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Telomere lengthening mechanisms in matrix-producing bone tumors:

A molecular genetic and cytogenetic study

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Abstract

Telomeren, die Enden linearer Chromosomen, sind essentiell für die Erhaltung der chromosomalen Stabilität und des replikativen Potentials. Aktivierung der reversen Transkriptase Telomerase ist vorherrschender Mechanismus, um Länge und Funktion der Telomeren zu regulieren. In ungefähr 90% aller malignen Tumoren kann hohe Telomeraseaktivität (TA) nachgewiesen werden, doch scheint diese nicht unersetzlich zu sein. „Alternative lengthening of telomeres" (ALT), Verlängerung der Telomeren in Abwesenheit detektierbarer TA, wurde in verschiedenen Organismen beschrieben und in immortalisierten und transformierten humanen Zellen nachgewiesen. In der vorgelegten Arbeit wurden die Häufigkeit von TA und Genexpression von Telomerase-Untereinheiten mit ALT in Matrix-produzierenden Knochentumoren. Dabei fiel auf, dass nur 50% der untersuchten Osteosarkome TA aufwiesen. Durch terminale Restriktionsfragment (TRF)-Analyse konnten in einem TA-negativen Osteosarkom und in Osteosarkom-Zellinien die ALT-typischen, stark verlängerten und heterogenen Telomeren nachgewiesen werden. In der Telomer-Fluoreszenz *in situ* Hybridisierung (T-FISH) zeigten sich in ALT-Zellinien im Gegensatz zu Zellinien mit TA zudem Telomeren in ungewöhnlichen Konfigurationen und Loci, dizentrische Markerchromosomen und signal-freie Chromosomen-Enden. Freie Enden induzieren End-zu-End Assoziationen und können zu Bruch-Fusions-Brücken Zyklen führen. Dies konnte in der multiplex-FISH (M-FISH)-Analyse durch eine signifikant höhere Anzahl komplexer Translokationen in Osteosarkom-Zellinien mit ALT im Vergleich zu TA nachgewiesen werden. Die Analyse unbalancierter chromosomaler Aberrationen in Osteosarkomen und Osteosarkom-Zellinien durch komparative genomischer Hybridisierung (CGH) konnte jedoch keine ALT-spezifischen Aberrationen aufdecken. In dieser Studie wird der Nachweis einer direkten Assoziation von ALT mit dysfunktionalen Telomeren und chromosomaler Instabilität geführt. Dies lässt vermuten, dass ALT kein Äquivalenz-Mechanismus für TA ist, was weitreichende Implikationen für die Tumorprogression haben könnte und wichtig für die Evaluation von neuen Therapiestrategien ist, die gegen Struktur und Funktion der Telomeren im Allgemeinen gerichtet ist.

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1. INTRODUCTION

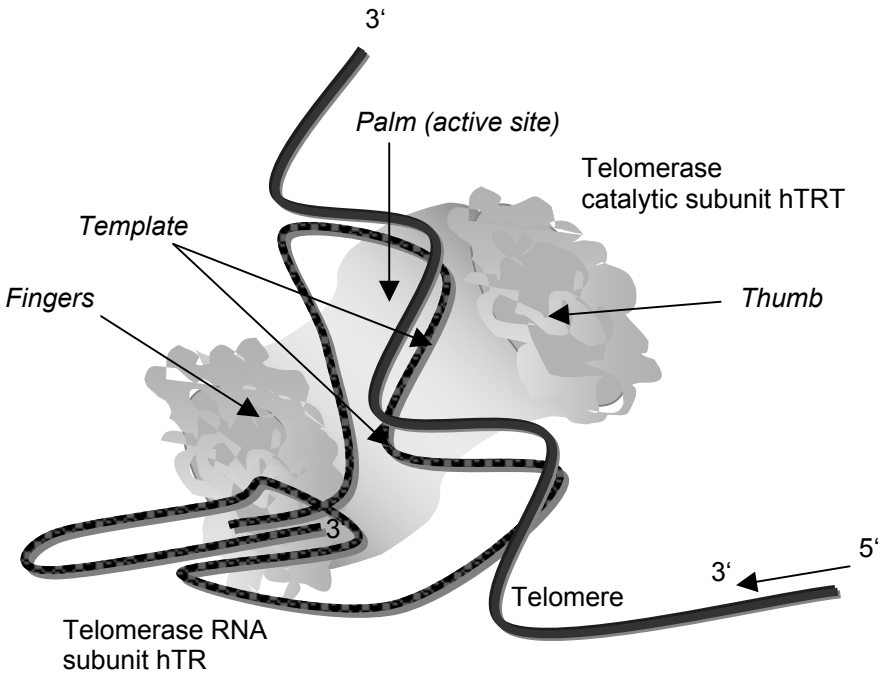
1.1 The functional complex of telomeres and telomerase

Telomeres (τελομερ, greek: „end-part“, “end-piece”), the physical ends of eukaryotic chromosomes, consist of protein-DNA complexes and are crucial in maintaining chromosomal stability and cell viability. The telomeric sequence is composed of tandem repeats containing a block of neighboring guanine nucleotides (Blackburn, 1991). In humans, this sequence is 5'-TTAGGG-3' (Moyzis R.K. et al., 1988). Functional telomeres prevent aberrant chromosomal recombination, end-to-end fusions and promote proper segregation of chromosomes during cell division (McClintock, 1941; Kirk et al., 1997; Hackett et al., 2001).

Complete replication of telomeres in most organisms is accomplished by the specialized reverse transcriptase telomerase, that was first isolated from the ciliate *Tetrahymena* (Greider and Blackburn, 1985; Greider and Blackburn, 1987). The ribonucleoprotein telomerase synthesizes repeats of telomeric DNA onto the guanine (G)-rich, 3'end of telomeres, thus creating a single-stranded 3' end-overhang. Telomerase is a multi-subunit enzyme (Figure 1). Essential for enzymatic activity are the reverse transcriptase protein catalytic subunit hTERT (Takakura et al., 1999; Ramakrishnan et al., 1998), and the endogenous RNA template hTR (Feng et al., 1995; Avilion et al., 1996) which is used for synthezising telomeric repeats onto the 3' ends of human chromosomes.

Expression of hTERT appears to be the rate-limiting step in telomerase activity (Bodnar et al., 1998; Meyerson et al., 1997)

Dysfunctional telomeres increase the mutation rate and genomic instability in telomerase-null yeast, suggesting that telomerase can prevent chromosomal instability (Hackett et al., 2001).



<u>Figure 1:</u> The ribonucleoprotein enzyme telomerase at the telomere
Shown are the two components essential for enzyme activity: the catalytic subunit hTERT and the RNA component hTR, providing the template for reverse transcription. Illustration adapted from *Saccharomyces cerevisiae* telomerase structure (Lingner et al., 1999).

1.2 Telomere shortening and activation of telomerase

Most somatic cells do not display telomerase activity and telomeres shorten progressively in a stochastic manner, partly due to the inability of conventional DNA polymerases to elongate the very end of the lagging strand during DNA synthesis, known as the "end-replication-problem" (Olovnikov, 1971; Watson, 1972), and also due to cell-cycle regulated 5' to 3' nuclease processing of the cytosine-rich strand (Wellinger et al., 1996; Makarov et al., 1997). Decrease in telomere length is thought to ultimately lead to proliferative failure in most dividing normal human cells *in vitro* (Allsopp et al., 1995; Harley et al., 1990; Kipling et al., 1999), Figure 2. Emerging immortalized cells usually display telomere maintenance by activation of telomerase (Counter et al., 1992).

Direct evidence for the key role of telomerase in unlimited cell proliferation and tumorigenesis was demonstrated by a) the ectopic expression of the enzyme in a variety of normal early passage, presenescent cells where it confers unlimited replicative potential *in vitro* (Bodnar et al., 1998; Vaziri and Benchimol, 1998); b) inhibition of telomerase in immortal cells leading to their proliferative crisis (Zhang et al., 1999; Hahn et al., 1999b); and c) combined expression of oncogenes and telomerase resulting in a tumorigenic phenotype (Hahn et al., 1999a). In most cases immortalization in transformed cells is concomitant with telomerase activation (Wright et al., 1989; Counter et al., 1992; Counter et al., 1998; Hahn et al., 1999a).

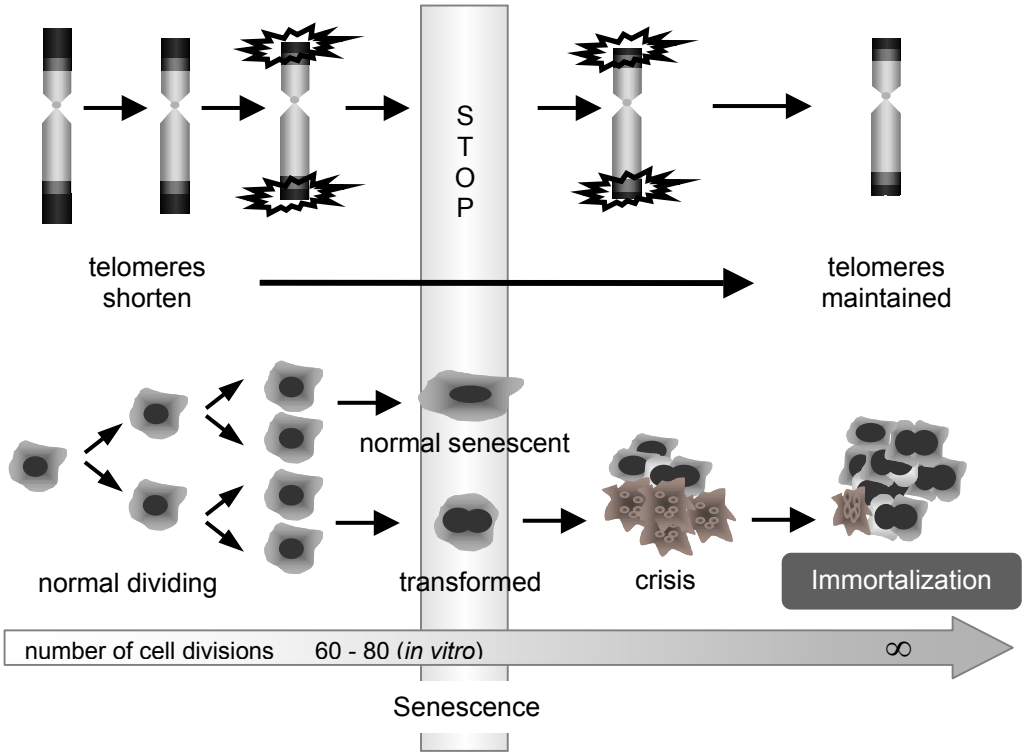


Figure 2: The concept of cellular senescence and immortalization
Telomeres in most normal somatic cells shorten with continuous cell divisions (*in vitro*). Loss of telomere function is thought to trigger a quiescent state termed senescence characterized by an enlarged, flattened cell morphology. Transformed cells bypass senescence and enter crisis, a state where high chromosomal instability leads to increased cell death. Emerging immortalized cells usually display stabilization of telomeres through activation of telomerase.

1.3 Telomerase activity and telomere maintenance in human tumors

Earlier investigations revealed telomerase activity in approximately 90% of human malignant tumors, but not in normal tissues (Kim et al., 1994; Shay and Bacchetti, 1997; Dhaene et al., 2000). For example, determination of telomerase activity can be used as a diagnostic marker in carcinomas of the bladder (Muller et al., 1996), colon (Tahara et al., 1999), lung (Yahata et al., 1998), as a sensitive diagnostic marker for malignancy in fine-needle aspirates of the breast (Poremba

et al., 1999) or as a prognostic molecular parameter in neuroblastoma (Poremba et al., 2000).

