 2005;65:7866-73

40. Jackson SR, Zhu CH, Paulson V, et al. Antiadhesive effects of GRN163L – an oligonucleotide N3¢!P5¢ thio-phosphoramidate targeting telomerase, Cancer Res 2007;67:1121-29

41. Bethesda, MD: clinicaltrials.gov. Available from: www.clinicaltrials.gov

42. Molckovsky A, Siu LL. First-in-class, first-in-human phase I results of targeted agents: highlights of the 2008 American society of clinical oncology meeting. J Hematol Oncol 2008;1:20

43. Blasco M. Telomere length, stem cells and aging. Nat Chem Biol 2007;3:640-9

44. Ning N, Li T, Zhao L, et al. TRF2 promotes multidrug resistance in gastric cancer cells. Cancer Biol Ther 2006;5:950-6

45. Sarraf SA, Harper JW. Telomeric TuRF1 wars. Dev Cell 2010;18:167-8

46. Zeng Z, Wang W, Yang Y, et al. Structural basis of selective ubiquitination of TRF1 by SCFFbx4. Dev Cell 2010;18:214-25

47. Yang Q, Zheng YL, Harris CC. POT1 and TRF2 cooperate to maintain telomeric integrity. Mol Cell Biol 2005;25:1070-80

48. Hockemeyer D, Sfeir AJ, Shay JW, et al. POT1 protects telomeres from a transient DNA damage response and determines how human chromosomes ends. EMBO J 2005;24:2667-78

49. Goldkorn A, Blackburn EH. Assembly of mutant-template telomerase RNA into catalytically active telomerase ribonucleoprotein that can act on telomeres is required for apoptosis and cell cycle arrest in human cancer cells. Cancer Res 2006;66:5763-71

50. Kim MM, Rivera MA, Botchina IL, et al. A low threshold of expression of mutant-template telomerase RNA inhibits human tumor cell proliferation. Proc Natl Acad Sci USA 2001;98:7982-7 • One of the first examples of the antitumor activity exerted by mutant telomerase templates.

51. Li S, Rosenberg JE, Donjacour AA, et al. Rapid inhibition of cancer cell growth induced by lentiviral delivery an expression of mutant-template telomerase RNA and antitelomerase short-interfering RNA. Cancer Res 2004;64:4833-40

52. Stohr BA, Blackburn EH. ATM mediates cytotoxicity of a mutant telomerase RNA in human cancer cells. Cancer Res 2008;68:5309-17

53. Bryan TM, Baumann P. G-quadruplexes: from guanine gels to chemotherapeutics. Methods Mol Biol 2010;608:1-16 • An up-to-date article on the structure and function of G-quadruplex structures. A brief description of the methodological approaches to probe G-quadruplexes is also included.

54. Chang CC, Kuo IC, Ling IF, et al. Detection of quadruplex DNA structures in human telomeres by a fluorescent carbazole derivative. Anal Chem 2004;76:4490-4

55. Granotier C, Pennarun G, Riou L, et al. Preferential binding of a G-quadruplex ligand to human chromosome ends. Nucleic Acids Res 2005;33:4182-90

56. Palumbo SL, Ebbinghaus SW, Hurley LH. Formation of a unique end-to-end stacked pair of G-quadruplexes in the hTERT core promoter with implications for inhibition of telomerase by G-quadruplex-interactive ligands. J Am Chem Soc 2009;131:10878-91 •• The first evidence, to our knowledge, of the presence of G-quadruplex-forming sequences within the core promoter of the human telomerase reverse transcriptase.

57. Gomez D, Lemarteleur T, Lacroix L, et al. Telomerase downregulation induced by the G-quadruplex ligand 12459 in A549 cells is mediated by hTERT RNA alternative splicing. Nucleic Acids Res 2004;32:371-9 •• The first evidence, to our knowledge, of the role of G-quadruplexes in regulating the alternative splicing of the human telomerase reverse transcriptase pre-mRNA.

58. Gomez D, Guedin A, Mergny JL, et al. A G-quadruplex structure within the 5¢-UTR of TRF2 mRNA represses translation in human cells. Nucleic Acids Res 2010;38:7187-98 .. The first evidence, to our knowledge, of the presence of G-

quadruplex-forming sequences within the mRNA sequence of the telomeric repeat binding factor 2.

59. Feuerhahn S, Iglesias N, Panza A, et al. TERRA biogenesis, turnoverand implications for function. FEBS Lett 2010;584:3812-18

60. Neidle S. Human telomeric G-quadruplex: the current status of telomeric G-quadruplexes as therapeutic targets in human cancer. FEBS J 2010;277:1118-25

61. Sun D, Thompson B, Cathers BE, et al. Inhibition of human telomerase by a G-quadruplex-interactive compound. J Med Chem 1997;40:2113-16

62. Di Antonio M, Doria F, Ricther SN, et al. Quinone methides tethered to naphthalene diimides as selective G-quadruplex alkylating agents. J Am Chem Soc 2009;131:13132-41 •• The first example, to our knowledge, of quinone methide tethered to naphthalene di-imides as G-quadruplex ligands with reversible alkylating properties.

63. Riou J-F, Guittat L, Mailliet P, et al. Cell senescence and telomere shortening induced by a new series of specific G-quadruplex DNA ligands. Proc Natl Acad Sci USA 2002;99:2672-7

64. Tauchi T, Shinya K, Sashida G, et al. Activity of a novel G-quadruplex-interactive telomerase inhibitor, telomestatin (SOT-095), against human leukemia cells: involvement of ATM-dependent DNA damage response pathways. Oncogene 2003;22:5338-47

65. Gomez D, Aouali N, Renaud A, et al. Resistance to senescence induction and telomere shortening by a G-quadruplex ligand inhibitor of telomerase, Cancer Res 2003;63:6149-53

66. Shammas MA, Reis RJS, Akiyama M, et al. Telomerase inhibition and cell growth arrest by G-quadruplex interactive agent in multiple myeloma. Mol Cancer Ther 2003;2:825-3367. Cookson JC, Dai F, Smith V, et al. Pharmacodynamics of the G quadruplex-stabilizing telomerase inhibitor 3,11-difluoro-6,8,13-trimethyl- 8H-quino[4,3,2-kl]acridinium methosulfate (RHPS4) in vitro: activity in human tumor cells correlates with telomere length and can be enhanced, or antagonized, with cytotoxic agents. Mol Pharmacol 2005;68:1551-8

68. Zhou JM, Zhu XF, Lu YJ, et al. Senescence and telomere shortening induced by novel potent G-quadruplex interactive agents, quindoline derivatives, in human cancer cell lines. Oncogene 2006;25:503-11

69. Shalaby T, von Beuren AO, Hurlimann M-L, et al. Disabling c-Myc in childhood medulloblastoma and atypical teratoid/rhabdoid tumor cells by the potent G-quadruplex interactive agent S2T1-6OTD. Mol Cancer Ther 2010;9:167-79

70. Folini M, Pivetta C, Zagotto G, et al. Remarkable interference with telomeric function by a G-quadruplex selective bisantrene regioisomer. Biochem Pharmacol 2010;79:1781-90

71. Incles CM, Schultes CM, Kempski H, et al. A G-quadruplex telomere targeting agent produces p16-associated senescence and chromosomal fusions in human prostate cancer cells. Mol Cancer Ther 2004;3:1201-6

72. Leonetti C, Amodei S, DAngelo C, et al. Biological activity of the G-quadruplex ligand RHPS4 (3,11-difluoro-6,8,13-trimethyl-8H-quino[4,3,2-kl] acridinium methosulfate) is associated with telomere capping alteration, Mol Pharmacol 2004;66:1138-46

73. Pennarun G, Granotier G, Gautier LR, et al. Apoptosis related to telomere inability and cell cycle alterations in human glioma cells treated by new highly selective G-quadruplex ligands. Oncogene 2005;24:2917-28

74. Rizzo A, Salvati E, Porru M, et al. Stabilization of quadruplex DNA perturbs telomere replication leading to the activation of an ATR-dependent ATM signaling pathway. Nucleic Acids Res 2009;37:5353-64

75. Salvati E, Leonetti C, Rizzo A, et al. Telomere damage induced by the G-quadruplex ligand RHPS4 has antitumor effect. J Clin Invest 2007;117:3236-47

76. Tsai Y-C, Qi H, Lin C-P, et al. A G-quadruplex stabilizer induces M-phase cell cycle arrest. J Biol Chem 2009;284:22535-43

77. Cuenca F, Moore MJB, Johnson K, et al. Design, synthesis and evaluation of 4,5-di-substituted acridone ligands with high G-quadruplex affinity and selectivity, together with low toxicity to normal cells. Bioorg Med Chem 2009;19:5109-13

78. Gowan S, Heald R, Stevens M, Kelland L. Potent inhibition of telomerase by small-molecule pentacyclic acridines capable of interacting with G-quadruplexes. Mol Pharmacol 2001;60:981-8

79. Temime-Smaali N, Guittat L, Sidibe A, et al. The G-quadruplex ligand telomestatin impairs binding of topoisomerase III alpha to G-quadruplex-forming oligonucleotides and uncaps telomeres in ALT cells. PLOS One 2009;4:1-14

80. Gavathiotis E, Heald RA, Stevens MF, et al. Drug recognition and stabilization of the parallel-stranded DNA quadruplex d(TTAGGGT)4 containing the human telomeric repeat. J Mol Biol 2003;334:25-36

81. Salvati E, Scarsella M, Porru M, et al. PARP1 is activated at telomeres upon G4 stabilization: possible target for telomere-based therapy. Oncogene 2010;29:6280-93

82. Hampel SM, Sidibe A, Gunaratman M, et al. Tetrasubstituted naphthalene di-imide ligands with selectivity for telomeric G-quadruplex and cancer cells. Bioorg Med Chem Lett 2010;20:6459-63

83. Lavrado J, Reszka AP, Moreira R, et al. C-11 diamino cryptolepine derivatives NSC748392, NSC748393, NSC748394: anticancer profile and G-quadruplex stabilization. Bioorg Med Chem Lett 2010;20:7042-5

84. Grand CL, Han H, Munoz RM, et al. The cationic porphyrin TMPyP4 down-regulates c-myc and human telomerase reverse transcriptase expression and inhibits tumor growth in vivo. Mol Cancer Ther 2002;1:565-73

85. Zhou JL, Lu YJ, Ou TM, et al. Synthesis and evaluation of quindoline derivatives as G-quadruplex inducing and stabilizing ligands and potential inhibitors of telomerase. J Med Chem 2005;48:7315-21

86. Liu JN, Deng R, Guo JF, et al. Inhibition of myc promoter and telomerase activity and induction of delayed apoptosis by SYUIQ-5, a novel G-quadruplex interactive agent in leukemia cells. Leukemia 2007;21:1300-2

87. Zhou WJ, Deng R, Zhang XY, et al. G-quadruplex ligand SYUIQ-5 induces autophagy by telomere damage and TRF2 delocalization in cancer cells. Mol Cancer Ther 2009;8:3203-13

88. Cimino-Reale G, Folini M, Orlotti NI, et al. Inhibition of autophagy sensitizes melanoma cells to a G-quadruplex stabilizer. Conference proceedings from EMBO Workshop on 'Telomeres and DNA damage response' 105, 2010

89. Cookson JC, Laughton CA. The levels of telomere-binding proteins in human tumors and therapeutic implications. Eur J Cancer 2009;45:536-50 . A recent survey of the expression levels of telomere-binding proteins in human tumor specimen.

90. Burger AM, Dai F, Schiltes CM, et al. The G-quadruplex interactive molecule BRACO-19 inhibits tumor growth, consistent with telomere targeting and interference with telomerase function. Cancer Res 2005;65:1489-96

91. Leonetti C, Scarsella M, Riggio G, et al. G-quadruplex ligand RHPS4 potentiates the antitumor activity of camptothecins in preclinical models of solid tumors. Clin Cancer Res 2008;14:7284-91 • An example of the in vivo chemosensitizing properties of a G-quadruplex ligand.

92. Gowan SM, Harrison JR, Patterson L, et al. A G-quadruplex-interactive potent small-molecule inhibitor of telomerase exhibiting in vitro and in vivo antitumor activity. Mol Pharmacol 2002;61:1154-62

93. Gunaratnam M, Green C, Moreira JB, et al. G-quadruplex compounds and cis-platin act synergistically to inhibit cancer cell growth in vitro and in vivo. Biochem Pharmacol 2009;78:115-22

94. Wu Y, Brosh RM Jr. G-quadruplex nucleic acids and human disease. FEBS J 2010;277:3470-88 •• An interesting review on the role of G-quadruplexes in human diseases.

95. Bryan TM, Jarstfer MB. Interrogation of G-quadruplex-protein interactions. Methods 2007;43:332-9

96. Schaffitzel C, Postberg J, Paeschke K, et al. Probing telomeric G-quadruplex DNA structures in cells with in vitro generated single-chain antibody fragments. Methods Mol Biol 2010;608:159-81

97. Gatto B, Palumbo M, Sissi C. Nucleic acid aptamers based on the G-quadruplex structure: therapeutic and diagnostic potential. Curr Med Chem 2009;16:1248-65

98. Gunaratnam M, Swank S, Haider SM, et al. Targeting human gastrointestinal stromal tumor cells with a quadruplex-binding small molecule. J Med Chem 2009;52:3774-83 •• An example of the multitarget properties of a G-quadruplex ligand.

Telomere Maintenance in Wilms Tumors: First Evidence for the Presence of Alternative Lengthening of Telomeres Mechanism.

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Abstract

Unlimited proliferative potential is a hallmark of cancer, and can be achieved through the activation of telomere maintenance mechanisms (TMMs). Most tumors activate telomerase, but a significant minority, mainly of mesenchymal origin, uses a recombination-based, alternative lengthening of telomeres (ALT) mechanism. We investigated the presence of ALT in 34 Wilms tumor (WT) samples from 30 patients by using two approaches: (i) the detection of ALT-associated promyelocytic leukemia (PML) nuclear bodies (APBs) by combined PML immunofluorescence and telomere fluorescence in situ hybridization and (ii) the assessment of terminal restriction fragment (TRF) length distribution by pulsed field gel electrophoresis.

In parallel, telomerase activity (TA) was determined by the telomeric repeat amplification protocol (TRAP) assay.