However, the specific association of telomerase activity with cancer progression is being challenged. Frequency and biologic importance of telomerase activity seem to be subjected to tumor-type dependent variation (Wynford-Thomas, 1999). Moreover, stem cells (Harle and Boukamp, 1996), stimulated lymphocytes (Broccoli et al., 1995), male germ cells (Holt and Shay, 1999), basal keratinocytes (Yasumoto et al., 1996) and stem or progenitor cells in different other highly proliferative tissues (Hiyama et al., 1996; Takubo et al., 1997) may also exhibit telomerase activity.

1.4 Telomere length regulation in telomerase-negative cells – alternative lengthening of telomeres (ALT)

Mechanisms of telomere regulation other than telomerase have been found in several eukaryotes. *Drosophila melanogaster* and related dipterans use retrotransposons in telomere length regulation (Mason and Biessmann, 1995). Homologous recombination is used for telomere elongation in the mosquito *Anopheles gambiae* (Roth et al., 1997). Rare survivors of telomerase-defective yeast are able to elongate their telomeres via recombination events, dependent on the DNA double-strand break repair gene RAD52, that are initiated either in the subtelomeric Y' regions or directly within the terminal telomere repeats (Lundblad and Blackburn, 1993; Teng and Zakian, 1999). Telomerase-null late-generation mouse embryonic stem cells were shown to contain amplified DNA of unknown

origin in the subtelomeric region, possibly due to recombination (Niida et al., 2000).

In human cells there is at least one mechanism of telomere lengthening apparently not requiring telomerase activity termed alternative lengthening of telomeres (ALT). ALT was first characterized in a descriptive way by the presence of heterogeneous and elongated telomeres in the absence of telomerase activity (Murnane et al., 1994; Bryan et al., 1995; Bryan et al., 1997a).

In studies of simian virus 40 (SV40) large T antigen induced transformation of human somatic cells it was observed that, in the course of continued cell divisions, telomeres progressively shorten as measured by Terminal Restriction Fragment (TRF) size employing Southern blotting and hybridizing with a telomere specific probe. Eventually, cells reached a state termed "crisis" accompanied by massive apoptosis and a peak in the number of dicentric chromosomes resulting from end-to-end fusions indicating chromosomal instability and telomere dysfunction (Counter et al., 1992; Ducray et al., 1999). An increase in anaphase bridges was seen in primary cultures as they approached crisis and was associated with extremely short telomeres (Counter et al., 1992).

Rare surviving immortalized cells emerging from crisis usually display activation of telomerase as detected with the PCR-based Telomere Repeat Amplification Protocol (TRAP)-assay for telomerase activity in whole cell lysates *in vitro* (Wright et al., 1995). Telomere length is eventually stabilized around a mean optimized length that varies between different cell types (usually 4 – 10 kilobases in normal and tumor human cells, reviewed in (Broccoli and Cooke, 1993).

However, a subset of postcrisis cells were found to have no detectable telomerase activity. A longer mean telomere length than in their precrisis counterparts indicated that net telomere elongation in the absence of telomerase activity had occured (Murnane et al., 1994; Bryan et al., 1995; Bryan et al., 1997a). A signal smear in TRF analysis revealed telomere lengths ranging from very short (approximately 2 kilobases) to extremely long (more than 40 kilobases). This characteristic pattern was maintained over many hundreds of population doublings, indicating that ALT is an efficient bypass for telomere maintenance by telomerase (Rogan et al., 1995).

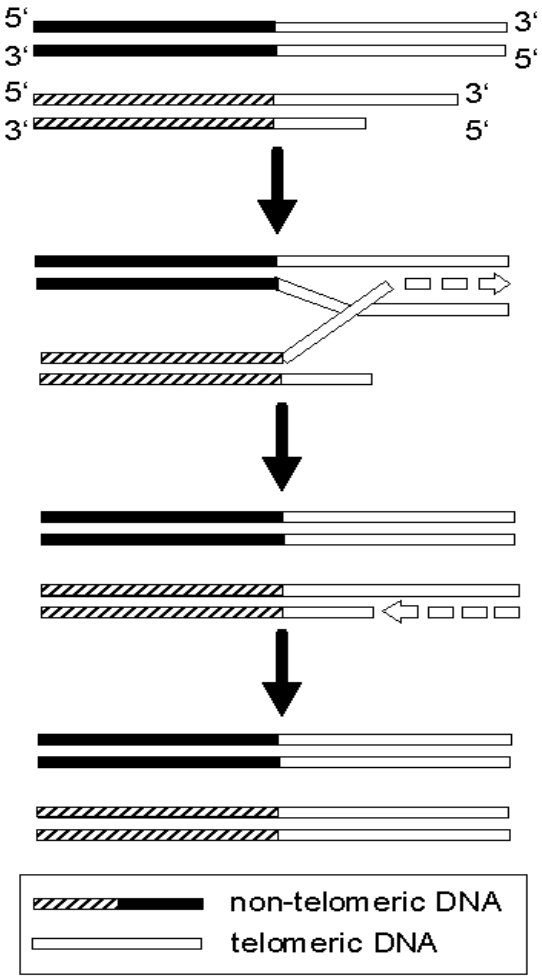


Figure 3: Recombination in telomere elongation.
Homologous recombination between telomeres and copy switching, adapted from Reddel et al., 2001.

Dunham et al. demonstrated that ALT in human cells can occur by means of homologous recombination and copy-switching (Dunham et al., 2000), Figure 3. They placed a plasmid tag in the subtelomeric region and within the telomeric repeats of immortalized human ALT or telomerase positive cell lines and demonstrated copying of DNA sequences from

telomere to telomere in the ALT cell line, but not in the cell line with telomerase activity. Since their assay was not designed to detect intratelomeric copying, it is possible, that such events occurred in addition to intertelomeric copying. In this context, it appears important to note that the G-rich 3' single stranded overhang of telomeres in humans and *Trypanosoma* is tucked far back into the telomeric tract, "invading" the duplex DNA by base pairing and forming a loop *in vitro* ("T-loop", Griffith et al., 1999; Greider, 1999; Munoz-Jordan et al., 2001), Figure 4. The proximal double-stranded telomeric sequence-invading single strand itself forms a displacement loop ("D-Loop"). Possibly, this "invaded" region itself can be used as a template for elongation of the invading 3' single strand in ALT cells (Reddel et al., 2001). The T-loop resembles the D-loop, a functional intermediate in DNA replication. It can

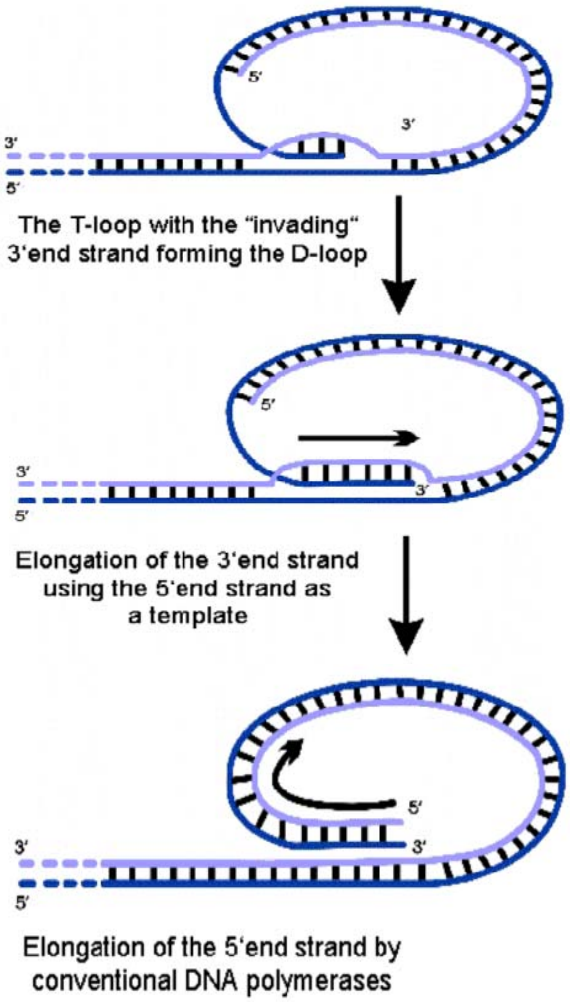


Figure 4: Intratelomeric recombination. The T-loop structure of the telomere may provide a template region for the G-rich 3'end single strand within the D-Loop.

be seen as a stalled D-loop requiring special circumstances to overcome its inactivation.

It appears that ALT is a sufficient mechanism in compensating for the lack of telomerase activity in cell survival in telomerase-deficient yeast (Lundblad and Blackburn, 1993) and in human cells during immortalization. However, the permissive molecular circumstances under which telomere lengthening without telomerase activity is being induced have only started to be understood (Opitz et al., 2001; Rizki and Lundblad, 2001). ALT has been detected in a subset of immortalized cells, tumor derived cell lines and tumor samples (reviewed in Reddel et al., 2001; Scheel and Poremba, 2002) and differs significantly in telomere morphology and likely telomere function (Scheel et al., 2001; Perrem et al., 2001; Ford et al., 2001) from telomere maintenance by telomerase.

In view of therapeutic approaches targeting telomere maintenance, the existence of ALT needs to be carefully evaluated. Telomerase inhibitors may be ineffective for tumors containing ALT cells and may increase the selection pressure to activate ALT in previously telomerase-positive tumors.

1.5 Telomere maintenance in matrix producing bone tumors

The purpose of this thesis was to evaluate the prevalence of telomerase activity and ALT in matrix producing bone tumors in regard to telomere morphology and function. Firstly, emphasis was laid on the analysis of the frequency of telomerase activity in osteo- and chondrosarcomas, and osteosarcoma cell lines by the telomere repeat amplification protocol (TRAP). Gene expression of the enzyme's

catalytic subunit hTERT and its endogenous RNA template hTR was performed by quantitative real-time rt-PCR.

Secondly, ALT was detected in a primary osteosarcoma and osteosarcoma cell lines by telomere length analysis using Terminal Restriction Fragment (TRF) size analysis based on a Southern blotting protocol. Analysis of telomere morphology and function was performed by telomere PNA-fluorescence *in situ* hybridization (T-FISH). Finally the occurrence of molecular cytogenetic aberrations characteristic for either telomerase activity or ALT was investigated. Analysis of unbalanced chromosomal aberrations was performed by comparative genomic hybridization (CGH) in osteosarcomas and osteosarcoma cell lines. Structural rearrangements were analyzed by 24-color multiplex fluorescence *in situ* hybridization (M-FISH) in osteosarcoma cell lines. As a guardian of genome integrity, p53 status in the osteosarcoma cell lines was analyzed. Cell lines OST, SJSA-1 and ZK-58, where p53 status was not found in the literature, were analyzed using a p53 GeneChip Assay.

2. MATERIAL AND METHODS

2.1 Material

2.1.1. Tumor material

Tumor specimens were derived from snap-frozen material of primary tumor biopsies or surgical resections and stored at the Gerhard-Domagk-Institute of Pathology, Münster at minus 80°C until use. Clinicopathological data comprised diagnosis, sex, age at diagnosis and tumor cell content in the obtained specimens. The patient‘s mean age at diagnosis was 21 ± 13 years for the osteosarcomas, 54 ± 13 years for the chondrosarcomas and 32 ± 18 years for the benign lesions. Tumor cell content in relation to the whole tissue area (matrix) evaluated by histologic study ranged from 5 - 100 % in malignant tumors and 5 - 80 % in the benign lesions.

2.1.2. Cell lines

Furthermore, 7 osteosarcoma cell lines were included in the study. Table 1 lists all osteosarcoma cell lines analysed. Furthermore, the Ewing's sarcoma cell line VH64 (van Valen et al., 1993) and the neuroblastoma cell lines Lan-5 (Negroni et al., 1991) and SK-PN-DW were included as positive controls for telomerase

activity. All cell lines were obtained from F. van Valen, Department of Experimental Orthopedics, Westfälische Wilhelms-Universität Münster.

Cell line	Reference
HOS	(Rhim et al., 1975a)
MNNG	(Rhim et al., 1975b)
OST	(McAllister et al., 1971)
SJSA-1	(Oliner et al., 1992)
Saos-2	(Banerjee et al., 1996)
U-2 OS	(Heldin et al., 1986)
ZK-58	(Sonnemann et al., 2001)

Table 1: Osteosarcoma cell lines and according references used in this study.

2.2 Methods

2.2.1 Protein preparation and telomerase assay

Background

The TRAP-assay (Telomeric Repeat Amplification Protocol) represents the standard method for analysis of the *in vitro* activity of the ribonucleoprotein enzyme complex telomerase (Kim et al., 1994). The TRAP-assay is based on a two-enzyme comprising system. In the first step, telomerase contained in the

cellular extracts adds hexamer repeats of telomeric sequence (TTAGGG) onto the 3'-end of an included oligonucleotide. Telomeric repeats synthesized by these means are then amplified by *taq*-polymerase in a regular Polymerase Chain Reaction (PCR), resulting in amplification products of 50, 56, 62, 68, etc. base pairs (bp). Electrophoretic separation of these products generates a characteristic ladder, increasingly sized by exactly six base pairs. A modified TRAP (Poremba et al., 1997; Poremba et al., 1998) was used for studying telomerase activity (TA). In detail, it comprises the following steps:

Extractions of cellular proteins

Tumor specimens were prepared as previously described (Poremba et al., 1998): 5 frozen tissue sections at a thickness of 10 µm were lysed in 100 µl of ice-cold CHAPS lysis buffer containing:

10 mM Tris-HCl, pH 7.5;

1 mM $MgCl_2$;

1 mM ethylene-glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA);

0,1 mM benzamidine;

5 mM β-mercaptoethanol;

0,5% 3-[3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS);

10% glycerol;

1 U/µl of RNAse inhibitor (Roche Diagnostics, Mannheim, Germany).

Lysates were pipeted into Fast-Prep tubes containing glass beads (Dianova, Hamburg). Homogenisation of the samples was achieved using a shaking incubator (Fast-Prep System, Dianova). Cell lines were grown to confluency in T25 cell culture flasks (GIBCO-BRL, Life Technologies, Burlington, USA) and washed with cold Phosphate Buffered Saline (PBS):

1,4 M NaCl;

50 mM KCl;

100 mM Na_2HPO_4;

15 mM K_2HPO_4;

in aqueous solution, adjusted to pH 7.4.

Cells were then suspended in cold PBS and pelleted by centrifugation for 5 minutes at a speed of 1.200 G at 4°C prior to protein extraction with 100 µl CHAPS-buffer on ice. Following these steps, the cell suspension was cooled on ice for 30 minutes, then centrifuged for 30 minutes at a speed of 12.000 G at 4°C. After centrifugation, the overlay was processed for photometric protein concentration assessment. The protein concentrations were measured by use of the Bradford Coomassie Protein Assay Reagent (BioRad, Hercules, CA, USA) on an ELISA Reader (Bio Rad) at 595 nm and adjusted to about 2 µg/µl.

Enzymatic reactions

1 µl of tissue or cell extract was suspended in a 24 µl reaction mix:

200 mM Tris-HCl, pH 8.3;

15 mM $MgCl_2$;

630 mM KCl;

0.5% Tween-20;

10 mM EGTA;

0.1% bovine serum albumin (BSA);

2.5 mM each of dATP, dTTP, dGTP, dCTP;

0.2 µl *taq*-Polymerase (5U/µl, Perkin-Elmer, Branchburg, NJ, USA);

19.8 µl PCR-grade H_2O;

0.15 µm/l fluorescence-labeled TS forward primer (5'-(Cy-5)-AATCCGTCGAGCAGAGTT-3') and

0.25 µl of CX reverse primer (5'-CCCTTACCCTTACCCTTACCCTTA-3').

Furthermore, an internal standard from a commercially available kit (TRAPeze telomerase detection kit, Oncor, Gaithersburg, MD, USA) was included, resulting in a 36 bp product which was co-amplified with telomerase-elongated products in a competitive manner to exclude *taq*-polymerase-inhibitors in the tissue and cell extracts. Cellular extracts of the telomerase positive Ewing's sarcoma cell line VH64 as a positive control, a heat-inactivated control (telomerase-positive control incubated at 85ºC for 10 minutes prior to reaction) and CHAPS-buffer as a negative control were included in each assay.

For activation of telomerase, samples were initially incubated at 30°C for 30 minutes to allow for telomerase-mediated extension from annealed TS-oligonucleotides. In the following PCR, the reaction mixture was immediately heated to 94°C for 5 minutes and then subjected to 30 PCR cycles with denaturation at 94°C for 30 seconds, annealing of primers at 55°C for 30 seconds, and elongation at 72°C for 45 seconds.

Electrophoretic separation and detection

Electrophoresis was performed utilizing denaturing 8% polyacrylamide gels on an automated laser-fluorescence sequencer (ALFexpress, Pharmacia, Freiburg, Germany). Gels were composed of:

8,5 ml Long Ranger Gel-Solution 50% (Biozym, Hess Oldendorf, Germany);

31,5 g urea;

9ml 10x TBE*;

deionized H_2O add 50 ml.

*diluted from 10x TBE in aqueous solution containing

0.89 M Tris base;

0.89 M Boric acid;

0.02 M disodium ethylenediaminetetraacetic acid (EDTA)

Briefly,

1 µl of PCR-product;

6.7 µl of loading buffer (90% formamide, 10% Blue dextran);

0.5 µm of a 12 bp standard;

1.5 µm of a 100 bp standard (both Pharmacia)

were mixed and denatured at 94°C for 5 minutes. Afterwards, 6 µl of this mix was loaded onto gels and subjected to electrophoresis at 45 W, 50°C and 2 seconds sampling, resulting in a running time of 480 minutes.

Semiquantitative analysis

Semiquantitative analysis was performed as described (Poremba et al., 1998) using the Fragment-Length-Manager 1.3 software (Pharmacia). Chromatogram peaks generated from photodetector signals were quantitated in terms of size (base pairs), peak height and peak area in relation to the positive telomerase control. Interpretation of proportions distinguished between absent TA, low (<30% of positive control), intermediate (30%-70% of positive control) and high TA (>70% of positive control). All TRAP data was analyzed in blind-trial fashion. For each tumor sample, the TRAP procedure was done twice and levels of telomerase activity proved to be consistent in all samples included in this study. To rule out the prevalence of telomerase inhibiting factors, mixed tissue samples that contained telomerase-negative and positive extracts were prepared.

2.2.2 Assessment of tissue sample preservation by RT-PCR of RNA content

In order to minimize the probability of false-negative results, with a lack of or low TA due to tissue degradation and necrosis, RNA derived from frozen sections was amplified by reverse-transcriptase PCR (RT-PCR) for glyceraldehyde dehydrogenase (GAPDH) as an indirect marker of tissue integrity. As described in greater detail in the later section 2.2.4, a 297 bp fragment of the human GAPDH gene was amplified with primers 5'-CACCCATGGCAAATTCCATGGC-3' and 5'-GCATTGCTGATGATCTTGAGGCT-3', correponding to GenBank positions 213-234 and 509-487, respectively (GenBank Accession Number M33197). For a small subset of tumors, no RNA was available. For those lesions, viability of the TRAP assay was verified by tumor cell count on hematoxylin-eosin stained histologic slides.

2.2.3 Isolation of RNA

Total RNA was isolated from fresh-frozen tissue by phenol-chloroform extraction and isopropanol precipitation using the TRISOLV system (Biozol, Eching, Germany) in accordance with the manufacturer's protocol. Briefly, approximately 5 frozen tissue sections at a thickness of 10 μm were homogenized in 1 ml of TRIZOL reagent (Biozol), a mono-phasic solution of phenol and guanidine isothiocyanate. Cell lines were grown to confluency in T25 cell culture flasks (GIBCO) washed with ice-cold PBS and directly lysed in the flask using 1 ml

TRIZOL. After phenol-chloroform extraction, RNA was isolated from the aqueous phase, treated with DNAse (Eurogentec, Seraing, Belgium) for 15 min at room temperature and purified by repeating the TRISOLV extraction protocol. Purity and integrity of RNA was assessed by 1% agarose gel eletrophoresis and spetrophotometric analysis of the optical density (OD) A_{260}/A_{280} ratio.

2.2.4 Relative quantitative real-time reverse transcription (RT)-PCR for expression of hTERT and hTR

c-DNA synthesis

cDNA was synthesized from approximately 2 µg of RNA using the FirstStrand Synthesis Kit (AmershamPharmacia, Freiburg, Germany). Briefly, a reaction mix of 20 µl was prepared containing

6.7 µl Bulk First-Strand (murine reverse transcriptase; porcine; RNAse/DNAse-free BSA; dATP, dCTP, dGTP and dTTP in aqueous buffer);
1.3 µl DTT 200 mM;
2.6 µl of *NotI*-d$(T)_{18}$ bifunctional primer at 2.5µg/µl
(5'-d[AACTGGAAGAATTCGCGGCCGCAGGAAT$_{18}$]-3');
4.4 µl of diethyl pyrocarbonate (DEPC) treated H_2O and
5 µl RNA (containing approximately 2 µg of RNA).

For reverse transcription, the reaction mix was incubated for 1 hour at 37°C.

Relative quantitative real-time PCR

Relative concentrations of cDNA samples were evaluated by quantitative RT-PCR of GAPDH performed on the LightCycler (Roche Diagnostics, Mannheim, Germany) followed by analysis of the gene expression of the telomerase catalytic subunit hTERT and the RNA component hTR. For amplification the cDNA, 5 µl aliquots of reverse-transcribed cDNA (containing approximately 500 ng of cDNA) were subjected to PCR.

A reaction mix of 20 µl was prepared containing a final concentration of 2-3 mM $MgCl_2$, 0.5 µM of each primer (see below), and 2 µl of ready-to-use LightCycler DNA Master SYBRGreen I (10x, containing TaqDNA polymerase, reaction buffer, dNTP mix with dUTP instead of dTTP, SYBRGreen I dye, and 10mM $MgCl_2$). For detection of hTERT and hTR, a hot-start PCR was performed by adding 0.16 µl/sample of TaqStart Antibody (Clontech, Heidelberg, Germany) to the amplification mixture prior to the addition of primers and template cDNA.

hTERT mRNA was amplified using the primers

hTERT-1 (5'-CGGAAGAGTGTCTGGAGCAA-3') corresponding to GenBank positions 1785-1804 and

hTERT-2 (5'-CATGGACTACGTCGTGGGAG-3') corresponding to positions 1961-1980 (GenBank Accession number AF018167).

hTR RNA was amplified using the primers

hTR-1 (5'-CCTAACTGAGAAGGGCGTAGGC-3') corresponding to GenBank positions 849-870 and

hTR-2 (5'-CTAGAATGAACGGTGGAAGGCG-3') corresponding to positions 961-940 (GenBank accession number AF047386).

The reaction conditions were initial denaturation at 95°C for 2 minutes followed by 35-40 cycles of denaturation at 95°C for 1 second, annealing at 60°C for 5 seconds and extension at 72°C for 6-8 seconds (depending on amplification length).