Based on APB expression, ALT was detectable in five samples as the sole TMM and in six samples in association with telomerase. Seventeen

samples only expressed TA and in six cases no known TMM was appreciable. Results of TRF length distribution were available in 32 cases, and a concordance between APB and TRF data in defining the ALT phenotype was found in 26/32 cases (81%). The study provides the first evidence of the presence of ALT in WT, and indicates that in a small but defined fraction of cases (about 15%) ALT is the only TMM that supports the development of WT.

INTRODUCTION

One of the hallmarks of cancer is a limitless proliferative capacity, which is strictly associated with the ability to maintain telomeres. Such an ability can be achieved through the activation of telomere maintenance mechanisms (TMMs). In 1994, Kim et al. made the landmark observation that telomerase activity (TA) is present in 85–90% of human cancers, whereas it is absent in most normal tissues. A minority of human cancers, ranging from 10 to 15%, is telomerase negative, and a fraction of them maintain telomeres through one or more mechanisms defined as alternative lengthening of telomeres (ALT). Several lines of evidence indicate that ALT involves a recombination-based mechanism (Dunham et al., 2000), and characteristics of ALT cells include long and heterogeneous telomeres and subnuclear structures, termed ALT-associated promyelocytic leukemia (PML) bodies (APBs). These structures contain telomeric DNA, telomere-specific binding proteins TRF1 and TRF2, and proteins involved in DNA replication and recombination (Yeager et al., 1999). The prevalence of the ALT mechanism in human cancers is still largely undetermined, since most of the studies carried out so far investigated only telomerase expression. Available data indicate that ALT is more frequently present in tumors of

mesenchymal and neuroepithelial origin than in tumors of epithelial origin, probably due to a stronger telomerase repression in normal mesenchymal and neuroepithelial cells than in epithelial tissues (Henson et al., 2002).

Wilms tumor (WT) or nephroblastoma is a pediatric malignancy of the kidney, and one of the most common solid cancers of childhood. WT is typically composed of a combination of epithelial, mesenchymal, and/or blastemal cells (Beckwith, 1983). Telomerase expression was investigated in WT; and, in different studies, high expression levels of the enzyme catalytic subunit TERT, or the telomerase RNA component TERC, were proposed as adverse prognostic factors (Dome et al., 1999, 2005; Wittmann et al., 2008). Furthermore, in the two studies on the expression of the enzyme activity, most of the WT samples investigated were TA-positive (Dome et al., 1999, 2005). Conversely, no information is currently available on the presence of ALT in WT.

In the present study, aimed at investigating the presence of ALT as the sole TMM or in association with TA, 34 WT samples were studied to provide a more complete description of the mechanisms involved in telomere maintenance in this disease.

MATERIALS AND METHODS

Study Population

Thirty-four WT samples from 30 patients treated at the Fondazione IRCCS – Istituto Nazionale Tumori (INT) of Milan from 2002 to 2008 were investigated for TMM. The specimens were consecutive with respect to the availability of frozen tissue (containing at least 80% of tumor ells as assessed by hematoxylin and eosin) obtained immediately after surgical resection and stored in the INT Tissue Bank and of adequate clinico-pathologic and follow-up information. In two patients with bilateral presentation and in two who suffered from tumor relapse, both lesions were available for analysis. The clinicopathologic features of patients and tumors are reported in Table 1. Among the present series of 30 patients, 20 were untreated and 10 had undergone preoperative chemotherapy. Patients were treated according to the Associazione Italiana Ematologia Oncologia (AIEOP) protocol. Overall, four patients suffered from tumor relapse whereas the remaining 26 patients were relapse-free at a median follow-up time of 46 months (range 9–76). The study was approved by the Ethics Committee of INT, and written informed consent to donate to the Institute the leftover tissue after diagnostic procedures was obtained from all patients' parents or legal guardians.

Molecular Studies

Tumor tissue was sampled by a pathologist at the time of surgery and flash frozen. A fragment of about 100 mg was cut from each lesion and further subdivided for APB detection and DNA, RNA, and protein extractions. A frozen section of each sample was stained with hematoxylin and eosin to check for the presence of tumor cells.

Detection of ALT-Associated Promyelocytic Leukemia Nuclear Bodies

APBs were assayed on frozen tissue sections by combined PML immunofluorescence and telomere fluorescence in situ hybridization (FISH), as previously described (Henson et al., 2005). Images were captured on a Nikon Eclipse E600 fluorescence microscope using ACT-1 (Nikon, Tokyo, Japan) image analysis software and processed using Adobe Photoshop Image Reader 7.0 software. APB status was determined according to previously defined criteria (Henson et al., 2005). The presence of an APB was defined by the localization of a telomeric DNA focus within a nuclear PML body. Sections were scored as APB+ if they contained APB in >0.5% of tumor cells, and a tumor was considered ALT+ when at least one section was APB+. To avoid false positives, an APB was considered to be present only when the telomeric DNA fluorescence within a PML body was more intense than that of telomeres, and a cell was not considered to contain APB if more than 25% of the co-localized foci occurred outside of the nucleus. To avoid false negatives, at least 2,000 tumor nuclei were examined, and the assay was repeated in the presence of negative results. Tumor sample from an ALT+ (U2OS) cell line was used as positive control for the APB assay.

TABLE 1. Clinicopathological Data and Telomere Maintenance Mechanisms (TMMs) in the Investigated Series of Wilms Tumors.

						TMM			
Sample	Gender	Age (months)	Stage	Pretreatement	Type	TA	hTERT	ALT	TRF
110 TR	F	37	3	NO	BES	+	+	-	-
111 TL[a]	F	13	3	NO	BES-DA	+	+	-	-
112 TR	M	44	3	NO	B	+	+	-	-
113 TR[a]	M	10	2	YES	BES	-	-	+	-
113 rel					BES	-	+	+	-
121 TR	F	43	1	NO	BES	+	+	-	-
125 TL	F	24	2	NO	BES	+	+	+	+
127 TL[a]	F	41	5	YES	BES	-	+	+	+
127 rel					N	-	+	+	Ne
128 TL	F	74	2	NO	E	-	-	-	-
139 TL	F	52	1	NO	B	+	+	+	-
146 TL	F	30	1	YES	BES	-	-	-	-
154 TL	M	57	2	YES	BES	+	+	-	-
159 TR	F	53	2	NO	S-DA	+	+	-	-
161 TR	M	14	5	YES	BES	+	+	+	+
161 TL					BES	+	+	+	+
163 TL	M	44	1	NO	E	+	+	-	-
176 TR	F	13	2	NO	BES	+	+	-	-
179 TL	M	15	1	NO	BES	-	-	-	+
187 TR	M	13	1	YES	BES	-	-	-	-
189 T	F	5	1	NO	BES	+	+	+	+
195 TL	F	16	1	NO	BES	+	+	-	-
196 TL	M	23	1	NO	BES	+	+	-	-
198 TR	M	4	1	NO	BE	+	+	+	-
201 TR	F	64	3	NO	BES	+	+	-	-
211 TL[a]	F	33	3	YES	BES	-	+	-	-
256 TR	F	20	1	NO	B	+	+	-	-
258 TL	F	63	3	NO	B	+	+	-	-
267 TL	F	6	1	NO	B	-	+	+	+
275 TL	F	44	2	NO	S	+	+	-	-
282 T	F	34	1	YES	BES	+	+	-	Ne
292 TR	F	16	5	YES	SE	-	-	-	+
292 TL					SE	+	+	-	-
320 TR	M	11	5	YES	SE	+	+	-	-

T: tumor, L: left side, R: right side, Rel: relapse; F: female, M: male, B: blastemal, E: epithelial, S: stromal, DA: diffuse anaplasia, N: necrosis; TMM: telomere maintenance mechanism, TA: telomerase activity, TERT: TERT transcript, APB: alternative lengthening of telomeres assessed on the presence of APB (ALT-associated promyelocytic leukemia nuclear bodies), TRF: alternative lengthening of telomeres assessed on the basis of terminal restriction fragment, Ne: not evaluable. a: Relapsed patient.

Telomere Length Analysis

Total DNA was isolated using QuicKpicK genomic DNA kit (BioNobile, Medi Diagnostici, Milan, Italy) and telomere length was assessed by pulsed-field gel electrophoresis as previously described (Villa et al., 2000). Autoradiographs were scanned (ScanJet IIcx/T; Hewlett Packard, Milan, Italy) and digitalized by Image Quant (Molecular Dynamics, Sunnyvale, CA). Each gel was standardized by inclusion of DNA from GM847 (ALT+) and HeLa (TA+) cell lines. ALT status was determined by calculating whether the mean, variance, and semi-interquartile range of the terminal restriction fragment (TRF) length distribution were greater than 16 kb, 1,000 kb2, and 4 kb, respectively. Tumors were classified as ALTþ when 2/3 or 3/3 of these criteria were met for unimodal or bimodal TRF length distributions, respectively. Statistical analysis of TRF length distributions was performed using Telometric software (Grant et al., 2001).

Telomerase Activity

TA was measured on 0.6 and 6 µg of protein by the telomeric repeat amplification protocol (TRAP) (Kim et al., 1994) using the TRAPeze kit (Intergen, Oxford, UK). A tumor was scored as TA+ when positive TRAP results were obtained for at least one protein concentration. In the case of tumors negative at both protein concentrations, the TRAP assay was repeated to avoid false negatives. The TA+ cell line HeLa was used as positive control for the TRAP assay. Each experiment always included one heat inactivated sample to exclude contaminations and avoid false positive results.

RNA Extraction and Quantitative Real-Time Reverse Transcription (RT)-PCR Analysis of TERT

Total cellular RNA was extracted from frozen samples with the TRIzol reagent (Life Technologies, Gaithersburg, MD). Total RNA (1 µg) from each sample was reverse-transcribed by random priming using the high capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). TERT expression was assessed by quantitative real-time RT-PCR using TaqMan gene expression assay (Assay ID: Hs00972656_m1, Applied Biosystems), and the b-2-microglobulin (b2M) housekeeping gene was amplified as internal control (Assay ID: Hs 99999907_m1, Applied Biosystems). Amplifications were run on the 7900HT Fast Real-Time PCR System (Applied Biosystems), and data were analyzed by Sequence Detector System 2.2.2 software (Applied Biosystems) using the comparative Ct method for relative quantification of expression levels. The cDNA obtained from TA+ Hela cell line was used as positive control.

Data Analysis

TMM measurements were performed by personnel blinded to patient data and clinical outcome (LV, RM), whereas clinical data were collected by personnel blinded to TMM results (FS, MT). The association between ALT and TA, as well as between ALT or TA and clinicopathologic features was assessed by the chi square test, adjusted for continuity when appropriate, or by Fisher's exact test. SAS software (SAS Institutes, Inc., Cary, NC) was used to perform statistical calculations, and a two-sided P value <0.05 was considered statistically significant.

RESULTS

Thirty-four WT samples obtained from 30 patients were characterized for TMMs. At least one TMM was detectable in 28/34 WTs (82%). Eleven of 34 (32%) cases were classified as ALT+ on the basis of APBs being present in at least 0.5% of 2,000 tumor cells (Fig. 1A). Among the ALT+ samples, APBs were detected in a variable, but always limited, percentage of cells, ranging from 0.5% to 2.0%. At least two separate sections were examined for APBs, and the results revealed no intratumor heterogeneity for ALT (data not shown). In 32 lesions, results of TRF analyses were also available (Fig. 1B).

Telomere length distribution of ALT+ samples are expected to be heterogeneous (range, <3 to >50 kb), whereas TA+ tumors usually display a more homogeneous distribution and a shorter average length of telomeres. Qualitative concordance between APB and TRF results in defining the ALT phenotype was found in 26/32 cases (81%). Twenty-three of 34 (68%) WT specimens were classified as TA+ based on positive TRAP results on at least one protein concentration (Fig. 2).

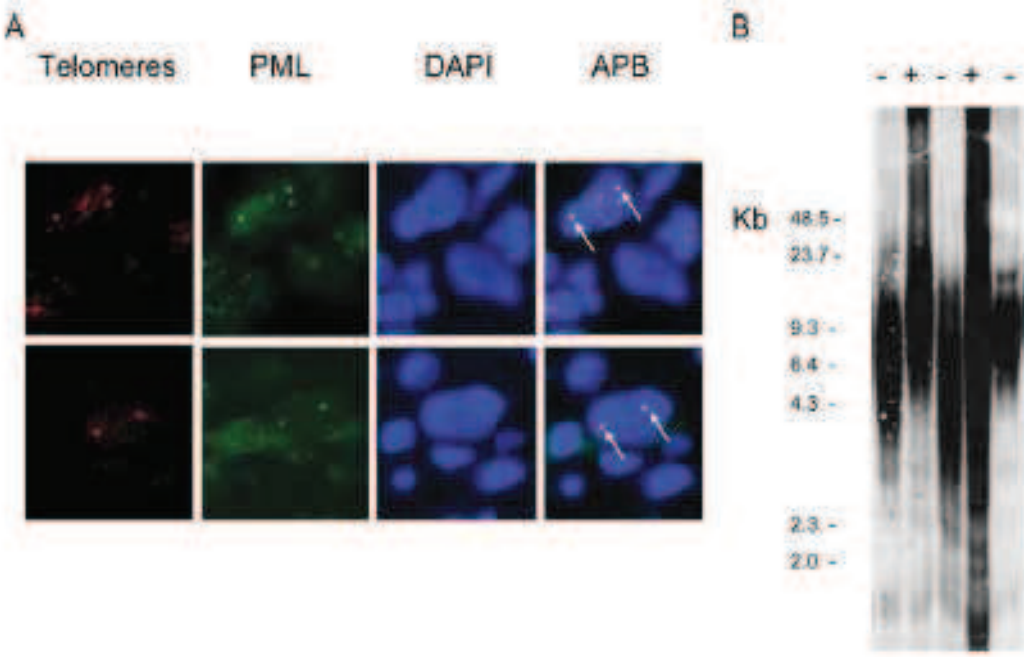

Figure 1. *ALT assays in Wilms tumors. A: APB assay: combined PML immunofluorescence and telomere FISH in a frozen section of ABP1 Wilms tumor. Telomere FISH was done using a Cy3-conjugated telomeric peptide nucleic acid probe (red stain). Indirect immunofluorescence was used for the PML protein (FITC label, green stain). Nuclei were counterstained with 40,6-diamidino-2-phenylindole (DAPI) (blue stain). The foci of telomeric DNA that co-localize with PML represent APB. B: TRF southern blot analysis. Telomere length distribution of a representative series of Wilms' tumors. The lengths of telomeres in ALT1 cells typically range from <3 to >50 kb. ALTcells typically have a more homogeneous distribution of telomere length and a shorter average length than ALT1 cells.*