Relative Quantification

Quantitative analysis was performed using the LightCycler Software employing a real-time fluorogenic detection system for a kinetic, rather than end-point approach as on conventional agarose or polyacrylamide gels. The generation of quantitative data was based on the different PCR kinetics of samples with different levels of target gene expression. We employed a relative quantification (Poremba et al., 2000, Scheel et al., 2001) in which the expression levels of the tumor samples and osteosarcoma cell lines were compared to the data from the Ewing's tumor telomerase positive cell line VH64 in a geometric dilution series (1:1, 1:2, 1:4, 1:8, 1:16, 1:32). The graph of the linear regression and calculation of the regression coefficient, r, served to confirm accuracy and reproducibility of this approach. For this approach, the identity and specificity of the PCR product was confirmed by melting curve analysis which is part of the LightCycler analysis program. The specific melting point of the PCR product was correlated with its molecular weight determined by agarose gel electrophoresis.

One-step RT-PCR

Evaluation of hTERT expression in the 8 osteosarcoma cell lines was also performed by using the LightCycler Telo*TAGGG*-Kit (Roche Diagnostics, Mannheim, Germany) which comprised a one-step RT-PCR in which relative quantification was realized by normalizing hTERT gene expression on expression of PBGD (porphobilinogen deaminase). Probes consisted of two different fluorescein-labeled short oligonucleotides that hybridized to an internal sequence of the amplified fragment during the annealing phase and emitted a fluorescence signal in close proximity through fluorescence resonance energy transfer (FRET).

For the amplification 2 µl prediluted aliquots containing approximately 200 ng of total RNA were subjected to first strand-synthesis and subsequent amplification in a final volume of 20 µl containing 1x ready-to-use Reaction Mix (0.1 µl reverse transcriptase; 1x hTERT Detection Mix or PBGD reaction mix and a ready-to-use primer and hybridization probe mixture specific for either hTERT or PBGD mRNA).

After an initial incubation at 60°C for 10 minutes to allow reverse transcription, samples were directly subjected to PCR. The reaction conditions were initial denaturation at 95°C for 30 seconds followed by 40 cycles of denaturation at 95°C for 1 second, annealing at 60°C for 5 seconds and extension at 72°C for 10 seconds.

Prediluted RNA standards for establishing a reference curve as well as a positive control RNA to ensure reliability and reproducibility were included in the kit. Again, identity and specificity of the PCR product was confirmed by melting curve analysis.

2.2.5 Extraction of DNA

Genomic DNA from fresh-frozen tissue and cell lines was prepared by proteinase K digestion followed by treatment with RNAse A according to the Puregene DNA extraction protocol (Biozym, Hess. Oldendorf, Germany). Genomic DNA was then isolated by phenol-chloroform extraction according to standard protocols and DNA concentration measured by spetrophotometric analysis of the OD A_{260}/A_{280} ratio.

2.2.6 Telomere Length Analysis

Background

Telomere lengths of cell lines and tumor samples were assessed by Terminal Restriction Fragment (TRF) analysis using the Telomere Length Assay Kit (Pharmingen, San Diego, USA). The measurement of telomeric length has traditionally been accomplished through Southern blotting. Briefly, for telomeric measurement, genomic DNA of the population of cells or from the tissue of interest was digested by two frequent cutting restriction enzymes which do not have a target sequence within the telomeric repeat. Thus, the chromosomal DNA was cut into small fragments except for the telomeres and subtelomeric regions which were left intact and comprised the TRFs. The cleaved DNA was then separated on a low percentage agarose gel, transferred onto a membrane and submitted to Southern blot analysis using a telomere-specific oligonucleotide probe conisisting of a series of hexamer telomeric repeats. After detection of the

position of the bybridized probe on the membrane, the average TRF length was then calculated from the position of the detected signal relative to the position of known size standards.

Restriction Enzyme Digestion

5 µg of genomic DNA was digested with 20 U (4U/µg genomic DNA) of HinfI/RsaI restriction enzyme mixture containing

10 U HinfI/Rsa per µl stored in

50 mM KCl;

10 mM Tris-HCl (pH 7.4);

0.1 mM EDTA;

1 mM DTT;

200 µg/ml BSA and

50% glycerol.

Digestions were performed at 37°C overnight in a total volume of 20-40 µl of 1x Restriction Enzyme Digestion Buffer, 10x stock buffer composed of

10 mM Tris-HCl;

10 mM $MgCl_2$;

50 mM NaCl;

1 mM DTT;

adjusted to pH 7.9.

Gel electrophoresis, transfer and hybridization:

Digested DNA with 1-2 µl loading dye (containing 0.25% xylene cyanole and 0.25% bromophenol blue in 50% glycerol, 10mM Tris, 1mM EDTA, pH 8) was loaded onto 0.6 % agarose gels. Gels were composed of:

0.6% agarose (Seakem LE agarose; FMC, Rockland, ME, USA);

0.4 µl/ml ethidium bromide (10 mg/ml);

1x TAE*

* diluted from 10x TAE in aqueous solution containing

0.4 M Tris base;

0.01 M EDTA-Na_2-salt;

0.2 M acetic acid.

Gels were run at 5V/cm for approximately 3 hours. BstEII- and HindIII digested λ DNA supplied with the kit was used as length standards.

Southern transfer blot onto a positively charged nylon membrane (Roche Diagnostics, Mannheim, Germany) was accomplished by alkaline vacuum blotting using the VacuGene XL blotting unit according to the manufacturer's protocol (Amersham Pharmacia). Briefly, gels were depurinated in 0.2 N HCl for 20 minutes and transfered onto membranes using alkaline buffer containing 0.1 M NaOH for approximately 1.5 hour.

Prior to hybridization, membranes were washed in 2x SSC (prepared from 20x SSC containing 1M NaCl and 1mM sodiumcitrate) for 10 minutes and

microwaved at 600 Watt for 2 minutes. Membranes were then hybridized overnight at 55°C with 5 ng/ml hybridzation buffer of an 51-mer biotinylated telomere probe (buffer and probe from Pierce Chemical Co., USA). Stringency washes were carried out by washing membranes three times for 5 minutes in 2x SSC/0.1% SDS (prepared from 20x SSC and 10% sodiumdodecylsulfate (SDS)) at room temperature.

Detection and analysis of TRFs:

Chemiluminescent detection was performed with Streptavidin conjugated to Horseradish Peroxidase (HRP) in a dilution of 1:300 in accordance to instructions supplied with the Telomere Length Assay kit. Exposure of an x-ray film (X-omat AR, Kodak, Rochester, New York, USA) to the membrane for 5 up to 20 minutes revealed telomere lengths as smears ranging from 1.9 to more than 23.1 kilobases (kb). TRF length analysis was performed on a densitrometric scan of the autoradiogram using an Agfa scanner (Agfa, Germany). The mean TRF length for each sample was calculated by integrating the signal intensity above background over the entire TRF distribution as a function of TRF length:

$L=\Sigma(OD_i \times L_i)/\Sigma(OD_i)$,

where OD_i and L_i are the signal intensity and TRF length repectively at position i on the gel image.

2.2.7 Telomere Fluorescence *in situ* Hybridization (T-FISH)

Background

T-FISH was employed to detect telomeres in metaphase chromosome spreads of osteosarcoma cell lines. Analysis was carried out using the DAKO Telomere FISH Kit/Cy3 (DAKO; Glostrup, Denmark). The kit utilized a peptide nucleic acid (PNA) probe conjugated to the fluorochrome Cy3. PNA is a synthetic DNA/RNA analogue binding to DNA/RNA in a sequence-specific manner obeying the Watson-Crick base pairing rules. In PNA the sugar phosphate backbone has been replaced by a neutral peptide/polyamide backbone. Primarily, this assay was used to analyse telomere morphology, however, hybridization using the PNA-probe is accurate enough to allow for quantification of telomere length (Lansdorp et al., 1996): the intensity of the fluorescent signal at the telomere directly correlates with telomere (Lansdorp et al., 1997).

Metaphase preparation

Metaphase spreads from the osteosarcoma cell lines OST, Saos-2, U2-OS, ZK-58, the neuroblastoma Lan-5 and a normal human lymphocyte culture were treated with colcemide and prepared according to standard procedures.

Briefly, cells were grown to half confluency in T25 flasks and then incubated with 2.5 ml of colcemide (Sigma) for 2 hours. Cells were then washed with ice-cold PBS, suspended in cold PBS and pelleted by centrifugation for 5 minutes at a speed of 1.200 G at 4°C. The pellet was resuspended in 1 ml PBS. 5 ml of KCL (75 mM in aqueous solution) warmed to 37°C were slowly added, while gently shaking the sample tube. 40 ml of KCL were then added and the cellular

suspension incubated at 37°C for 8 minutes.1 ml of freshly prepared fixative (3 parts methanol and 1 part iced acetic acid) was added. After centrifugation for 5 minutes at a speed of 1.200 G at room temperature, the pellet was resuspended in 3 ml fixative, gently homogenized and filled up to 25 ml with fixative. Following incubation at room temperature for 30 minutes, the suspension was again pelleted by centrifugation and the pellet resuspended with 25 ml fixative. This step was repeated three more times and finally the pellet resuspended in 1-4 ml fixative to achieve an optimal chromosomal solution to prepare the microscopy slides. The suspension was then dripped onto the slides and the metaphase preparations stored in 70% ethanol at 4°C.

Hybridization

The metaphase spreads were immersed in Tris-buffered Saline (TBS, diluted from 10x TBS in aqueous solution containing 0.2 M Tris base; 1.5 M NaCl) for 5 minutes prior to fixation in 3.7 % formaldehyde in TBS for 2 minutes, washes in TBS for 2x5 minutes and treatment with proteinase K (DAKO, Glostrup Denmark) for 10 minutes. After further washes in TBS for 2x5 minutes, slides were dehydrated with a cold ethanol series (70%, 85% and 96%) and airdried in a vertical position. 10 µl of Cy3-conjugated telomere specific PNA probe in hybridization solution (supplied with the kit) containing 70% formamide was added and DNA was denatured by heat for 3 minutes at 80°C. After hybridizing in the dark for 30 min, slides were briefly immersed in Rinse Solution (supplied with the kit) and then washed at 65°C for 5 minutes in Wash Solution (supplied with the kit). After rinsing with TBS slides were counterstained with 4',6-Diamidino-2-

phenylindole (DAPI), and mounted with antifade solution (VectaShield, Vector laboratories Inc., Burlingame, CA, USA).

Microscopy and Digital Image Analysis

Separate digitized gray level images of DAPI and Cy3 fluorescence were taken with a charge coupled device (CCD) camera (Cohu 6X-924) connected to a Leica DMRBE microscope. The image processing was carried out by use of Applied Image Software.

2.2.8 Comparative Genomic Hybridization (CGH)

Background

For CGH, equal amounts of biotin-labeled tumor DNA were visualized with avidin-fluorescein (FITC) and digoxigenin-labeled normal reference DNA was visualized with anti-digoxigenin-rhodamine (TRITC) hybridized to normal metaphase chromosomes. Fluorescence images were evaluated quantitating the signal intensities of the different fluorochromes as gray levels along the single chromosomes. The over-and underrepresented DNA segments reflecting gains and losses of genetic material in the tumor samples were then quantified by computation of FITC/TRITC ratio images and average ratio profiles.

Experimental Procedure

CGH analysis, microscopy and digital image analysis was performed as previously described (Brinkschmidt et al., 1998). With minor modifications, the

procedure follows the protocol established by du Manoir et al. (du et al., 1993). Briefly, tumor or cell line DNA was labeled with biotin-16-dUTP (Roche Diagnostics, Mannheim, Germany) and reference DNA from a lymphocyte culture of a healthy male donor was labeled with digoxigenin-11-dUTP (Roche Diagnostics, Mannheim, Germany) in a standard nick reaction. For CGH, 500 ng of the tumor DNA, 500 ng of reference DNA and 30 µg of human Cot 1 DNA (Gibco) were used for hybridization on target metaphase spreads (46,XY) as described elsewhere. Post-hybridization washes were carried out to a stringency of 50% formamide/2x SSC at 45°C and 0.1x SSC at 60°C. Biotinylated and digoxigenated sequences were detected simultaneously, using avidin-FITC (Roche Diagnostics, 1:200) and anti-digoxigenin-TRITC (Roche Diagnostics, 1:40). The slides were counterstained with DAPI and mounted in an antifade solution (Vectashield).