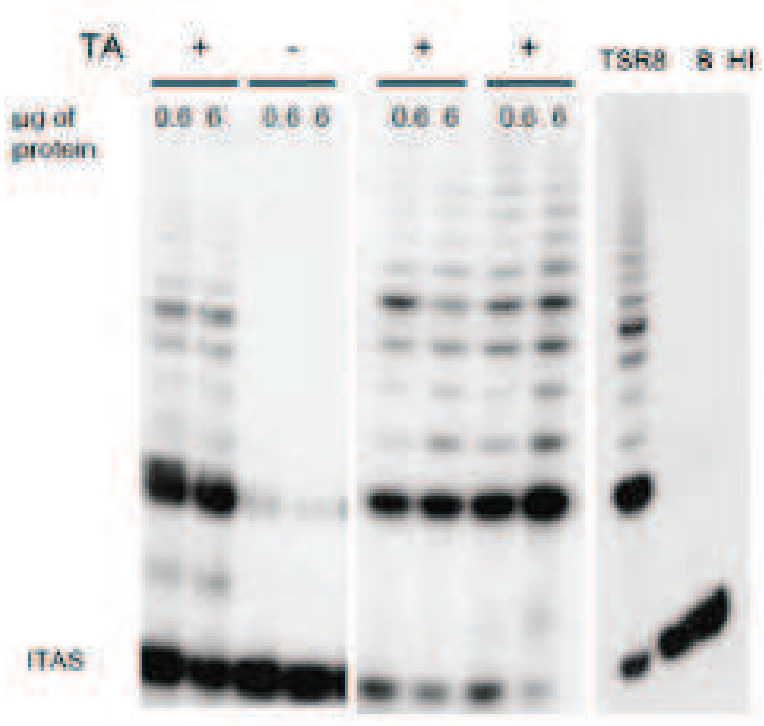

***Figure 2**. A representative TRAP experiment showing telomerase activity in Wilms tumor. The presence (1) or absence (2) of telomerase activity (TA) is reported for each sample using 0.6 and 6 µg of protein. The location of the internal amplification standard (ITAS) is reported. Positive control (TSR8) and negative control (heat inactivated sample, HI) as well as blank control (B) are shown.*

Expression of the catalytic component of telomerase TERT was found in all TA+ specimens and in 5/11 of TA- cases. Overall, based on the presence of APBs, five cases expressed ALT as the sole TMM (ALT+/TA-) with a mean percentage of APB+ cells of 1.1% (range, 0.5–2.0), and six cases concomitantly expressed both TMM (ALT+/TA+) with a mean percentage of APB positive cells of 0.9% (range, 0.65-1.09). Seventeen samples only presented telomerase activation and six did not express any known TMM.

In four patients, more than one lesion was available for investigation. In the two cases in which both primary tumor and relapse were studied, the TMM phenotype was concordant (Table 1, samples 113 and 127). In two cases with bilateral disease presentation, a difference in TMM status was found in one case (Table 1, sample 292), in which the right and left tumors were ALT-/TA- and ALT-/TA+, respectively, while in the other case (Table 1, sample 161) both lesions were ALT+/TA+. TMM results as a function of patient clinicopathological data are summarized in Table 1. TA and ALT (as defined by APB expression) were both unrelated to gender, age (namely older or younger than 24 months at diagnosis), and stage (1–5). Conversely, the presence of TA was more frequently observed in tumors from untreated patients compared to samples obtained from patients who underwent preoperative chemotherapy ($P = 0.042$). The presence of APB proved to be unrelated to previous treatment. The small number of tumor relapses (four cases: one case TA+/ALT-, one case TA-/ALT-, two cases TA-/ALT+) did not allow the analysis of the clinical outcome as a function of TMM.

DISCUSSION

Telomere biology of pediatric tumors has been an object of investigation in recent years for the possible prognostic and therapeutic implications (Shalaby et al., 2010). Telomerase is expressed in most pediatric embryonal tumors and leukemia, whereas it is less consistently expressed in sarcomas and brain tumors. In general, a high level of telomerase expression correlates with aggressive disease and adverse patient outcome (for a review, see Tabori and Dome, Cancer Investigation, 2007). Among pediatric cancers, ALT presence has been

described in a significant percentage of osteosarcomas (Ulaner et al., 2003; Sanders et al., 2004; Henson et al., 2005) and in a limited fraction of neuroblastoma (Henson and Reddel, 2010). Thus far, only a few studies have investigated the mechanisms by which telomeres are maintained in WT, and they focused exclusively on TA or on the expression levels of TERT and TERC, in the search for new prognostic biomarkers. TA, as determined by the TRAP assay (which provides a functional readout of the protein), has been investigated in two studies. The enzyme's activity was detected in 58/72 (80%) and 157/164 (96%) cases, respectively, but without, any predictivity for relapse (Dome et al., 1999, 2005). In the same studies, the expression levels of TERT mRNA and TERC was evaluated, and detectable levels were found in 97–99% and 100% of samples, respectively (Dome et al.,1999, 2005). Although the first study showed that TERT expression levels was an independent predictor of recurrence (Dome et al., 1999), the second study, involving a larger cohort of patients, revealed that levels of TERC expression was an even stronger predictive factor (Dome et al., 2005). In a different study, among 40 markers proposed for the classification and/or prediction of outcome in WT, which were investigated in 102 WTs and validated in an independent set of 74 WT cases, TERT turned out to be a potentially relevant marker of relapse and death (Wittmann et al., 2008). To the best of our knowledge, WT has never been investigated for presence of the ALT mechanism. Different published data showing a higher frequency of ALT+ cases in tumors of mesenchymal and neuroepithelial origin (Henson et al., 2002), together with the pattern of gene expression in WT (which mimics the earliest committed stage in the metanephric mesenchymal-epithelial transition [Li et al., 2002]), prompted us to analyze the presence of ALT and, for comparative

purposes, TA in WT. In the study, we took advantage of a mono-institutional series of WTs, which can be reasonably assumed to be uniform in terms of tumor specimen collection and processing, in order to avoid any possible variability in the results due to different sample handlings.

Interestingly, 32% (11/34) of investigated WTs showed activation of the ALT mechanism. In five cases ALT was the sole mechanism responsible for telomere maintenance in the tumor. This finding could be clinically relevant as telomerase inhibitors come into use in clinical trials, since not all WTs would be susceptible to such therapies.

As regards telomerase, the enzyme activation was found to be present in 23 out of 34 studied samples. The frequency of tumors positive in the TRAP assay we detected in our case series (68%) was somewhat lower than that previously reported (80–96%) (Dome et al., 1999, 2005). Such a lower incidence may be due to methodologic differences or to the timing of nephrectomy, i.e., preoperative chemotherapy, that was administered approximately to one-third of our patients, since a trend in favor of a more frequent telomerase activation was observed in untreated compared to treated tumors.

A concomitant presence of the two TMMs was observed in six WT samples (18%). Although a simultaneous activation of TA and ALT has already been observed in other neoplasms, such as osteosarcoma (Ulaner et al., 2003), liposarcoma (Montgomery et al., 2004; Johnson et al., 2005; Costa et al., 2006), glioblastoma multiforme (Hakin-Smith et al., 2003), and diffuse malignant peritoneal mesothelioma (Villa et al., 2008), it is yet not known whether both TMMs coexist in the same neoplastic cell or whether the two distinct mechanisms represent different ALT+ and TA+ tumor subpopulations. Evidence obtained in

ALT cells engineered to express hTERT suggests that the two TMMs can function concurrently in most (Cerone et al., 2001; Grobelny et al., 2001; Perrem et al., 2001; Stewart et al., 2001) but not all models investigated (Ford et al., 2001).

The assay we used to investigate TERT mRNA measures the total amount of transcripts and does not discriminate among the alternatively spliced isoforms. The evidence that some cases, which are TA- in the TRAP assay, express the TERT transcript confirms that the presence of the TERT mRNA is necessary but not sufficient to activate the enzyme. Furthermore, we cannot exclude the presence of a mutated TERT gene or imbalances in the different transcripts, which have been demonstrated to affect TA in other tumor types (Villa et al., 2001; Brambilla et al., 2004; Zaffaroni et al., 2005; Villa et al., 2008).

Although telomerase and ALT appear equivalent in their ability to support immortalization, their contribution to tumor progression may differ. Our preliminary evidence that one locally relapsing tumor (sample 113) showed ALT as the sole TMM would indicate that ALT+ WTs may nevertheless be highly malignant, as previously observed in other tumor types (Hakin-Smith et al, 2003; Ulaner et al., 2003; Costa et al., 2006). Six cases (18%), despite being informative in the different assays used in the study, seemed to possess no known TMM. Although we cannot exclude that this results could be due to the sensitivity of our assays, the existence of ALT-/TA- lesions has already been observed in other tumor types (Hakin-Smith et al., 2003; Ulaner et al., 2003; Costa et al., 2006), suggesting the presence of a not yet identified TMM in these lesions, or, alternatively, that the acquisition of a constitutively active TMM is not a stringent requirement for this subset of tumors, in agreement with experimental data that showed that TMM activation is not always

required for malignant transformation of normal human cells (Seger et al., 2002). A further possibility is that, based on the presence of APBs, the ALT phenotype is underestimated, since, at least in liposarcomas, some tumors activate the ALT mechanism in the absence of APBs (Jeyapalan et al., 2008). In conclusion, this study provides the first evidence of the presence of ALT in approximately one-third of WTs, indicating that in a small but significant fraction of cases (about 15%) ALT is the only TMM that supports WT development.

REFERENCES

Beckwith JB. 1983. Wilms' tumor and other renal tumors of childhood: A selective review from the National Wilms' Tumor Study Pathology Center. Hum Pathol 14:481–492.

Brambilla C, Folini M, Gandellini P, Daprai L, Daidone MG, Zaffaroni N. 2004. Oligomer-mediated modulation of hTERT alternative splicing induces telomerase inhibition and cell growth decline in human prostate cancer cells. Cell Mol Life Sci 61:1764–1774.

Cerone MA, Londono-Vallejo JA, Bacchetti S. 2001. Telomere maintenance by telomerase and by recombination can coexist in human cells. Hum Mol Genet 10:1945–1952.

Costa A, Daidone MG, Daprai L, Villa R, Cantu` S, Pilotti S, Mariani L, Gronchi A, Henson JD, Reddel RR, Zaffaroni N. 2006. Telomere maintenance mechanisms in liposarcomas: Association with histologic subtypes and disease progression. Cancer Res 66:8918–8924.

Dome JS, Chung S, Bergemann T, Umbricht CB, Saji M, Carey LA, Grundy PE, Perlman EJ, Breslow NE, Sukumar S. 1999. High telomerase reverse transcriptase (hTERT) messenger RNA level correlates with tumor recurrence in patients with favourable histology Wilms' tumor. Cancer Res 59:4301–4307.

Dome JS, Bockhold CA, Li SM, Baker SD, Green DM, Perlman EJ, Hill DA, Breslow NE. 2005. High telomerase RNA expression level is an adverse prognostic factor for favorable-histology Wilms' tumor. J Clin Oncol 23:9138–9145.

Dunham MA, Neumann AA, Fasching CL, Reddel RR. 2000. Telomere maintenance by recombination in human cells. Nat Genet 26:447–450.

Ford LP, Zou Y, Pongracz K, Gryaznov SM, Shay JW, Wright WE. 2001. Telomerase can inhibit the recombination-based pathway of telomere maintenance in human cells. J Biol Chem 276:32198–32203.

Grant JD, Broccoli D, Muquit M, Manion FJ, Tisdall J, Ochs MF. 2001. Telometric: A tool providing simplified, reproducible measurements of telomeric DNA from constant field agarose gels. Biotechniques 31:1314 -1316.

Grobelny JV, Kulp-McEliece M, Broccoli D. 2001. Effects of reconstitution of telomerase activity on telomere maintenance by the alternative lengthening of telomeres (ALT) pathway. Hum Mol Genet 10:1953–1961.

Hakin-Smith V, Jellinek DA, Levy D, Carroll T, Teo M, Timperley WR, McKay MJ, Reddel RR, Royds JA. 2003. Alternative lengthening of telomeres and survival in patients with glioblastoma multiforme. Lancet 361:836–838.

Henson JD and Reddel RR. 2010. Assaying and investigating alternative lengthening of telomeres activity in human cells and cancers. FEBS Lett 584:3800–3811.

Henson JD, Neumann AA, Yeager TR, Reddel RR. 2002. Alternative lengthening of telomeres in mammalian cells (Review). Oncogene 21:598–610.

Henson JD, Hannay JA, McCarthy SW, Royds JA, Yeager TR, Robinson RA, Wharton SB, Jellinek DA, Arbuckle SM, Yoo J, Robinson BG, Learoyd DL, Stalley PD, Bonar SF, Yu D, Pollock RE, Reddel RR. 2005. A robust assay for alternative lengthening of telomeres in tumors shows the significance of alternative lengthening of telomeres in sarcomas and astrocytomas. Clin Cancer Res 11:217–225.

Jeyapalan JN, Mendez-Bermudez A, Zaffaroni N, Dubrova YE, Royle NJ. 2008. Evidence for alternative lengthening of telomeres in liposarcomas in the absence of ALT-associated PML bodies. Int J Cancer 122:2414–2421.

Johnson JE, Varkonyi RJ, Schwalm J, Cragle R, Klein-Szanto A, Patchefsky A, Cukierman E, von Mehren M, Broccoli D. 2005. Multiple mechanisms of telomere maintenance exist in liposarcomas. Clin Cancer Res 11:5347–5355.

Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL, Shay JW. 1994.

Specific association of human telomerase activity with immortal cells and cancer. Science 266:2011–2015.

Li CM, Guo M, Borczuk A, Powell CA, Wei M, Thaker HM, Friedman R, Klein U, Tycko B. 2002. Gene expression in Wilms' tumor mimics the earliest committed stage in the metanephric mesenchymal-epithelial transition. Am J Pathol 160:2181–2190.

Montgomery E, Argani P, Hicks JL, DeMarzo AM, Meeker AK. 2004. Telomere lengths of translocation associated and nontranslocation-associated sarcomas differ dramatically. Am J Pathol 164:1523–1529.

Perrem K, Colgin LM, Neumann AA, Yeager TR, Reddel RR. 2001. Coexistence of alternative lengthening of telomeres and telomerase in hTERT-transfected GM847 cells. Mol Cell Biol 21:3862–3875.

Sanders RP, Drissi R, Billups CA, Daw NC, Valentine MB, Dome JS. 2004. Telomerase expression predicts unfavorable outcome in osteosarcoma. J Clin Oncol 22:3790–3797.

Seger YR, Garcı´a-Cao M, Piccinin S, Cunsolo CL, Doglioni C, Blasco MA, Hannon GJ, Maestro R. 2002. Transformation of normal human cells in the absence of telomerase activation. Cancer Cell 2:401–413.

Shalaby T, Hiyama E, Grotzer MA. 2010. Telomere maintenance as therapeutic target in embryonal tumours. Anticancer Agents Med Chem 10:196–212.

Stewart JL, Baird KM, Farr CJ. 2001. All's well that ends well. Trends Cell Biol 11:279–280.

Tabori U, Dome JS. 2007. Telomere biology of pediatric cancer. Cancer Invest 25:197–208.

Ulaner GA, Huang HY, Otero J, Zhao Z, Ben-Porat L, Satagopan JM, Gorlick R, Meyers P, Healey JH, Huvos AG, Hoffman AR, Ladanyi M. 2003. Absence of a telomere maintenance mechanism as a favorable prognostic factor in patients with osteosarcoma. Cancer Res 63:1759–1763.

Villa R, FoliniM, Perego P, Supino R, Setti E, Daidone MG, Zunino F, Zaffaroni N. 2000. Telomerase activity and telomere length in human ovarian cancer and melanoma cell lines: Correlation with sensitivity to DNA damaging agents. Int J Oncol 16:995–1002. Villa R, Porta CD, Folini M, Daidone MG, Zaffaroni N. 2001. Possible regulation of telomerase activity by transcription and alternative splicing of telomerase reverse transcriptase in human melanoma. J Invest Dermatol 116:867–873.

Villa R, Daidone MG, Motta R, Venturini L, De Marco C, Vannelli A, Kusamura S, Baratti D, Deraco M, Costa A, Reddel RR, Zaffaroni N. 2008. Multiple mechanisms of telomere maintenance exist and differentially affect clinical outcome in diffuse malignant peritoneal mesothelioma. Clin Cancer Res 14:4134–4140.

Wittmann S, Wunder C, Zirn B Furtwa¨ngler R, Wegert J, Graf N, Gessler M. 2008. New prognostic markers revealed by evaluation of genes correlated with clinical parameters in Wilms' tumors. Genes Chromosomes Cancer 47:386–395.

Yeager TR, Neumann AA, Englezou A, Huschtscha LI, Noble JR, Reddel RR. 1999. Telomerase-negative immortalized human cells contain a novel type of promyelocytic leukemia (PML) body. Cancer Res 59:4175–4179.

Zaffaroni N, Villa R, Pastorino U, Cirincione R, Incarbone M, Alloisio M, Curto M, Pilotti S, Daidone MG. 2005. Lack of telomerase activity in lung carcinoids is dependent on human telomerase reverse transcriptase transcription and alternative splicing and is associated with long telomeres. Clin Cancer Res 11:2832–2839.

Telomere maintenance mechanisms in malignant peripheral nerve sheath tumors: expression and prognostic relevance

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Abstract

The study aimed at investigating the prevalence of the two known telomere maintenance mechanisms (TMMs), telomerase activity (TA) and alternative lengthening of telomeres (ALT), and assessing their prognostic relevance in malignant peripheral nerve sheath tumors (MPNST). In 57 specimens obtained from 49 patients with MPNST (35 sporadic and 14 neurofibromatosis type 1-related), TA was determined using the telomeric repeat amplification protocol and ALT was detected by assaying both ALT-associated promyelocytic leukemia bodies (APB) and terminal restriction fragment (TRF) length distribution. TA or ALT (as detected by the presence of APB) alone was found in 24.6% or 26.3% of the lesions, respectively, whereas 6 cases (10.5%) were TA+/ALT+. A concordance between APB and TRF results in defining the ALT status

was observed in 44 of 57 cases (77.2%; *P*<0.0001). TA was found to be more frequently expressed in samples from patients with neurofibromatosis type 1-related MPNST than in those with sporadic disease (60% vs 29.4%, *P*=0.087). In the overall series, TA proved to be prognostic for 5-year disease-specific death (Hazard Ratio, 3.78; 95% CI, 1.60-8.95; *P*=0.002). These findings held true even when adjusted for the concomitant presence of neurofibromatosis type 1 syndrome (Hazard Ratio, 4.22; 95% CI, 1.804-9.874; *P*=0.001) and margin status after surgery (Hazard Ratio, 5.78; 95% CI, 2.19-15.26; p<0.001). Conversely, ALT failed to significantly affect clinical outcome of MPNST patients either using APB expression (Hazard Ratio,1.25; 95% CI 0.54-2.89; *P*=0.605) or TRF distribution (Hazard Ratio 0.57; 95% CI 0.17-1.96; *P*=0.375) as the detection approach. Our results indicate for the first time that both known TMMs, TA and ALT, are present in MPNST and differentially affect patient prognosis.

Keywords: telomere maintenance mechanisms, telomerase, alternative lengthening of telomeres, malignant peripheral nerve sheath tumors, schwannoma.

Introduction

Peripheral nerve sheath tumors constitute a group of relatively rare soft tissue neoplasms which includes benign lesions deriving from specific neural tissues (neurofibromas and schwannomas) and malignant peripheral nerve sheath tumour (MPNSTs) that most commonly involve major nerve trunks (1). MPNSTs are highly aggressive malignancies that account for 3-10% of all soft tissue sarcomas (2). Approximately 50-60% of MPNSTs occur in the setting of neurofibromatosis type 1 (NF1), a hereditary tumor syndrome, and are the leading cause of NF1-related mortality (3,4). The remainder of MPNSTs develop sporadically. In patients with NF1, MPNST usually arise in the presence of a neurofibroma or, very rarely, a schwannoma (5).

Complete surgical excision is the mainstay of MPNST therapy and represents the primary curative modality. Adjuvant chemotherapy and radiation therapy are also often used. However, despite the aggressive treatment, local recurrence and metastases are common, leading to a poor prognosis for MPNST patients, with 5-year survival rates ranging from 20-50%. These finding strongly indicate an urgent need for improved therapeutic approaches able to significantly impact on the disease outcome (6).

MPNST pathogenesis is poorly understood due mainly to its complex histopathology. A number of molecular and genetic alterations have been found in relation to these tumors (7-10). However, there is no defined molecular signature for MPNST development, although recent work in the field of MPNST molecular pathobiology has identified several altered receptor tyrosine kinase-mediated intracellular signal transduction cascades, posing the possibility of utilizing personalized, targeted therapeutics for the disease (11).

The activation of a telomere maintenance mechanism (TMM) is essential for tumor cells to counteract the normal limit on cell proliferation resulting from progressive telomere shortening that normally accompanies each round of cell division (12). Indeed, limitless proliferative potential is a hallmark of cancer cells. There are two known TMMs: telomerase (13) and alternative lengthening of telomeres (ALT) (14). Telomerase is a ribonucleoprotein complex containing an RNA subunit, hTR, that provides the template for the synthesis of telomeric DNA by the catalytic subunit, hTERT (15). Approximately 85-90% of all tumors express telomerase (16), whereas most of the remaining tumors, mainly those of mesenchymal and neuroepithelial origin (17-24), rely on ALT to maintain their telomeres.

Although the molecular details of ALT are incompletely understood, previous studies have shown that it is consistent with a recombination-dependent DNA replication mechanism (14). Characteristics of ALT cells include an extremely heterogeneous telomere length distribution, ranging from very short to more than 50 kb, and the presence of subnuclear structures termed ALT-associated promyelocytic leukemia (PML) bodies (APBs) (14). PML bodies are normal nuclear domains of unknown function that contain the PML protein. In ALT cells, a subset of the PML bodies contains telomeric chromatin (telomeric DNA and the telomere binding proteins TRF-1, TRF-2, TIN2 and RAP1) as well as other proteins involved in DNA replication, recombination and repair (14). Another specific feature of ALT mechanisms is the presence of C-circles (self-priming circular telomeric DNA), which may represent molecular intermediates of the ALT mechanism and whose specificity for assessing ALT activity in biological samples has recently been demonstrated (25).

Because the presence of an active TMM is an almost universal feature of cancer, and normal cells do not have sufficient levels of TMM activity to counteract telomere shortening, in recent years TMMs have been suggested as attractive new targets for anti-cancer therapies (26,27), particularly for those tumor types, such as MPNST, that are refractory to conventional therapeutic interventions. In this context, it is important to know whether individual MPNSTs use telomerase or ALT to maintain their telomeres. In a previously published study, telomerase activity was found to be present in about 60% of MPNST samples derived from NF1-affected patients (28). However, no information is currently available concerning the presence of ALT in MPNSTs. Taking advantage of a relatively large mono-institutional series of MPNST patients with long follow-up, in this study we propose to investigate the prevalence of telomerase activity (TA) and ALT, and whether they contribute to clinical progression in this disease.

Materials and methods

Study Population. A total of 57 lesions taken from 49 adult patients (median age 40 years, range 18-90) treated with curative intent at the Fondazione IRCCS Istituto Nazionale dei Tumori of Milan (INT) from November 1990 to January 2007 were available for analysis. Fourteen patients had NF1 with an associated MPNST, and 35 were classified as having sporadic tumors because they had no clinical signs or family history of NF1. The specimens, stored in the Institutional Tissue Bank, were consecutive with respect to the availability of frozen tissue for TMM studies and adequate clinicopathologic and follow-up information. Six patients developed recurrence and/or metastasis during the follow-up period, and the corresponding 8 lesions were collected and included in

the analysis. Patient and tumor characteristics are summarized in Table 1. The median follow-up of the entire group, was 46 months (range: 3 to 240). During the follow-up period, 23 patients died for cancer-related causes. Fourteen benign schwannoma lesions, obtained from 14 patients who underwent surgery, were included in our research for comparative purposes. The study was approved by the Institutional Review Board of INT, and each patient provided written informed consent to donate to the Institute the tissues left over after diagnostic procedures.

Table 1. Patient and tumor characteristics

	No. of patients
Total	49 (100%)
Gender:	
Female	20 (41.0%)
Male	29 (59.0%)
Syndrome:	
Sporadic	34 (69.4%)
Site	
Trunk	17 (34.7%)
Type of lesion at first presentation	
Primary	42 (85.7%)
Recurrence	4 (8.2%)
Metastasis	3 (6.1%)
Size (cm)	
Grade (FNCLCC):	
1	5 (10.2%)
2	14 (28.6%)
3	23 (46.9%)
Missing	7 (14.3%)
Post-surgery treatment	
Surgery only	20 (40.8%)
Chemotherapy	11 (22.4%)
Radiotherapy	11 (22.4%)
Chemotherapy + radiotherapy	5 (10.2%)
Missing	2 (4.1%)

Molecular studies. Normal and tumor tissues were sampled by a pathologist at the time of surgery, flash-frozen in liquid nitrogen, and stored at -80 ℃. Diagnosis and sampling adequacy were pathologically confirmed on H&E-stained slides. A fragment of 70 to 100 mg was cut from each lesion and further subdivided for APB detection, protein extraction for telomerase activity (TA) assay and DNA extraction for telomere length assessment and array-CGH.

Detection of APB. Frozen sections were cut to 5 to 7 µm thickness, fixed in 1:1 methanol/acetone, processed to detect APB by combined PML immunofluorescence and telomere fluorescence in situ hybridization (21), and independently scored by two observers. Images were captured on a Nikon Eclipse Ti fluorescence microscope using Volocity 5.3 (Perkin-Elmer) image analysis. APB status was determined according to previously defined criteria (21). The presence of an APB was defined by the localization of a telomeric DNA focus within a nuclear PML body; sections were scored as APB positive if they contained APB in ≥0.5% of tumor cells; and a tumor was considered ALT+ when at least one section was APB positive. To avoid false positives, an APB was considered to be present only when the telomeric DNA fluorescence within a PML body was more intense than that of telomeres, and a cell was not considered to contain APB if >25% of the colocalized foci occurred outside the nucleus. To avoid false negatives, at least 2,000 tumor nuclei were examined, and the assay was repeated in the presence of negative results. An ALT+ (U2OS) cell line was used as a positive control. In order to assess whether APB were present in tumor cells and not in admixed stromal cells, we simultaneously performed indirect immunofluorescence for NF1 protein on the frozen sections

using a commercial antibody (sc-67, Santa Cruz Biotechnology, Santa Cruz, CA).

Telomerase activity detection assay. TA was measured on 0.6 and 6 µg of protein by the telomeric repeat amplification protocol (TRAP) (29), with the TRAPeze kit (Intergen Company). A tumor was scored as TA+ when positive TRAP results were obtained for at least one protein concentration. In the case of tumors negative at both protein concentrations, the TRAP assay was repeated to avoid false negatives.

Terminal restriction fragment (TRF) analysis. Total DNA was isolated isolated using QuicKpicK genomic DNA kit (BioNobile), digested with *Hinf*I restriction enzyme (Promega) and further processed by pulsed-field gel electrophoresis as previously described (30). ALT status was determined by calculating whether the mean, variance, and semi-interquartile range of TRF length distribution were greater than 16 kb, 1,000 kb^2, and 4 kb, respectively. Tumors were classified as ALT+ when two of three or three of three of these criteria were met for unimodal or bimodal TRF length distributions, respectively. Statistical analysis of TRF length distributions was done with Telometric software (31).