Microscopy and Digital Image Analysis

Separate digitized gray level images of DAPI, FITC and rhodamine fluorescence were taken with a CCD camera connected to a Leica DMRBE microscope. The image processing was carried out by use of Applied Image Software. Average green-red ratios were calculated for each chromosome in 5-10 metaphases.

Statistical Thresholds and Controls

Chromosomal regions with CGH ratio profiles surpassing the 50% CGH thresholds (upper threshold 1.25, lower threshold 0.75) were defined as loci with copy number gains or losses. Based on experiments with normal control DNA, these thresholds have been proved to eliminate false-positive results. As the tumor

specimens and normal DNA were not sex matched, entire X and Y chromosomes were excluded.

2.2.9 Multiplex-Fluorescence *in situ* Hybridization (M-FISH)

Background

M-FISH is a filter-based multicolor karyo-typing technology that allows for the visualization of the 22 human autosomes and the two gonosomes in 24 different colors, so that each chromosome is identifiable by its own color. M-FISH is based on the combinatorial labeling strategy (Nederlof et al., 1989). The combinatorial labeling strategy provides the simplest way to label probes in a multiplex fashion as each probe fluorochrome is either completely absent or present. Since the number of useful boolean combinations of N fluors is 2N-1, at least 5 distinguishable fluorochromes are needed for combinatorial labeling to uniquely identify all 24 chromosome types in the human genome using chromosome painting probe sets. Thus, structural (=translocations) and numerical (=deletions/gains) aberrations of chromosomal material can be detected.

Hybridization

Metaphase spreads from the osteosarcoma cell lines MNNG, Saos-2 and ZK-58 were prepared according to standard protocols. M-FISH was performed as described (Speicher et al., 1996) (Eils et al., 1998) with minor modifications. Briefly, 5 pools of whole chromosome painting probes (kindly provided by M. Ferguson-Smith, Cambridge, UK) were amplified and labeled by DOP-PCR using

5 different fluorochromes FITC, Cy3, Cy3.5, Cy5 and Cy5.5, respectively). About 100 ng of each chromosome painting probe was precipitated in the presence of 30 µg Cot-1 DNA, resolved in 10 µl hybridization mixture (15% dextrane sulfate, 2x SSC) and hybridized for 48 hours.

Microscopy and Digital Image Analysis

Microscopic evaluation was performed using a Leica DMRXA-RF8 microscope (Leica, Wetzlar, Germany) equipped with a Sensys CCD camera (Photometrics, Tucson, AZ) with a Kodak KAF 1400 chip. Images for each fluorochrome were aquired separately using highly specific filter sets (Chroma Technology Corp., Brattleboro, VT) and processed using the Leica MCK software (Leica Microsystems Imaging Solutions Ltd., Cambridge, UK).

2.2.10 GeneChip p53 assay

Background

The GeneChip p53 assay (Affymetrix, Santa Clara, CA, USA) was used for the analysis of the coding region of the human p53 tumor suppressor gene (exons 2–11). The assay identifies missense mutations and single-base deletions while detecting mutant p53 in a background of wild type p53. Briefly, this assay was performed using a DNA chip with short-oligonucleotide-probes on the array arranged in sets of five. Each probe in the set was designed to be complementary to the reference sequence except for a mismatch position, representing each of the four possible nucleotides (A,C,G,T) and a single base deletion. Assay conditions

were optimized, so that hybridization of the fluorescently labeled DNA target to the probe best matching its sequence resulted in a higher fluorescence intensity relative to the other hybridization targets.

Experimental Procedure

DNA extracted from osteosarcoma cell lines OST, SJSA-1 and U2-OS was sequenced using the GeneChip p53 assay (Affymetrix) according to the manufacturer's protocol. p53 exons 2-11 from each cell line DNA were amplified as 10 separate amplicons in a decaplex polymerase-chain reaction (PCR). Each 100-µl PCR contained

PCR Buffer II (Perkin Elmer);

2.5 mM $MgCl_2$ (Perkin Elmer);

0.2 mM of each dNTP (Sigma);

5 µl of the p53 Primer Set (Affymetrix);

10 U of AmpliTaq Gold (Perkin-Elmer); and

250 ng of genomic DNA.

PCR was carried out on MJ Research, Inc., thermocyclers under the following reaction conditions: initial denaturation at 95°C for 10 minutes, followed by 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds, with a final extension at 72°C for 10 minutes. 45-µl volumes of tumor or reference amplicons were subjected to fragmentation using 0.25 U of GeneChip Fragmentation Reagent (Affymetrix) at 25°C for 15 minutes in 0.4 mM EDTA (Gibco), 2.5 U of calf intestine alkaline phosphatase (Amersham-Pharmacia

Biotech), and 0.5 mM Tris·acetate (pH 8.2) (Teknova) before heat inactivation at 95°C for 10 minutes.

The fragmented tumor or reference DNAs (50 µl) were 3'-end labeled with fluorescein-N6-dideoxy-ATP. 25 U of terminal transferase (Promega), TdTase Buffer (Promega), 10 µM fluorescein-N6-ddATP (DuPont/NEN), and the fragmented DNA were incubated at 37°C for 45 minutes in a 100-µl reaction followed by heat inactivation at 95°C for 5 minutes. The fluorescein-labeled DNAs were hybridized to the GeneChip p53 probe arrays using the GeneChip Fluidics Station 400. 0.5-ml reaction volumes containing

6X SSPE (0.9 M NaCl, 0.06 M NaH_2PO_4, 6 mM EDTA) (BioWhitakker);
0.05% Triton X-100 (Sigma);
1 mg of acetylated BSA (Sigma):
2 nM Control Oligonucleotide F1 (Affymetrix); and
the labeled DNAs

were hybridized at 45°C for 30 minutes. Each probe array was washed twice with Wash Buffer A (3X SSPE, 0.005% Triton X-100) at 35°C prior to being scanned on a HP GeneArray Scanner (Hewlett-Packard). The captured light signals were analyzed using GeneChip Software.

3. RESULTS

3.1 Frequency of TA and subunit gene expression in matrix producing bone tumors

68 bone tumors were assayed for TA by the TRAP assay. 15/29 osteosarcomas (52%) compared to 16/23 chondrosarcomas (70%) showed TA. 6/16 histologically different benign lesions (38%), all of chondrogenic origin, presented minimal TA (Table 2).

22/68 tumors (9 osteosarcomas, 9 chondrosarcomas and 4 benign bone tumors) were assayed for telomerase core component (hTERT, hTR) expression by quantitative real-time rt-PCR. 8/11 tumors with TA (73%) were positive for hTERT gene expression and 9/11 tumors lacking TA (82%) had no detectable hTERT expression. Notably, the level of hTERT expression did not necessarily correspond to the TRAP score: one osteosarcoma with medium (++) TA displayed relatively high hTERT expression, whereas dedifferentiated chondrosarcoma with high telomerase activity (+++) also displayed only low hTERT expression scores (Table 2). Taken together, in 17/22 tumors (77%), positivity in the TRAP assay corresponded to hTERT gene expression (Table 3).

All tumors revealed hTR expression with a tendency to higher expression in telomerase positive tumors, consistent with the literature (Ito et al., 1998; Avilion et al., 1996), Table 4 and Figure 5.

Cell lines MNNG, HOS, OST and SJSA-1 displayed high TA with varying, but significantly higher levels of hTERT and hTR gene expression when compared to

Saos-2, which exhibited low TA and hTERT/hTR gene expression. The osteosarcoma cell lines U2-OS and ZK-58 had no detectable TA in the TRAP assay and no hTERT expression. U2-OS presented marginal hTR expression and ZK-58 had no detectable hTR expression (Table 5, also with CGH results and p53 status).

For the osteosarcoma cell lines, hTERT expression was assayed for using both our own relative quantitative PCR (Poremba et al., 2000, Scheel et al., 2001) approach and the TeloTAGGG-Kit (Roche Diagnostics, Figure 5). Our approach consists of a two-step RT-PCR with our own primer design for hTERT. hTERT levels are then quantified relative to the levels of the housekeeping gene GAPDH. The TeloTAGGG-Kit employs a one-step RT-PCR. hTERT are quantified levels relative to PBGD, a different housekeeping gene. Importantly, relative levels of hTERT gene expression with both approaches proved to be consistent.

Entity	Number	Telomerase Activtiy	%
Total number	**68**	**37**	**54**
Malignant tumors	**52**	**31**	**60**
Osteosarcoma	**29**	**15**	**52**
high grade	25	13	52
low grade	2	0	0
post chemo	4	1	25
relapse	2	0	0
not further class.	2	1	50
Chondrosarcoma	**23**	**16**	**70**
GI	6	5	83
GII	13	7	54
GIII	4	4	100
Benign lesions*	**16**	**6***	**38**
En/Chondroma	7	5	71
Eosinophilic granuloma	3	0	0
Giant cell tumor	1	0	0
Desmoplastic fibroma	1	0	0
Aneurysmatic bone cyst	2	0	0
Chondrogenic metaplasia	1	1	100
Synovial chondromatosis	1	0	0

*Consistently low telomerase activity (+).

Table 2: **Frequency of telomerase activity in matrix producing bone tumours.**

Case#	Age	Grade	Histopathological Diagnosis	TuCell %	TRAP	GAPDH	hTERT	hTR
Osteosarcomas								
1	15	III	osteoblastic	65	0	34	0	10
2	6	III	osteoblastic, giant cells	n.a.	0	48	0	6
3	9	III	osteoblastic/chondroblastic	n.a.	(+)	27	3	24
4	34	III	osteoblastic	n.a.	(+)	16	6	59
5	41	III	osteoblastic	80	++	42	100	22
6	15	III	chondroblastic/fibroblastic	90	0	12	0	24
7	15	III	pleomorph: osteobl/fibroblastic	70	0	16	0	100
8	12	III	n.a.	50	0	21	0	31
9	57	III	osteoblastic		+	8	0	19
Chondrosarcomas								
10	38	I	n.a.	15	+	18	0	7
11	30	I		10	(+)	37	0	3
12	73	II		n.a.	0	13	0	8
13	65	II	partially myxoid	10	0	11	0	6
14	54	II	myxoid	15	(+)	22	18	7
15	55	III		30	(+)	55	3	5
16	59	II		15	+	17	8	9
17	63	II		8	+	81	4	2
18	74	III	dedifferentiated	95	+++	100	6	24
Benign Lesions								
19	61	-	Chondrogenic metaplasia	n.a.	(+)	11	0	15
20	68	-	Synovial Chondromatosis	80	0	50	1	6
21	10	-	Eosinophilic granuloma	75	0	9	0	7
22	30	-	Giant cell tumor, second. bone cyst	90	0	41	0	2

Table 3: Telomerase subunits gene expression in matrix producing bone tumours

Case: case number, Age: age at diagnosis, Grade: tumor grade, Histopathological diagnosis: classification, TuCell%: tumor cell content on histologic slides, TRAP: telomerase activtiy measured by the TRAP assay, GAPDH: GAPDH expression, hTERT, hTR: hTERT and hTR expression relative to GAPDH, n.a.: not available.

	hTERT+	hTERT-	Entity	n	hTERT+/TA+ hTERT-/TA-
TA+	3	1	Osteosarcoma	9	8 (89%)
TA-	0	5			
TA+	5	2	Chondrosarcoma	9	7 (78%)
TA-	0	2			
TA+	0	1	Benign lesions	4	2 (50%)
TA-	1	2			
TA+	8	3	Σ = sum	22	17 (77%)
TA-	2	9			