Array CGH. The analysis was performed on genomic DNA derived from 31 MPNST (16 NF1-related, 15 sporadic) lesions, using the Agilent's Human Genome CGH Array 44K (Agilent Technologies). Total DNA was isolated with QuicKpicK genomic DNA kit (BioNobile). For each CGH hybridization, 1 µg of genomic DNA from the experimental sample and suitable reference DNA (female XX or male XY, Promega) was digested with *Alu*I and *Rsa*I restriction enzyme (Promega). Labeling reactions were performed using Agilent Genomic DNA Enzymatic Labeling Kit according to the manufacturer's instructions with a modified dNTP pool containing Cy5-dUTP (for the experimental sample) or Cy3-dUTP (for

the reference). Labeled samples and references were subsequently filtered by using a Microcon YM-30 column (Millipore). Specific activity was calculated for each sample as a ratio between dyes pmol and µg of DNA, then experimental and reference targets for each hybridization were pooled and mixed with human Cot-1 DNA (Invitrogen). The samples were loaded in an hybridization chamber, with a clean Gasket Slide and a Microarray Slide (Agilent Technologies). Then hybridization was carried out for 24 hours at 65 ℃ in a rotatin g oven. The arrays were then disassembled and washed in specific buffers (Agilent Technologies) according to manufacturer's instructions, then slides were dried and scanned using Agilent Scanner (Agilent Technologies). Microarray images were extracted and analyzed using Agilent Feature Extraction Software. Data from arrays were analyzed using Agilent CGH Analytic Software 3.4 (Agilent Technologies).

Statistical analysis. The clinical end point of this study was disease-specific survival, and the time of its occurrence was computed from the date of surgery to the time of death or censored at the date of the last recorded follow-up for living patients. Survival curves were estimated by means of the Kaplan-Meier product limit method (32), and the Cox proportional hazards model (33) was used to calculate hazard ratios (HR) and their 95% confidence interval (95% CI). Fisher's and χ^2 exact tests were used to assess the relationship between TMM status and clinico-pathological features. All P values were two-sided, and values ≤0.05 were considered statistically significant. The agreement between APB and TRF data was assessed by kappa statistics.

Results

Fifty-seven frozen tumor samples obtained from 49 patients with MPNST were assayed for the presence of TMM (Table 2). TA was detected by the TRAP assay (Fig. 1A), and tumors were defined as ALT+ on the basis of APB presence in at least 10 (0.5%) of 2,000 tumor cells (Fig. 1B). Thirty-five lesions (61.4%) expressed at least one TMM. Specifically, 14 lesions (24.6%) were TA+/ALT-, 15 (26.3%) were TA-/ALT+, and 6 (10.5%) were defined as TA+/ALT+ due to the concomitant expression of APB and TA. A consistent fraction of tested samples (22/57, 38.6%) did not express any known TMM.

In ALT-positive samples, APBs were observed in a variable but always limited fraction of cells, ranging from 0.5% to 5.7% (mean value, 1.29%). Specifically, the percentage of APB-positive cells ranged from 0.59 to 5.7% (mean value, 1.4%) and from 0.5 to 2.15% (mean value, 0.99) in TA-/ALT+ and TA+/ALT+ group, respectively. For all 57 samples, results of TRF analysis (Fig. 1C) were also available and we found concordance between APB and TRF results in defining the ALT status in 44 cases (77.2%; kappa= 0.437; 95% CI, 0.215-0.659; P<0.0001). Specifically, 8 lesions (14.0%) were defined as ALT+ and 36 (63.2%) were defined as ALT- with both detection methods, while the remaining 13 lesions (22.8%) were defined as ALT+ on the basis of APB expression but did not show a TRF length distribution suggestive of ALT.

Table 2: Telomerase activity and ALT mechanisms in MPNST lesions.

Case		Lesion		TA	APB	TRF length
1	NF1	1	R	+	-	-
2	NF1	2A	M	+	-	-
		2B	M	+	+	-
3	NF1	3A	R	+	+	-
		3B	R	-	-	-
4	NF1	4A	P	+	-	-
		4B	R	-	+	-
5	NF1	5A	P	-	+	-
		5B	R	-	-	-
6	NF1	6	P	+	-	-
7	NF1	7	P	-	-	-
8	NF1	8	P	+	-	-
9	NF1	9	P	-	+	+
10	NF1	10	P	+	-	-
11	NF1	11	P	+	-	-
12	NF1	12	P	+	+	-
13	NF1	13	P	-	-	-
14	NF1	14	P	-	-	-
15	NF1	15	P	-	+	+
16	sporadic	16	P	-	-	-
17	sporadic	17	P	-	+	+
18	sporadic	18A	R	+	-	-
		18B	R	-	+	-
		18C	R	-	-	-
		18D	R	-	-	-
19	sporadic	19	R	-	+	-
20	sporadic	20	P	-	-	-
21	sporadic	21	M	+	+	-
22	sporadic	22	P	-	+	-
23	sporadic	23A	P	-	-	-
		23B	M	-	+	+
24	sporadic	24	P	+	+	-
25	sporadic	25	P	-	-	-
26	sporadic	26	M	+	-	-
27	sporadic	27	P	-	+	+
28	sporadic	28	P	-	-	-
29	sporadic	29	P	-	+	+
30	sporadic	30	P	+	-	-
31	sporadic	31	P	-	+	-
32	sporadic	32	P	-	-	-
33	sporadic	33	P	-	+	-
34	sporadic	34	P	+	-	-
35	sporadic	35	P	+	-	-
36	sporadic	36	P	+	+	+
37	sporadic	37	P	-	-	-
38	sporadic	38	P	-	-	-
39	sporadic	39	P	+	-	-
40	sporadic	40	P	-	-	-
41	sporadic	41	P	-	-	-
42	sporadic	42	P	-	+	+
43	sporadic	43	P	-	-	-
44	sporadic	44	P	-	+	-
45	sporadic	45	P	-	-	-
46	sporadic	46	P	+	-	-
47	sporadic	47	P	-	-	-
48	sporadic	48	P	-	-	-
49	sporadic	49	P	-	-	-

NF1: Neurofibromatosis type 1, P: primary disease, R: recurrent locoregional tumor, M: metastatic lesion, TA: telomerase activity, APB: alternative lengthening of telomeres assessed on the presence of APB (ALT-associated promyelocytic leukaemia nuclear bodies), TRF: alternative lengthening of telomeres assessed on the basis of terminal restriction fragment length distribution.

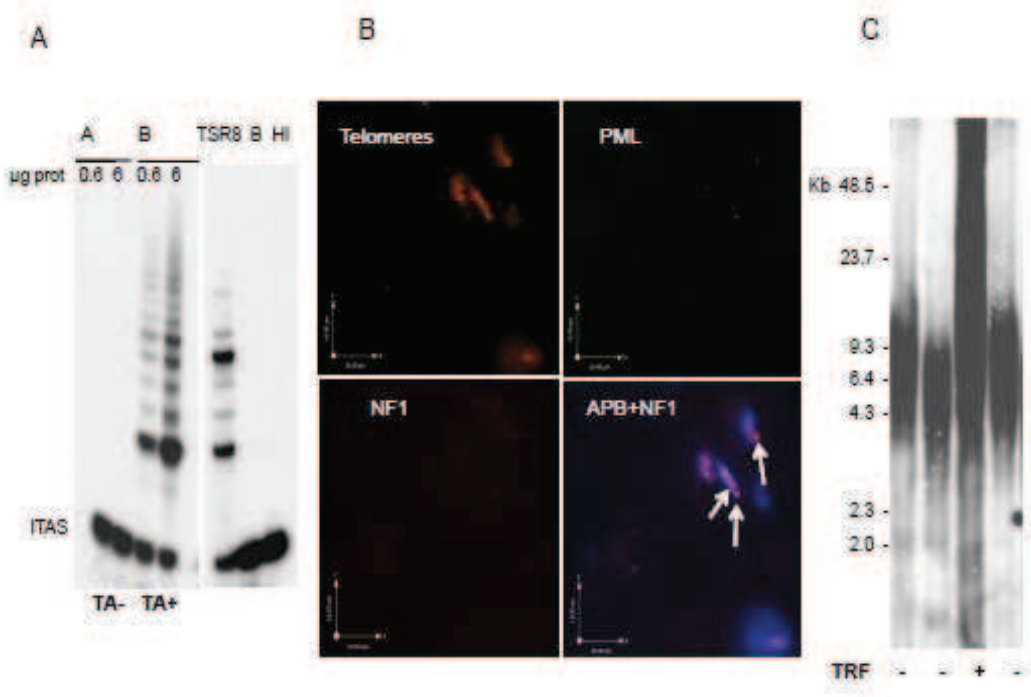


Figure 1. *(**A**) Two representative specimens of MPNST in which telomerase activity was detected by the TRAP assay using 0.6 and 6 µg of protein. The location of the internal amplification standard (ITAS) is indicated. (**B**) APB assay in MPNST: combined PML immunofluorescence and telomere fluorescence in situ hybridization in a frozen section of a representative ABP-positive MPNST sample. Indirect immunofluorescence was used for the PML protein (FITC label, green stain); telomere FISH was done using a Cy3 conjugated telomeric peptide nucleic acid probe (red stain); nuclei were counterstained with 4',6-diamidino-2-phenylindole, DAPI (blue stain); the foci of telomeric DNA that colocalize with PML represent APBs (white arrows). (**C**). Terminal restriction fragment (TRF) profiles of four representative MPNST specimens.*

The presence of ALT, as determined on the basis of APB presence, was unrelated to gender (*P*=0.966), age (*P*=0.201), tumor location (*P*=0.826), grade (*P*=0.999), size of the lesion (p=1.000) and NF1 syndrome (*P*= 0.964). Such findings were also observed when ALT was determined on the basis of TRF analysis (data not shown). As regards TA, it did not prove to correlate with gender (*P* = 0.298), age (*P*=0.636), tumor location (*P*= 1.000), grade (*P* = 0.603) and size of the lesion (P=0.885). However, TA was found to be more frequently expressed in samples from patients with NF1-related MPNST than in those with sporadic disease (60.0 *vs* 29.4%, *P*= 0.087).

As far as the 14 benign schwannoma lesions are concerned, none of them expressed TA whereas 2 lesions were scored as ALT+ on the basis of APBs (0.52% and 1.11% APB-positive cells), although they did not show a TRF distribution pattern consistent with ALT.

Array CGH data, obtained in a subset of 31 MPNST samples, both sporadic and NF1-associated, exhibited significant DNA copy number changes, with variations in size, span and nature of the aberrations on the whole genome (data not shown). In particular, relevant to TMMs, we could detect a copy number gain of hTERT gene on chromosome 5 in 20/31 (65%) samples, with a similar percentage in NF1-associated (69%) and sporadic (60%) MPNSTs (Fig. 2A). When considering the prevalence of hTERT aberration in relation to the TMM operating in the tumor, we found a slightly higher frequency of copy number gain of hTERT gene in TA+/ALT- tumors (7/10, 70%) that in TA-/ALT+ tumors (5/9, 56%). In addition, aCGH results for NF1 gene on chromosome 17q11 revealed no significative differences between NF1-associated and sporadic tumors with a copy number loss of NF1 gene in 9/16 (56%) of NF1 related tumors and in 8/15 (53%) sporadic lesions (Fig. 2B).

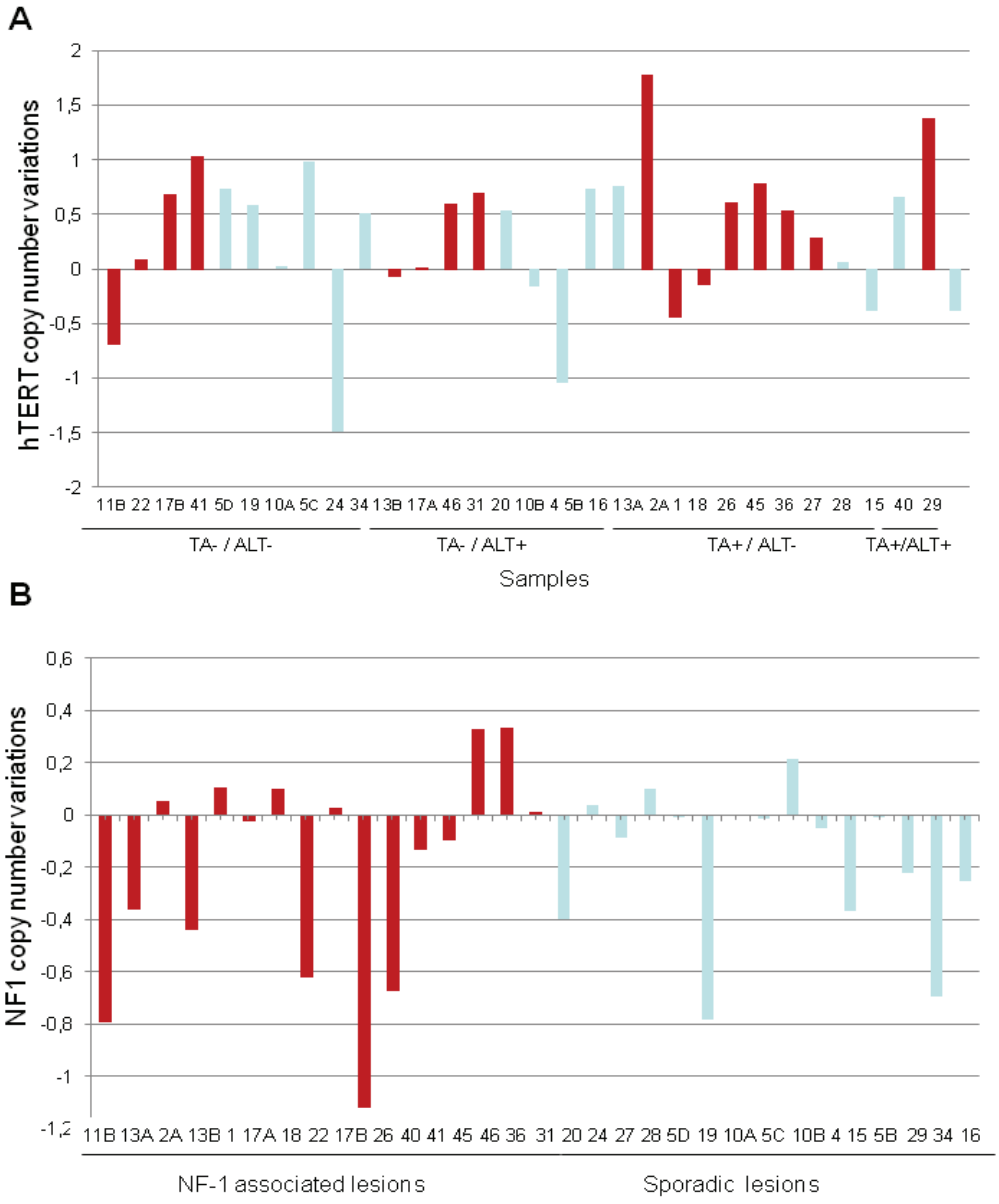


***Figure 2**. Copy number variation of hTERT gene (**A**) and NF1 gene (**B**) in a subset of 31 MPNSTs. Samples arising in the context of NF1 syndrome are highlighted in red.*

Clinical outcome was analyzed on the overall series of 49 MPNST patients. After 5 years of follow-up, 22 patients had died of the disease, and only 1 additional patient died in the 5 to 10 years interval, which corresponds to survival estimates of 47% and 39%, respectively. From the survival analyses, we found a correlation between the degree of surgical excision and disease specific survival in univariable analysis (Margin status: positive (negative): HR 4.11, 95% CI 1.67-10.10, p=0.002) (Table S1).

Conversely, age, presence of NF1 syndrome, location of the tumor, size, and grade did not show any statistically significant correlation with patients' prognosis (Table S1). As far as the prognostic relevance of TMMs is concerned, for 6 patients who experienced progressive disease (recurrence or metastasis), and whose ALT/TA status changed during the course of the disease, the appearance of any TMM defined the final phenotype (e.g., if metachronous lesions were ALT+ and ALT-, the patient would be categorized as ALT+). At 5 years of follow-up, TA proved to be significantly associated with disease-specific mortality. Specifically, results obtained from univariable analysis showed that patients with TA+ tumors had a significantly lower probability of being alive than patients with TA− (Hazard Ratio [HR], 3.78; 95% CI 1.60-8.95; *P* = 0.002) (Fig.3A). Such findings held true also when adjusted for the presence of NF1 syndrome (HR, 4.22; 95% CI, 1.804-9.847; *P* = 0.001) and for the margin status after surgical excision (HR, 5.78; 95% CI, 2.19-15.26; p<0.001) (Table S1). Conversely, ALT alone did not prove to be associated with disease-specific mortality either using APB expression (HR,1.25; 95% CI 0.54-2.89; *P*=0.605) (Fig. 3B) or TRF distribution analysis (HR, 0.57; 95% CI 0.17-1.96; *P*=0.375) to define ALT status.

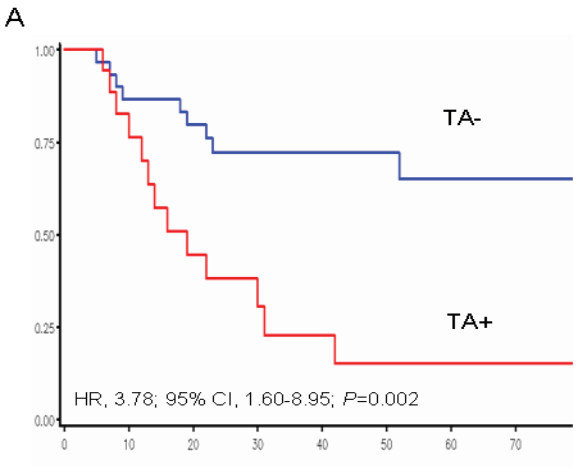
Table S1

Univariable analysis

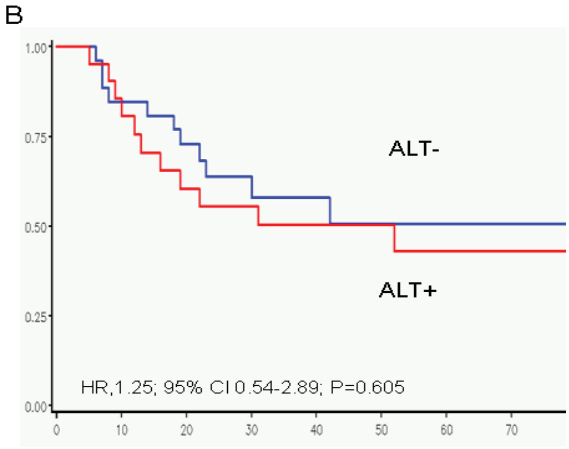
Category (reference)	**HR**	**95%CI**	**p**
TA: present (absent)	3.78	1.60-8.95	0.002
APB: present (absent)	1.25	0.54-2.89	0.605
TRF: present (absent)	0.57	0.17-1.96	0.375
Age: ≤40 years (>40 years)	0.97	0.42-2.26	0.949
Syndrome: NF1 (sporadic)	0.96	0.40-2.63	0.956
Tumor site: trunk (extremity)	0.88	0.36-2.17	0.788
Tumor size: >5cm (≤5cm)	5.75	0.77-43.11	0.089
Margin status: positive (negative)	4.11	1.67-10.10	0.002
Tumor grade: grade 2 (grade 1)	1.88	0.22-16.12	0.563
grade 3 (grade 1)	3.47	0.45-26.77	0.233
grade 3 (grade 1+2)	2.11	0.79-5.67	0.138

Bivariable analysis adjusted for margin status

Category (reference)	**HR**	**95%CI**	**p**
TA: present (absent)	5.78	2.19-15.26	<0.001
APB: present (absent)	1.36	0.57-3.25	0.495
TRF: present (absent)	0.63	0.20-2.02	0.436



Patients at risk at:		
month	0	60
TA-	30	7
TA+	19	2



Patients at risk at:		
month	0	60
ALT-	28	4
ALT+	21	5

Figure 3. *Disease-specific survival as a function of TA (**A**) and ALT (**B**) in MPNST patients.*

Discussion

This is the first report of a comparative analysis of the expression and clinical relevance of the two currently known TMMs, ALT and TA, in a monoinstitutional series of MPNSTs, including both sporadic and NF1-associated tumors. Overall, TA was detected in 35.1% of the lesions, although it was found to be more frequently expressed in samples from patients with NF1-associated MPNST than in those with sporadic disease (60.0 *vs* 29.4%). This observation is consistent with previous data from Mantripragada *et al*. (28), who recorded TA in 61% of MPNST samples derived from NF1-affected patients. The authors also showed that all TA+ lesions were high grade MPNSTs. Conversely, in our case series we were able to detect TA also in a low grade sporadic MPNST lesion (out of 5 grade 1 lesions tested). Through a genome–wide high resolution analysis of DNA copy number alterations in NF1-associated MPNSTs and benign tumors, the same research group (29) was able to reveal amplification of the hTERT gene in malignant tumors but not in benign lesions. Array CGH results we generated in a subset of 31 MPNSTs showed that such an amplification was also present in sporadic MPNSTs, in a percentage of cases superimposable to that found in NF1-associated tumors (60% *vs* 69%). The similar genetic alterations we observed in sporadic and NF1 associated MPNST (as outlined in hTERT and NF1 gene) are not surprising. Indeed Brekke et al. (Brekke HR, Ribeiro FR, Kolberg M, Agesen TH, Lind GE, Eknaes M, Hall KS, Bjerkehagen B, van den Berg E, Teixeira MR, Mandahl N, Smeland S, Mertens F, Skotheim RI, Lothe RA. Genomic changes in chromosomes 10, 16, and X in malignant peripheral nerve sheath tumors identify a high-risk patient group. J Clin Oncol. 2010 Mar 20;28(9):1573-82) recently suggested that the genetic events leading to

MPNST are comparable in NF-related and sporadic tumors. It is noteworthy that the observed amplification of hTERT gene is in agreement with gene up-regulation in MPNSTs, as previously detected by real-time RT-PCR (34). No clear correlation between hTERT copy number gain and TA was evident since such a genomic alteration was also found in half ALT+ lesions as well as in a high percentage of TA-/ALT- tumors.

ALT was found in 36.8% of cases when APB expression was used as the detection approach, and the percentage of ALT+ tumors decreased to 14% when the ALT phenotype was determined on the basis of TRF length distribution. The incomplete overlapping of the results obtained with the two methods is not surprising. In fact, while the APB assay allows the analysis of individual tumor cells, the TRF pattern could be misleading due to the admixture of normal and tumor cells present in the specimens. However, based on available data, the concordance rate between the two assays seem to be dependent on the tumor type. Specifically, the 77.2% concordance rate between the two approaches in defining MPNST specimens as ALT+ or ALT- is superimposable to that (77.6%) we previously observed in a series of 85 liposarcoma specimens that were comparatively assayed for ALT with the two detection methods (35). Conversely, Henson *et al.* (21) reported a complete agreement in the results of the two assays in glioblastoma multiforme.

In six patients, more than one lesion was available for TMM investigation. In three cases in which both primary tumor and subsequent lesions were studied, the TA status was concordant (Table2, case 2, 5 and 23), while for the other 3 patients TA status changed during the course of the disease (Table2, case 3, 4 and 18).

As far as ALT status is concerned, the presence of APB varied in all the patients with metachronous lesions, while TRF phenotype remained stable in 5 out of 6 patients (Table2).

The observation that MPNSTs utilize either TA or ALT to maintain their telomeres suggest that subsets of tumors can undergo different pathways of tumorigenesis. In this context, we previously demonstrated that distinct gene expression signatures can distinguish tumors and cell lines that are TA+ from those that are ALT+ (36).

As already reported for other tumor types (17,18,20,22,23), in a few MPNSTs we found a concomitant expression of TA and ALT confirming the possibility that the two TMMs can coexist in the same lesion. However, at present it is unclear whether TA and ALT can be present within the same tumor cell or whether a given tumor lesion may contain distinct ALT+ and TA+ subpopulations. On the other hand, we found that a significant percentage of MPNSTs (~40%) possessed no apparent TMM despite being informative for the different assays, suggesting that the presence of a constitutively active TMM is not a stringent requirement for a subset of MPNSTs, or, alternatively, that these tumors use a mechanism that has not yet been identified. Moreover, such a lack of any known TMM, previously observed also in subsets of other tumor types (17, 18, 22, 23), is in accord with experimental data suggesting that TMM acquisition is not always required for malignant transformation of normal human cells (37). However, the possibility that the lack of TMM expression observed in a high percentage of the tested MPNSTs may be related, at least in part, to the sensitivity of the assays used cannot be excluded.

The activation of a TMM is able to overcome replicative senescence induced by cell division-associated telomere attrition. In a

previous study, carried out in a series of mesenchymal tumors, including 16 MPNSTs, and aimed to understand the contribution of senescence signaling to the biology of these tumors, we used a senescence scoring approach based on expression profiling of well-defined senescence markers as a means to evaluate latent senescence pathways. Specifically, a DNA damage associated signature (DAS) and a modified secretory senescence signature (mSS) were used. Results we obtained indicated that, at an individual tumor level, DAS and mSS scores were not correlated, suggesting that senescence phenotypes may be differentially activate during transformation (38).

Concerning benign schwannomas, we did not found evidence of TA in any of the 14 samples tested, in agreement with previous data by Chen *et al.* (39) who reported as TA- all 30 schwannoma samples considered in their study. The authors also characterized the specimens in term of TRF length distribution analysis and could identify 4 lesions having elongated telomeres: 3 of them showed an aggressive clinicopathological behaviour. In addition, one patient died of the disease, and one experienced a clinical recurrence. In our case series, no schwannoma lesion could be defined as ALT+ on the basis of TRF results, but two lesions were defined as ALT+ based on APB expression. Unfortunately, since no follow-up data are available for these patients, we cannot estimate the possible relevance of ALT phenotype on the clinical outcome.

As far as the prognostic relevance of TMMs in MPNST is concerned, TA proved to be prognostic for disease-specific survival. Specifically, patients with TA+ tumors had a significantly lower probability of being alive at 5 years after surgery than patients with TA- tumors. In addition, TA proved to be a strong prognostic discriminant of

increased mortality even when adjusted for the concomitant presence of NF1 syndrome and for margin status after surgical excision. Results available in the literature indicate an association between telomerase activity/expression and poor prognosis in different tumor types, including breast, non-small cell lung cancer, gastric and colorectal cancer, and neuroblastoma (40), although not all the published studies confirmed such an association.

In our series of MPNST patients, ALT failed to significantly affect clinical outcome, independently of the detection method used. Based on available information, the association of ALT with patient prognosis seems to be disease related. In glioblastoma multiforme, a better survival for patients with a ALT+ tumor was consistently observed in three studies in which ALT phenotipe was detected by TRF analysis or the presence of APB (17, 21, 24). On the opposite, in liposarcoma patients, we found that ALT proved to be a stronger prognostic discriminant of increased mortality than TA in both univariable and multivariable analysis (22). Again, we reported a negligible role for ALT in the prognosis of patients with diffuse malignant peritoneal mesothelioma (23). A possible tentative explanation for the different outcomes may be that ALT activation results from different sets of tumor-specific genetic changes that are correlated with a better or worse prognosis as a function of the tumor type.

The lack of reliable therapeutic options, other than complete surgery, for MPNST together with our observation that this tumor may utilize either TA or ALT to maintain telomeres, suggests the opportunity to consider TMMs as new therapeutic targets for the disease. In this context, the characterization of the TMM operating in individual tumors could allow the identification of patients suitable for treatment with anti-

telomerase drugs which are currently used in the clinical practice (41). In addition, preclinical evidence indicating that ALT+ tumor models are sensitive to compounds that induce telomere dysfunction by binding to G-quadruplex structures in telomeric DNA and inhibit tumor growth through a mechanism largely independent of the presence of active telomerase (27), would suggest the possibility, in the near future, to treat ALT+ MPNST patients with these compounds as soon as they reach the clinical setting.