Table 4: Correlation of telomerase activity and hTERT gene expression in malignant bone tumors:

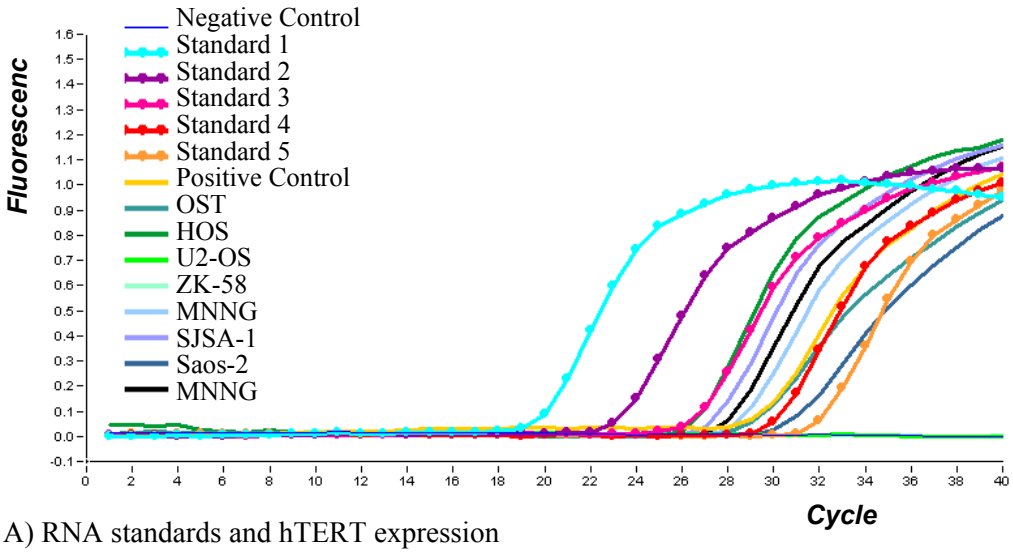
Tumors examined:	**22**
- with telomerase activity (TA+)	11
- without telomerase activity (TA-)	11

cell lines	TA	A) hTERT	B) hTERT	hTR	Mean telomer [kb]	*CGH*	p53 status
MNNG	+++	8,1	51,5	9,9	3,0	33	Point mutation Codon 156 (Radig et al., 1998)
HOS	+++	13,5	86,2	25,5	3,5	18	Point mutation Codon 152(Romano et al., 1989)
OST	+++	0,4	28,3	100,0	3,5	37	Wild type+
SJSA-1	+++	15,6	100,0	42,5	3,5	23	Wild-type+
Saos-2	+	0,2	0,1	0,01	23,0	33	large deletion(Chen et al., 1990)
U-2 OS	0,0	0,0	0,0	0,0	23,0	33	Wild type, 7 fold MDM2 amplification (Landers et al., 1997)
ZK-58	0,0	0,0	0,0	0,01	23,0	29	Wild-type+

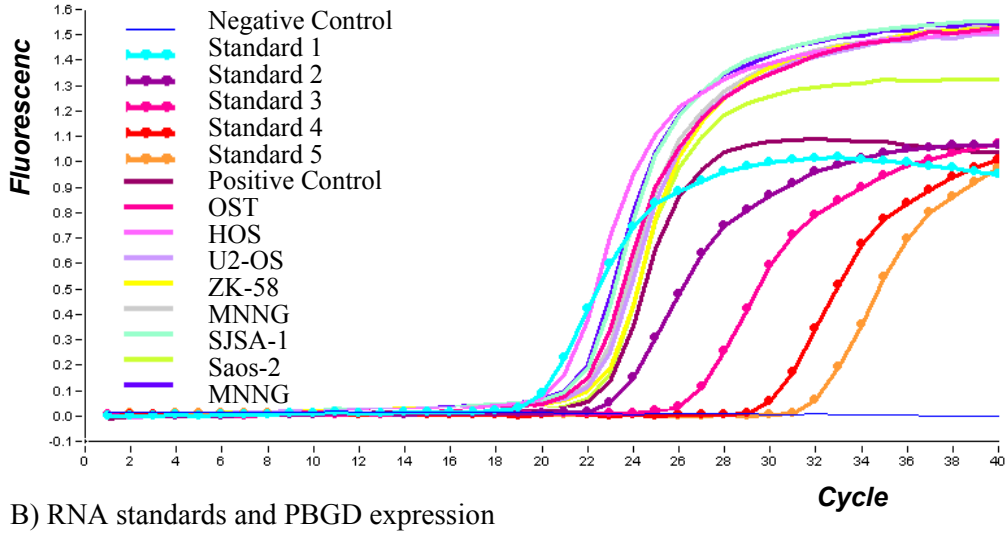
+determined using the p53 Gene Chip (Affymetrix)

Table 5: Telomere dynamics in osteosarcoma cell lines

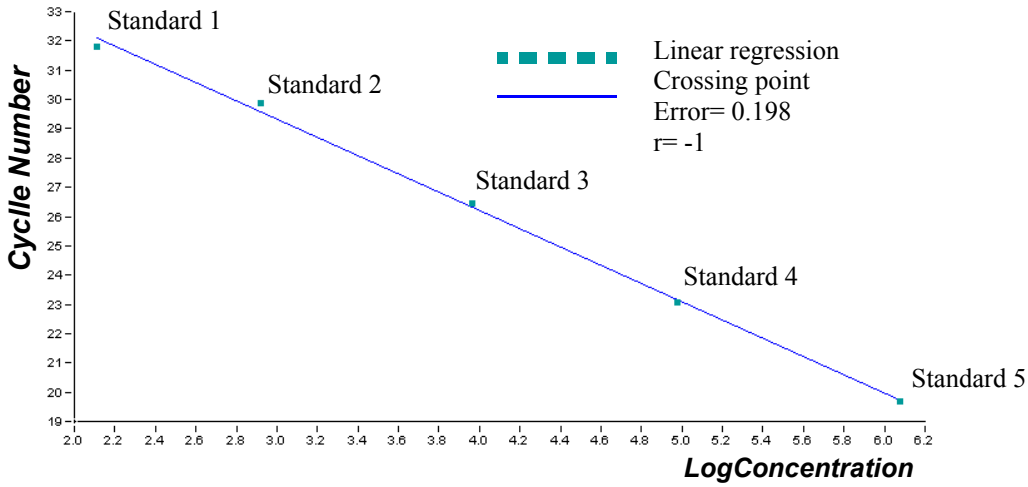
TA by TRAP: telomerase activity assayed by the TRAP assay. A) hTERT, hTERT expression relative to PBGD expression (TeloTAGGG kit, Roche Diagnostics), B) hTERT, hTR: hTERT and hTR expression normalized on GAPDH expression; values relative to highest relative gene expression which was assigned relative value 100 [%]. Mean telomeres: mean telomere length in kilobases [kb]. CGH: total number of molecular cytogenetic aberrations (gains and losses of chromosomal material) assayed by Comparative Genomic Hybridization (CGH).



A) RNA standards and hTERT expression



B) RNA standards and PBGD expression



C) Linear regression of RNA standards

Figure 5: Relative quantitative real-time PCR (Telo*TAGGG*-hTERT Kit, Roche Diagnostics). Relative values for hTERT expression are obtained by the hTERT/PBGD ratio. Negative and positive controls and linear regression of the RNA standards indicate accuracy and reproducibility of the analysis.

3.2 ALT, TA and telomere morphology

The 4/7 osteosarcoma cell lines with high TA (HOS, MNNG, OST, SJSA-1) as well as the neuroblastoma cell line SK-PN-DW (high TA in the TRAP assay), displayed short telomeres of a relatively homogeneous length. For these cell lines, a mean telomere length of 3.5 kb was calculated, determined by the strongest hybridization signal (Figure 6 and Table 5).

Osteosarcoma cell lines U2-OS and ZK-58 did not display TA and hTERT expression. Telomere length analysis of these cell lines by Southern blotting revealed a hybridization signal smear stretching from < 2 - 23 kb with the strongest hybridization signal present at 23 kb, thus representing the ALT phenotype (Bryan et al., 1997a).

Osteosarcoma cell line Saos-2 exhibited low TA in the TRAP assay, marginal hTERT and hTR expression, but elongated and heterogeneous telomeres. The strongest hybridization signal was found at 23 kb, consistent with the ALT phenotype. Thus, we assigned this cell line to represent an intermediate ALT-type.

To exclude that ALT is a cell culture artifact, DNA of two primary high grade osteoblastic osteosarcomas was included for telomere length analysis. One tumor without TA and without hTERT expression revealed elongated and heterogeneous telomeres with a mean length of 23 kb, representing the ALT telomeric phenotype. The other tumor, with intermediate TA and high hTERT expression, had telomeres with a mean length of 4 kb.

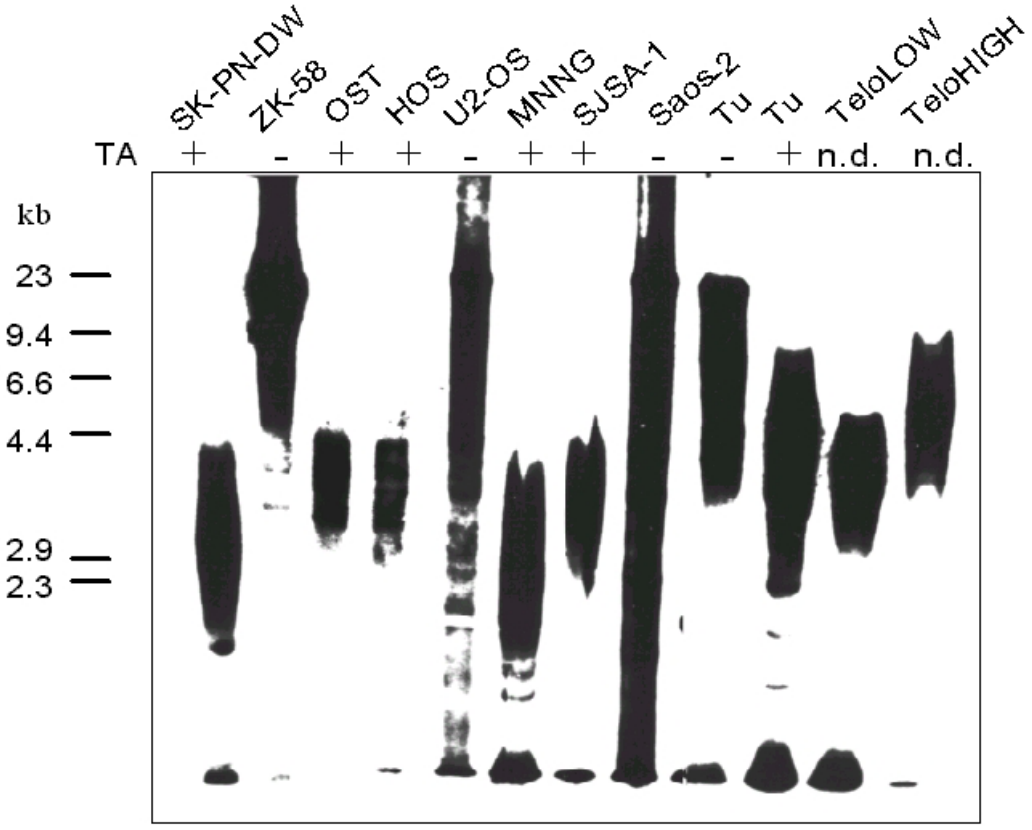


Figure 6: Telomere length and morphology

Telomere Restriction Fragment (TRF) analysis, telomere lengths of the neuroblastoma cell line SK-PN-DW, the seven osteosarcoma cell lines included in this study (ZK-58, OST, HOS, U2-OS, MNNG, SJSA-1, Saos-2) and 2 primary high grade, highly cellular osteoblastic osteosarcomas (Tu). TA, telomerase activity; kb, kilobases. Cell lines (ZK-58, U2-OS) and the tumor without detectable telomerase activity display elongated and hetereogeneous telomeres characteristic for ALT. Cell line Saos-2 displays marginal telomerase activity in the TRAP assay and the elongated ALT telomeres in TRF analysis, therefore termed to be of intermediate ALT-type. Telo LOW and Telo HIGH are HinfI/RsaI digested control DNAs with a mean length of 3.3 kb and 11.3 kb, respectively.