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References

1. Abreu E, Aubert S, Wavreille G, Gheno R, Canella C, Cotten A. Peripheral tumor and tumor-like neurogenic lesions. Eur J Radiol. 2011 May 9. [Epub ahead of print] PubMed PMID: 21561733.
2. Perrin RG, Guha A. Malignant peripheral nerve sheath tumors. Neurosurg Clin N Am 2004;15:203–216.
3. Ferner RE, Gutmann DH. International consensus statement on malignant peripheral nerve sheath tumors in neurofibromatosis. Cancer Res. 2002 Mar 1;62(5):1573-7.
4. Evans DG, Baser ME, McGaughran J, Sharif S, Howard E, Moran A. Malignant peripheral nerve sheath tumours in neurofibromatosis 1. J Med Genet. 2002 May;39(5):311-4.
5. Woodruff JM, Selig AM, Crowley K, Allen PW. Schwannoma (neurilemoma) with malignant transformation. A rare, distinctive peripheral nerve tumor. Am J Surg Pathol. 1994 Sep;18(9):882-95.
6. Grobmyer SR, Reith JD, Shahlaee A et al. Malignant Peripheral Nerve Sheath Tumor: Molecular Pathogenesis and Current Management Considerations Journal of Surgical Oncology 2008; 97:340–349.
7. Holtkamp N, Atallah I, Okuducu AF, et al. MMP-13 and p53 in the progression of malignant peripheral nerve sheath tumors. Neoplasia. 2007 Aug;9(8):671-7.
8. Birindelli S, Perrone F, Oggionni M, et al. Rb and TP53 pathway alterations in sporadic and NF1-related malignant peripheral nerve sheath tumors. Lab Invest. 2001 Jun;81 (6):833-44.
9. Upadhyaya M, Kluwe L, Spurlock G, et al. Germline and somatic NF1 gene mutation spectrum in NF1-associated malignant peripheral nerve sheath tumors (MPNSTs). Hum Mutat. 2008 Jan;29(1):74-82.
10. Mantripragada KK, de Ståhl TD, Patridge C, et al. Genome-wide high-resolution analysis of DNA copy number alterations in NF1-associated malignant peripheral nerve sheath tumors using 32K BAC array. Genes Chromosomes Cancer. 2009 Oct; 48(10):897-907.
11. Katz D, Lazar A, Lev D. Malignant peripheral nerve sheath tumour (MPNST): the clinical implications of cellular signalling pathways. Expert Rev Mol Med. 2009 Oct 19;11:e30.

12. Gilson E, Géli V. How telomeres are replicated. Nat Rev Mol Cell Biol. 2007 Oct;8(10):825-38.
13. Blackburn EH. Telomeres and telomerase: the means to the end (Nobel lecture). Angew Chem Int Ed Engl. 2010 Oct 4;49(41):7405-21.
14. Cesare AJ, Reddel RR. Alternative lengthening of telomeres: models, mechanisms and implications. Nat Rev Genet. 2010 May;11(5):319-30.
15. Mitchell M, Gillis A, Futahashi M, Fujiwara H, Skordalakes E. Structural basis for telomerase catalytic subunit TERT binding to RNA template and telomeric DNA. Nat Struct Mol Biol. 2010 Apr;17(4):513-8.
16. Shay JW, Bacchetti S. A survey of telomerase activity in human cancer. Eur J Cancer. 1997 Apr;33(5):787-91. Review.
17. Hakin-Smith V, Jellinek DA, Levy D, et al. Alternative lengthening of telomeres and survival in patients with glioblastoma multiforme. Lancet. 2003 Mar 8;361(9360):836-8.
18. Ulaner GA, Huang HY, Otero J, et al. Absence of a telomere maintenance mechanism as a favourable prognostic factor in patients with osteosarcoma. Cancer Res 2003;63:1759–63
19. Ulaner GA, Hoffman AR, Otero J, et al. Divergent patterns of telomere maintenance mechanisms among human sarcomas: sharply contrasting prevalence of the alternative lengthening of telomeres mechanism in Ewing's sarcomas and osteosarcomas. Genes Chromosomes Cancer 2004;41:155–62.
20. Johnson JE, Varkonyi RJ, Schwalm J, et al. Multiple mechanisms of telomere maintenance exist in liposarcomas. Clin Cancer Res. 2005 Aug 1;11(15):5347-55.
21. Henson JD, Hannay JA, McCarthy SW, et al. A robust assay for alternative lengthening of telomeres in tumors shows the significance of alternative lengthening of telomeres in sarcomas and astrocytomas. Clin Cancer Res 2005;11:217-25.
22. Costa A, Daidone MG, Daprai L, et al. Telomere maintenance mechanisms in liposarcomas: association with histologic subtypes and disease progression. Cancer Res. 2006 Sep 1;66(17):8918-.
23. Villa R, Daidone MG, Motta R, et al. Multiple mechanisms of telomere maintenance exist and differentially affect clinical outcome in diffuse malignant peritoneal mesothelioma. Clin Cancer Res. 2008 Jul 1;14(13):4134-40.
24. McDonald KL, McDonnell J, Muntoni A, et al. Presence of alternative lengthening of telomeres mechanism in patients with glioblastoma identifies a less

aggressive tumor type with longer survival. J Neuropathol Exp Neurol. 2010 Jul;69(7):729-36.

25. Henson JD, Cao Y, Huschtscha LI, et al. DNA C-circles are specific and quantifiable markers of alternative-lengthening-of-telomeres activity. Nat Biotechnol. 2009 Dec;27(12):1181-5.
26. Shay JW, Keith WN. Targeting telomerase for cancer therapeutics. Br J Cancer. 2008 Feb 26;98(4):677-83. Epub 2008 Jan 29. Review.
27. Folini M, Venturini L, Cimino-Reale G, Zaffaroni N. Telomeres as targets for anticancer therapies. Expert Opin Ther Targets. 2011 May;15(5):579-93.
28. Mantripragada KK, Caley M, Stephens P, et al. Telomerase activity is a biomarker for high grade malignant peripheral nerve sheath tumors in neurofibromatosis type 1 individuals. Genes Chromosomes Cancer. 2008 Mar; 47(3):238-46.
29. Kim NW, Piatyszek MA, Prowse KR, et al. Specific association of human telomerase activity with immortal cells and cancer. Science 1994;266:2011-5.
30. Villa R, Folini M, Perego P, et al. Telomerase activity and telomere length in human ovarian cancer and melanoma cell lines: correlation with sensitivity to DNA damaging agents. Int J Oncol. 2000 May;16(5):995-1002.
31. Grant JD, Broccoli D, Muquit M, Manion FJ, Tisdall J, Ochs MF. Telometric: a tool providing simplified, reproducible measurements of telomeric DNA from constant field agarose gels. Biotechniques. 2001 Dec;31(6):1314-6, 1318.
32. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. J Am Stat Assoc 1958;53: 457-81.
33. Cox DR. Regression models and life tables. JR Stat Soc B1972;34:187-220.
34. Lévy P, Vidaud D, Leroy K, et al. Molecular profiling of malignant peripheral nerve sheath tumors associated with neurofibromatosis type 1, based on large-scale real-time RT-PCR. Mol Cancer. 2004 Jul 15;3:20.
35. Venturini L, Motta R, Gronchi A, Daidone M, Zaffaroni N. Prognostic relevance of ALT-associated markers in liposarcoma: a comparative analysis. BMC Cancer. 2010 Jun 3;10:254.
36. Lafferty-Whyte K, Cairney CJ, Will MB, et al. A gene expression signature classifying telomerase and ALT immortalization reveals an hTERT regulatory network and suggests a mesenchymal stem cell origin for ALT. Oncogene. 2009 Oct 29;28(43):3765-74.

37. Seger YR, Garcia-Cao M, Piccinin S, et al. Transformation of normal human cells in the absence of telomerase activation. Cancer Cell 2002;2:401–13.

38. Lafferty-Whyte K, Bilsland A, Cairney CJ, et al. Scoring of senescence signalling in multiple human tumour gene expression datasets, identification of a correlation between senescence score and drug toxicity in the NCI60 panel and a pro-inflammatory signature correlating with survival advantage in peritoneal mesothelioma. BMC Genomics. 2010 Oct 1;11:532.

39. Chen HJ, Cho CL, Liang CL, Lu K, Lin JW. Implication of telomere length as a proliferation-associated marker in schwannomas. J Surg Oncol. 2002 Oct;81(2):93-100.

40. Hiyama E, Hiyama K. Telomerase as tumor marker. Cancer Lett. 2003 May 15;194(2):221-33. Review.

41. Harley CB. Telomerase and cancer therapeutics. Nat Rev Cancer. 2008 Mar;8(3):167-79. Review.

PART III

Supplementary table 1
Characteristics of peritoneal mesothelioma specimens included for miRNA expression profiling.

	subtype	TA	ALT
MP10	Epithelial	+	-
MP14	Epithelial	-	+
MP18	Epithelial	+	-
MP19	Epithelial	+	-
MP2	Epithelial	+	-
MP20	Epithelial	+	-
MP21	Epithelial	+	+
MP22	Epithelial	+	-
MP25	Epithelial	+	-
MP27	Epithelial	-	+
MP28	Biphasic	+	-
MP29	Epithelial	-	+
MP3	Biphasic	+	-
MP31	Epithelial	-	+
MP32	Epithelial	-	+
MP33	Epithelial	-	+
MP36	Epithelial	-	-
MP37	Epithelial	-	-
MP39	Epithelial	-	-
MP4	Epithelial	+	-
MP41	Biphasic	-	-
MP42	Epithelial	+	-
MP43	Epithelial	+	-
MP46	Epithelial	-	-
MP48A	Epithelial	-	-
MP51	Epithelial	+	+
MP52	Epithelial	-	-
MP53	Epithelial	+	-
MP54	Epithelial	-	-
MP55	Epithelial	-	+
MP57	Epithelial	+	-
MP58	Epithelial	-	-
MP59	/	-	+
MP60	Epithelial	+	-
MP62	Epithelial	+	+
MP63	Epithelial	-	-
MP64	Epithelial	+	-
MP65	Epithelial	+	-
MP66	/	-	-
MP67	Epithelial	+	-
MP68	Epithelial	+	-
MP69	Epithelial	-	-
MP70A	Epithelial	-	-
MP71	Epithelial	-	-
MP72	Epithelial	-	-
MP73	Epithelial	+	-
MP74	Epithelial	+	-
MP76	Epithelial	-	+
MP7A	/	+	-
MP8A	Epithelial	+	-

Supplementary table 2

miRNA which are differentially expressed in peritoneal mesothelioma among TA-positive and TA-negative classes. Number of miRNAs significant at 0.05 level of the univariate test: 84, sorted by p-value of the univariate test. FDR: false discovery rate.

	Parametric p-value	FDR	Geom mean of intensities in class TA-	Geom mean of intensities in class TA+	Fold-change	Gene symbol
1	9,52E-05	0,0488	1100,15	858,25	1,28	hsa-miR-1305
2	9,59E-05	0,0488	245,54	182,69	1,34	hsa-miR-380*
3	0,0012936	0,378	215,22	181,83	1,18	HS_177
4	0,001585	0,378	435,7	347,91	1,25	HS_40
5	0,0018575	0,378	1149,88	923,53	1,25	hsa-miR-548a-3p
6	0,0030268	0,436	673,41	580,91	1,16	hsa-miR-609
7	0,0030576	0,436	939,92	755,28	1,24	hsa-miR-1257
8	0,0036501	0,436	1008,98	1911,7	0,53	hsa-miR-200b*
9	0,0040117	0,436	453,06	345,22	1,31	hsa-miR-1231
10	0,0042876	0,436	1256,95	1008,88	1,25	HS_152
11	0,0063244	0,506	1105,79	872,33	1,27	HS_19
12	0,0064986	0,506	165,22	138,94	1,19	hsa-miR-122*
13	0,0081354	0,506	14641,83	12479,86	1,17	hsa-miR-34a*
14	0,0083139	0,506	322,46	280,37	1,15	hsa-miR-1262
15	0,0083324	0,506	951,28	801,43	1,19	HS_150
16	0,0097529	0,506	803,48	707,74	1,14	HS_268
17	0,0114723	0,506	3452,93	2768,71	1,25	HS_275
18	0,0115634	0,506	578,11	501,25	1,15	HS_145.1
19	0,0124729	0,506	183,03	157,14	1,16	hsa-miR-1261
20	0,0132116	0,506	38054,89	34839,16	1,09	hsa-miR-100
21	0,0133077	0,506	1166,18	1019,92	1,14	hsa-miR-519b-3p
22	0,0134169	0,506	217,9	251,41	0,87	hsa-miR-589*
23	0,013476	0,506	510,32	389,53	1,31	hsa-miR-548m
24	0,0137887	0,506	783,76	667,06	1,17	solexa-7509-112
25	0,0147717	0,506	288,27	259,61	1,11	hsa-miR-943
26	0,0153529	0,506	1187,58	996,66	1,19	HS_184
27	0,015627	0,506	1987,68	3430,44	0,58	hsa-miR-429
28	0,0166338	0,506	612,45	531,59	1,15	hsa-miR-1207-5p
29	0,0168759	0,506	4721,28	7219,19	0,65	hsa-miR-200b
30	0,0185308	0,506	25395,02	29684,39	0,86	hsa-miR-1280
31	0,0186846	0,506	1389,48	1175,32	1,18	hsa-miR-194*
32	0,0187286	0,506	8946,76	7885,13	1,13	HS_96
33	0,0187922	0,506	4483,61	2734,44	1,64	hsa-miR-136*
34	0,0192058	0,506	12461,33	14043,52	0,89	hsa-miR-339-5p
35	0,0202519	0,506	530,97	472,21	1,12	hsa-miR-518c*
36	0,0202856	0,506	209,22	185,39	1,13	hsa-miR-875-3p
37	0,0205458	0,506	726,95	434,1	1,67	hsa-miR-876-3p

38	0,0218162	0,506	486,05	435,5	1,12	hsa-miR-525-5p
39	0,0221374	0,506	257,08	219,77	1,17	hsa-miR-297
40	0,0225651	0,506	1805,41	1160,01	1,56	hsa-miR-376a*:9.1
41	0,0227294	0,506	1318,76	936,76	1,41	hsa-miR-485-5p
42	0,0228816	0,506	2162,63	1769,64	1,22	hsa-miR-1267
43	0,0246552	0,506	262,61	363,04	0,72	HS_5.1
44	0,0251764	0,506	264,44	234,02	1,13	hsa-miR-525-3p
45	0,0260759	0,506	491,89	643,16	0,76	hsa-miR-1228
46	0,026207	0,506	2551,13	1456,46	1,75	hsa-miR-136
47	0,026225	0,506	336,13	283,07	1,19	hsa-miR-186*
48	0,0263388	0,506	250,28	343,76	0,73	HS_287
49	0,026655	0,506	130,87	168,31	0,78	HS_58
50	0,0269067	0,506	929,68	794,66	1,17	hsa-miR-641
51	0,0269738	0,506	6161,36	5549,49	1,11	hsa-miR-129-5p
52	0,0272279	0,506	2341,55	1757,78	1,33	hsa-miR-369-5p
53	0,0276099	0,506	1666	1342,63	1,24	hsa-miR-1225-3p
54	0,027747	0,506	2962,66	2176,96	1,36	hsa-miR-125b-1*
55	0,0277875	0,506	190,71	216,98	0,88	HS_162
56	0,0278092	0,506	1398,4	1100,18	1,27	HS_81
57	0,0302248	0,523	499,49	449,32	1,11	hsa-miR-367*
58	0,0303167	0,523	914,49	1242,46	0,74	hsa-miR-215
59	0,0306111	0,523	305,46	329,12	0,93	hsa-miR-548g
60	0,0310285	0,523	1203,89	1012,57	1,19	hsa-miR-449b
61	0,0313481	0,523	32494,34	35156,01	0,92	hsa-miR-148a
62	0,0318912	0,524	2086,94	3165,55	0,66	hsa-miR-1275
63	0,0325853	0,527	194,45	154,93	1,26	hsa-miR-135a*
64	0,0332359	0,529	116,15	127,18	0,91	hsa-miR-767-3p
65	0,0348689	0,546	1069,72	689,65	1,55	hsa-miR-369-3p
66	0,0363088	0,56	717,15	600,22	1,19	hsa-miR-1184
67	0,0390608	0,579	19964,74	23945,98	0,83	hsa-miR-224
68	0,0391414	0,579	2878	2427,31	1,19	HS_244
69	0,0392193	0,579	508,76	449,38	1,13	hsa-miR-222*
70	0,0411315	0,592	876,72	751,33	1,17	hsa-miR-1286
71	0,0422127	0,592	3126,75	2108,13	1,48	hsa-miR-382
72	0,042213	0,592	408,55	360,13	1,13	hsa-miR-583
73	0,0424946	0,592	393,13	357,23	1,1	hsa-miR-190
74	0,0430213	0,592	2774,26	2124,73	1,31	hsa-miR-493*
75	0,0439799	0,594	1326,84	1050,31	1,26	hsa-miR-218-1*
76	0,0457048	0,594	597,27	766,63	0,78	hsa-miR-92a-1*
77	0,0458423	0,594	2537,08	1615,22	1,57	hsa-miR-379
78	0,0465555	0,594	541,9	468,18	1,16	hsa-miR-596
79	0,046902	0,594	8228,26	9480,15	0,87	hsa-miR-1228*
80	0,0476679	0,594	295,38	267,88	1,1	hsa-miR-30b*
81	0,047976	0,594	8592,05	11687,88	0,74	hsa-miR-200a
82	0,0480201	0,594	116,06	102,11	1,14	hsa-miR-630
83	0,0487252	0,594	1033,62	1389,16	0,74	hsa-miR-296-3p
84	0,0489885	0,594	2360,06	2895,99	0,81	hsa-miR-550*

Supplementary table 3

miRNA which are differentially expressed in peritoneal mesothelioma among ALT-positive and ALT-negative classes. Number of miRNAs significant at 0.05 level of the univariate test: 61, sorted by p-value of the univariate test. FDR: false discovery rate.

	Parametric p-value	FDR	Geom mean of intensities in class ALT-	Geom mean of intensities in class ALT+	Fold-change	Gene symbol
1	0,00063	0,641	4316,23	2906,86	1,48	hsa-miR-188-5p
2	0,003956	0,764	386,62	599,33	0,65	hsa-miR-380
3	0,004502	0,764	2228,27	4164,72	0,54	hsa-miR-382
4	0,00467	0,764	13731,25	11641,35	1,18	hsa-miR-339-5p
5	0,006003	0,764	4050,26	5409,09	0,75	hsa-miR-624*
6	0,006972	0,764	619,42	425,76	1,45	hsa-miR-1228
7	0,007661	0,764	265,79	303,42	0,88	hsa-miR-943
8	0,008873	0,764	95,38	114,67	0,83	hsa-miR-520f
9	0,009797	0,764	1865,46	2745,29	0,68	hsa-miR-369-5p
10	0,012024	0,764	719,54	879,27	0,82	HS_303_b
11	0,012323	0,764	5570,33	4121,41	1,35	hsa-miR-339-3p
12	0,012775	0,764	212,58	264,78	0,8	hsa-miR-520c-3p,hsa-miR-520f
13	0,013137	0,764	595,56	890,95	0,67	hsa-miR-377*
14	0,015728	0,764	4199,19	5663,32	0,74	hsa-miR-889
15	0,017245	0,764	6270,05	4739,88	1,32	hsa-miR-16-1*
16	0,020526	0,764	462,96	414,11	1,12	hsa-miR-220c
17	0,020714	0,764	1766,83	3222,42	0,55	hsa-miR-379
18	0,022943	0,764	150,06	188,82	0,79	hsa-miR-1255a
19	0,023495	0,764	2461,52	3911,72	0,63	hsa-miR-539
20	0,023933	0,764	234,18	184,49	1,27	HS_163
21	0,024811	0,764	138,46	189,82	0,73	HS_286_a
22	0,025004	0,764	146,24	196,7	0,74	hsa-miR-519a
23	0,025387	0,764	400,41	648,62	0,62	hsa-miR-379*
24	0,027358	0,764	3606,66	6079,1	0,59	hsa-miR-432
25	0,027624	0,764	236,72	201,97	1,17	HS_23
26	0,02897	0,764	188,76	160,46	1,18	HS_8
27	0,029565	0,764	5915,11	4481,04	1,32	hsa-miR-501-3p
28	0,030469	0,764	770,95	1412,44	0,55	hsa-miR-409-5p
29	0,031759	0,764	276,5	328,72	0,84	hsa-miR-1255b
30	0,031874	0,764	11815,38	14945,96	0,79	hsa-miR-218
31	0,032461	0,764	3488,47	2417,65	1,44	HS_32
32	0,033339	0,764	1867,68	1394,25	1,34	hsa-miR-128a:9.1
33	0,033559	0,764	138,48	104,61	1,32	hsa-miR-374b
34	0,033892	0,764	1930,57	3307,63	0,58	hsa-miR-299-5p
35	0,035733	0,764	140,22	118,6	1,18	HS_112
36	0,036555	0,764	105,44	123,53	0,85	hsa-miR-630

37	0,036653	0,764	4243,28	3117,34	1,36	hsa-miR-125a-3p
38	0,036735	0,764	479,28	803,17	0,6	hsa-miR-655
39	0,037193	0,764	211,98	254,94	0,83	hsa-miR-1226*
40	0,03781	0,764	1290,14	876,3	1,47	hsa-miR-15a*
41	0,038041	0,764	6471,84	4307,77	1,5	hsa-miR-149
42	0,038333	0,764	4445,22	6339,43	0,7	hsa-miR-511
43	0,038551	0,764	1682,54	3083,35	0,55	hsa-miR-136
44	0,040952	0,764	1989	1058,24	1,88	hsa-miR-203
45	0,041816	0,764	1589,36	1171,7	1,36	hsa-miR-345
46	0,041962	0,764	508,91	1043,81	0,49	hsa-miR-144*
47	0,043748	0,764	5951,52	4535,57	1,31	hsa-miR-874
48	0,045003	0,764	772,29	1253,54	0,62	hsa-miR-369-3p
49	0,04551	0,764	183,72	134,84	1,36	HS_53
50	0,045813	0,764	10453,09	8950,39	1,17	hsa-miR-532-3p
51	0,046191	0,764	281,16	179,12	1,57	hsa-miR-552
52	0,046294	0,764	567,99	385,72	1,47	hsa-miR-129*
53	0,046494	0,764	3276,11	5399,27	0,61	hsa-miR-494
54	0,047112	0,764	614,97	454,01	1,35	hsa-miR-1234
55	0,047421	0,764	4442,76	6333,12	0,7	hsa-miR-363
56	0,047643	0,764	2027,29	3629,82	0,56	hsa-miR-410
57	0,047681	0,764	322,8	298,18	1,08	hsa-miR-548g
58	0,047933	0,764	105,35	132,14	0,8	hsa-miR-516b
59	0,048526	0,764	3688,82	2786,99	1,32	hsa-let-7e*
60	0,049021	0,764	1064,84	592,7	1,8	hsa-miR-33b
61	0,049686	0,764	426,29	530,72	0,8	hsa-miR-1197

Supplementary table 4

Characteristics of MPNST specimens included for miRNA expression profiling.

case	Syndrome	Lesion	SITE	TA	APB
S0290	sporadic	P	extremities	-	-
S0762R	sporadic	R	extremities	-	+
S0767M	NF1	M	extremities	+	-
S0779	NF1	P	trunk	-	+
S0784	NF1	M	extremities	+	+
S0802R	NF1	R	extremities	+	-
S0900	sporadic	R	extremities	+	-
S1223	sporadic	P	trunk	-	+
S1228	sporadic	P	trunk	-	-
S980R	sporadic	R	extremities	-	-
S1313	NF1	P	trunk	+	-
S0298	sporadic	P	extremities	-	+
S1383	NF1	P	trunk	-	-
S1743	NF1	R	trunk	-	-
S1768	sporadic	P	trunk	+	-
S1792	sporadic	P	extremities	+	-
S1209	NF1	P	extremities	+	-
S1222	sporadic	P	extremities	-	-
S1315	NF1	P	extremities	-	-
S1488	sporadic	P	extremities	-	-
S1502	sporadic	P	trunk	+	-
S1538	sporadic	P	extremities	-	-
S0306	sporadic	P	extremities	+	+
S0530R	NF1	R	extremities	+	+
S0600M	sporadic	M	extremities	+	+
S0656M	NF1	R	extremities	-	-
S0737	sporadic	P	extremities	-	+
S738M	sporadic	M	extremities	+	-
S0758	NF1	P	trunk	+	-

NF1: neurofibromatosis type 1 syndrome; P:primitive lesion, R: recurrent lesion, M:metastatic lesion.

Supplementary table 5

miRNA which are differentially expressed in malignant peripheral nerve sheat tumors among TA-positive and TA-negative classes. Number of miRNAs significant at 0.01 level of the univariate test: 32, sorted by p-value of the univariate test. FDR: false discovery rate.

	Parametric p-value	FDR	Geom mean of intensities in class TA-	Geom mean of intensities in class TA+	Fold-change	Unique id
1	0,000156	0,063	522,51	2015,43	0,26	hsa-miR-182
2	0,000177	0,063	5254,81	3443,42	1,53	hsa-miR-424
3	0,000327	0,0734	4414,07	2538,53	1,74	hsa-miR-146a
4	0,000412	0,0734	2758,94	1706,65	1,62	hsa-miR-29c
5	0,000674	0,0835	1763,55	224,28	7,86	HS_72
6	0,000726	0,0835	728,23	202,16	3,6	HS_75.1
7	0,000821	0,0835	2593,52	472,67	5,49	HS_35
8	0,001133	0,0952	3675,96	2414,73	1,52	hsa-miR-22
9	0,00136	0,0952	868,72	222,19	3,91	HS_4.1
10	0,001483	0,0952	3417,85	4686,14	0,73	hsa-miR-484
11	0,001565	0,0952	996,81	487,61	2,04	hsa-miR-34a*
12	0,001605	0,0952	4620,44	6151,3	0,75	hsa-miR-181a
13	0,002665	0,146	1912,46	1001,87	1,91	hsa-miR-660
14	0,003102	0,155	3506,79	5600,01	0,63	hsa-miR-181b
15	0,003261	0,155	3490,45	1893,87	1,84	hsa-miR-29b
16	0,003808	0,169	521,7	948,44	0,55	HS_188
17	0,004172	0,175	787,28	1771,64	0,44	hsa-miR-18a*
18	0,004548	0,175	4542,03	5713,39	0,79	hsa-miR-15b
19	0,00467	0,175	1014,56	433,09	2,34	HS_232
20	0,005089	0,181	1216,51	604,3	2,01	hsa-miR-450b-5p
21	0,006002	0,199	2771,28	1866,48	1,48	hsa-miR-769-5p
22	0,006152	0,199	306,4	193,96	1,58	HS_47
23	0,006701	0,201	2160,92	3042,32	0,71	hsa-miR-877
24	0,006915	0,201	1397,74	2751,52	0,51	hsa-miR-486-5p
25	0,007051	0,201	1879,75	3214,74	0,58	hsa-miR-410
26	0,007908	0,209	710,22	311,36	2,28	hsa-miR-142-5p
27	0,00812	0,209	284,16	171,43	1,66	hsa-miR-548b-3p
28	0,008218	0,209	281,6	184,84	1,52	hsa-miR-146b-3p
29	0,008876	0,212	776,77	494,76	1,57	HS_114
30	0,008917	0,212	1291	546,35	2,36	hsa-miR-339-3p
31	0,009242	0,212	643,55	241,79	2,66	HS_157
32	0,009565	0,213	293,28	185,56	1,58	HS_201

Supplementary table 6

miRNA which are differentially expressed in malignant peripheral nerve sheat tumors among ALT-positive and ALT-negative classes. Number of miRNAs significant at 0.01 level of the univariate test: 15, sorted by p-value of the univariate test. FDR: false discovery rate.

	Parametric p-value	FDR	Geom mean of intensities in class ALT-	Geom mean of intensities in class ALT+	Fold-change	Unique id
1	0,003064	0,997	651,82	1272,6	0,51	solexa-3044-295
2	0,003652	0,997	704,15	1238,65	0,57	hsa-miR-591
3	0,005083	0,997	951,86	382,62	2,49	hsa-miR-187
4	0,011661	0,997	901,21	1222,92	0,74	HS_150
5	0,01339	0,997	3093,64	3963,3	0,78	hsa-miR-10b
6	0,017482	0,997	1477,06	895,85	1,65	hsa-miR-1275
7	0,020607	0,997	430,2	300,02	1,43	hsa-miR-125b-1*
8	0,023983	0,997	382,27	220,37	1,73	hsa-miR-760
9	0,036499	0,997	1742,87	820,63	2,12	hsa-miR-135b
10	0,038781	0,997	3716,13	2975,44	1,25	hsa-miR-19b
11	0,041564	0,997	362,95	496,79	0,73	hsa-miR-554
12	0,044384	0,997	1777,59	2589,25	0,69	solexa-4793-177
13	0,045377	0,997	195,02	357,21	0,55	HS_169
14	0,045447	0,997	496,7	335,54	1,48	solexa-603-1846
15	0,047947	0,997	3764,44	2960,95	1,27	hsa-miR-1274a

Supplementary figure 1

Volcano plot showing miRNA differentially expressed between TA+ and TA- peritoneal mesothelioma. Micro-RNA with p<0.05 (blue) are considered statistically significantly differentially expressed. miR-380* is higlighted in red.