3.3 Fluorescence *in situ* hybridization with a telomere specific PNA-probe (T-FISH)

Twenty metaphases each of neuroblastoma cell line Lan-5 and osteosarcoma cell line OST, both with high TA, ALT cell lines U2-OS, ZK-58 and intermediate Saos-2 and a normal human lymphocyte culture were examined. Aberrations present in at least 50% (10 metaphases) are described.

Chromosomes of Lan-5, OST (Figure 7) and the human normal lymphocyte culture (data not shown) essentially showed four fluorescent hybridization signals at telomeric positions of similar fluorescent intensity.

In contrast, in Saos-2, U2-OS and ZK-58, fluorescence intensity differed greatly in chromosomes of the same metaphase and between chromosome ends (Figure 8). Hybridization spots with a higher and a lower intensity than in Lan-5, OST and the lymphocyte culture were found. Large, dicentric marker chromosomes indicating end-to-end fusion were present. Telomeric repeats were found at unusual loci and configurations. Some chromosomes had no detectable telomeric repeats at one or both ends (signal-free ends), others displayed from one up to four signals at chromosome ends. Additionally, telomeric signals at ectopic and intrachromosomal centromeric positions were detected.

Among chromosomes with aberrant telomere morphology, chromosomes with a „normal“ telomeric phenotype were found ruling out the possibility of failed or unspecific hybridization. All slides were hybridized in the same assay and the procedure was performed twice with consistent results.

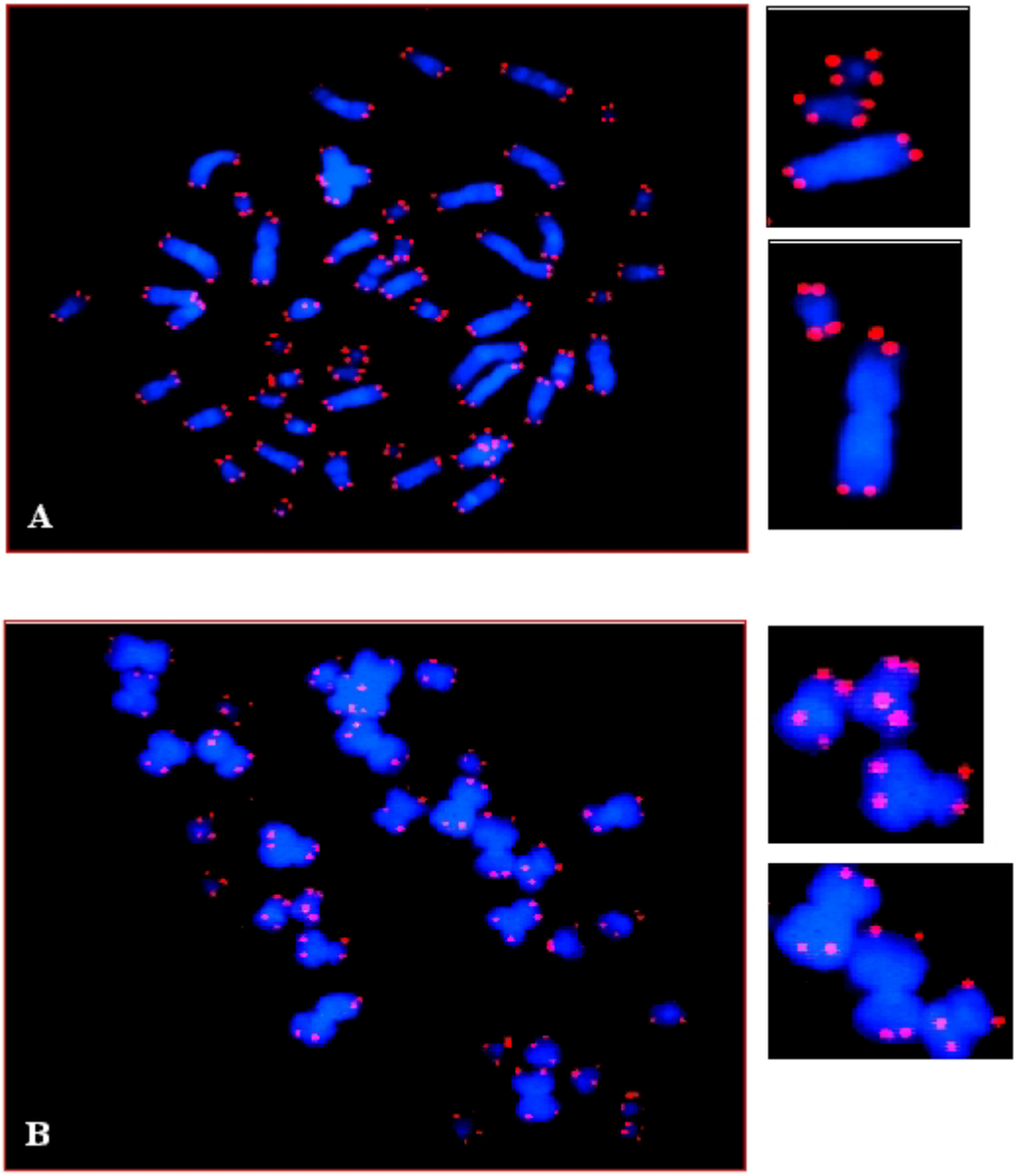


Figure 7: Telomere fluorescence *in situ* hybridization (T-FISH)
Metaphase spreads of cell lines with high telomerase activity. Telomere lengths are homogeneous, no marker chromosomes are seen: A), metaphase spread and representative chromosomes of neuroblastoma cell line Lan-5. B), metaphase spread and representative chromosomes of osteosarcoma cell line OST.

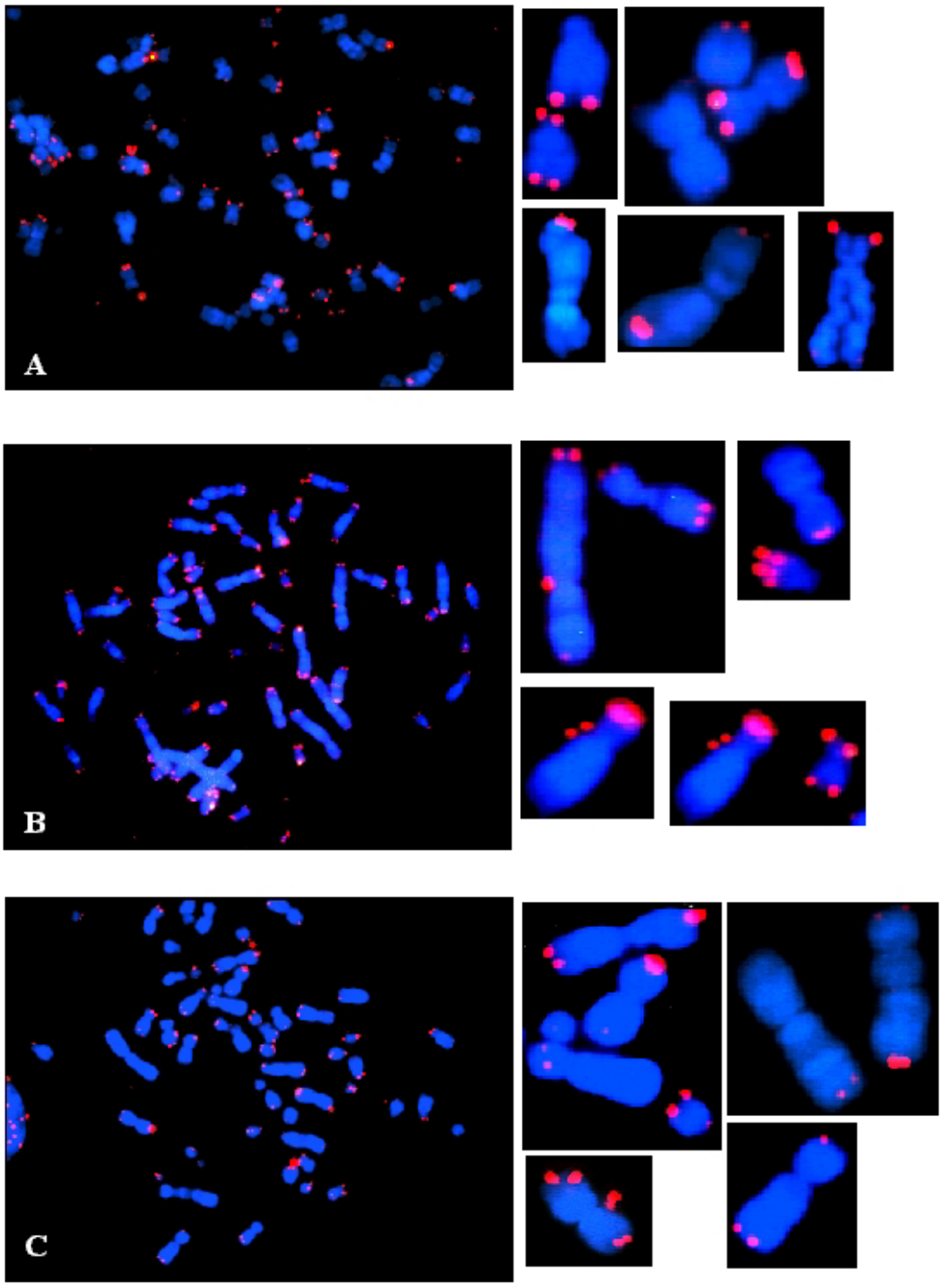


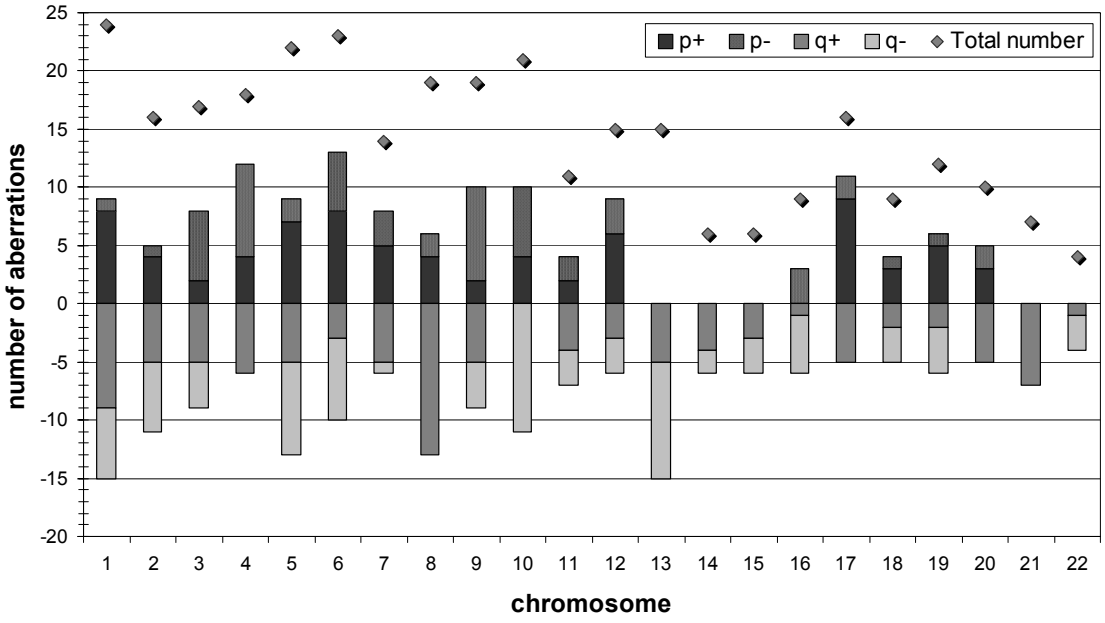
Figure 8: Telomere fluorescence *in situ* hybridization (T-FISH)
Metaphase spreads and representative chromosomes and marker chromosomes with atypical telomeres of osteosarcoma cell lines A), U2-OS, ALT; B), Saos-2, intermediate ALT-type; C), ZK-58, ALT.

3.4 Comparative Genomic Hybridization (CGH)

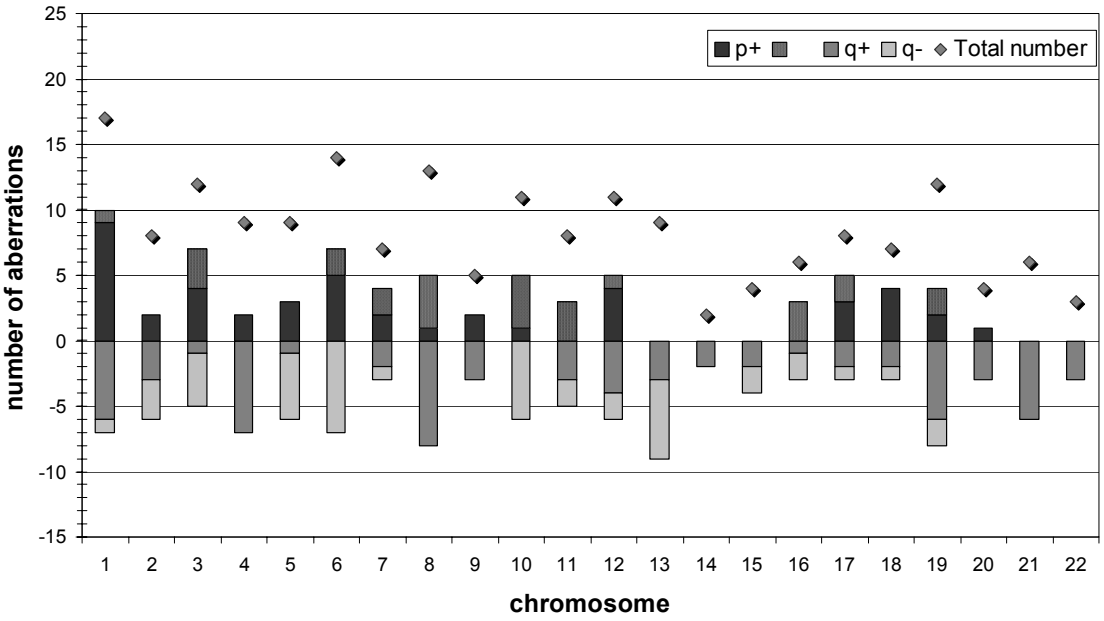
The total number of molecular cytogenetic aberrations in the 24 analyzed osteosarcomas ranged from 3 up to 35 aberrations with a mean total number of aberrations of 19 ± 9. The 7 osteosarcoma cell lines analyzed revealed a mean total number of 29 ± 7 aberrations ranging from 18 to 33 aberrations per cell line (Table 5).

However, number and distribution of gains and losses of genetic material was heterogeneous and no loci were shared by all tumors and/or cell lines, or by tumors grouped according to their telomerase status. Thus, no aberrations were found to be significant discriminators for osteosarcomas with or without telomerase activity (Figure 9).

In this regard, emphasis was laid on the analysis of chromosomal regions which are related to the regulation of TA. There is evidence for a cellular telomerase repressor at chromosome 3p (Tanaka et al., 1998; Shay, 1999). The hTERT gene was recently mapped to chromosome 5p15.33 by fluorescence in situ hybridization (Bryce et al., 2000). Tumors were grouped into 13 tumors with and 11 tumors without TA. The Chi-square test and Fisher's Exact test for the parameters of gains/amplifications and losses on chromosome 3p and 5p, respectively, revealed no differences between both groups.



A) Osteosarcomas with TA (n=13)



B) Osteosarcomas without TA (n=11)

Figure 9: Comparative genomic hybridization (CGH)
Shown is the distribution and total number of all molecular cytogenetic aberration in the 24 analysed ostesarcoma tumor samples with p+: gain of genetic material on the short chromosomal arm (for example, by amplification), p-: loss on the short arm (for example, by deletions), q+: gain on the long arm, q-:loss on the long arm.

3.5 24-color multiplex fluorescence *in situ* hybridization (M-FISH)

Metaphases of 3 osteosarcoma cell lines (ZK-58 n=10, Saos-2 n=5, MNNG n=3) were analyzed for structural (= rearrangements) and numerical (= aneuploidy) chromosomal aberrations.

In the osteosarcoma cell line Saos-2 with a modal number of 52 chromosomes, 37 chromosomes (71.2%) were affected by structural aberrations, whereby a high ratee of complex rearrangements composed of three up to five different chromosomes was found (12/37), Figure 10.

Very similar results were achieved by M-FISH analysis in the cell line ZK-58. Structural abnormalities were detected in 69.8% (37/53) of the chromosome, whereby 10 chromosomes showed complex rearrangements composed of three to five different chromosomes (Figure 11).

In contrast, in the osteosarcoma cell line MNNG, M-FISH detected structural aberrations only in 29.9% (18/60) of the chromosomes, whereby three chromosomes consisted of three different chromosomal segments (Figure 9). Additionally, M-FISH identified a trisomy for chromosomes 1, 2, 5 and 22. Thus, in contrast to ALT cell lines Saos-2 and ZK-58 this telomerase positive cell line demonstrated a pseudotriploid karyotype.

When comparing ZK-58 (no TA, elongated telomeres) and Saos-2 (low TA, elongated telomeres) with MNNG (high TA, short telomeres) using the Wilcoxon test, the proportion of complex rearranged chromosomes (with three up to six chromosomes involved) as well as the overall number of rearrangements was significantly higher in ZK-58 and Saos-2 (both p=0.009). In contrast, the number of trisomies was significantly higher in MNNG (p=0.0001), Figure 12.

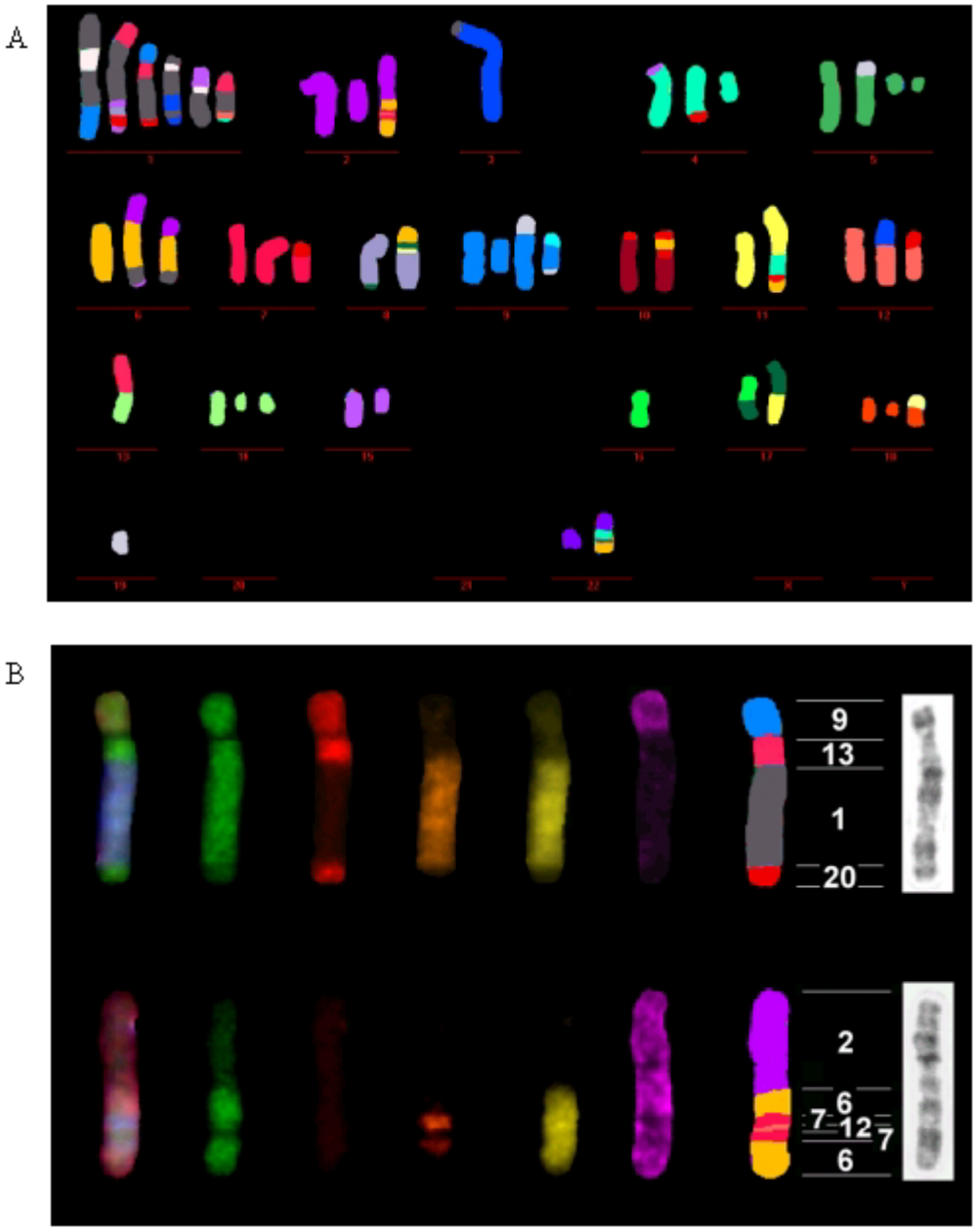
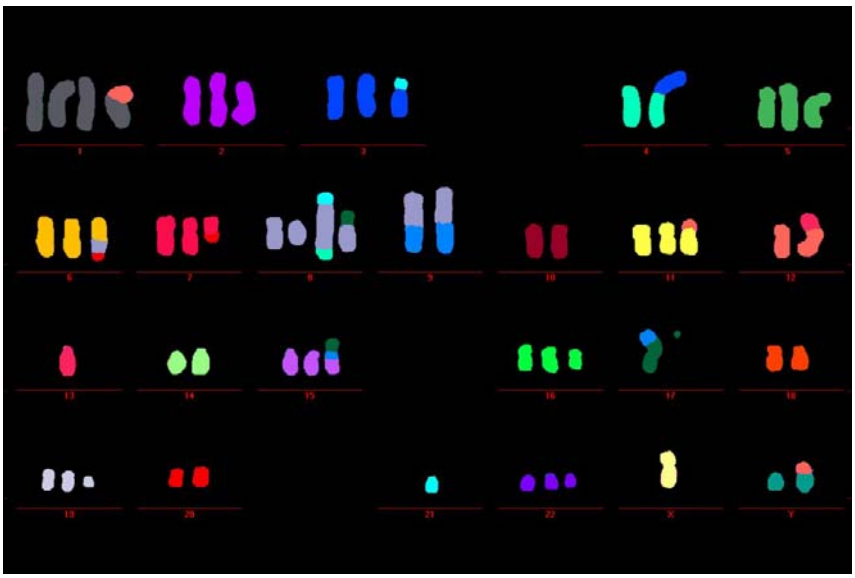
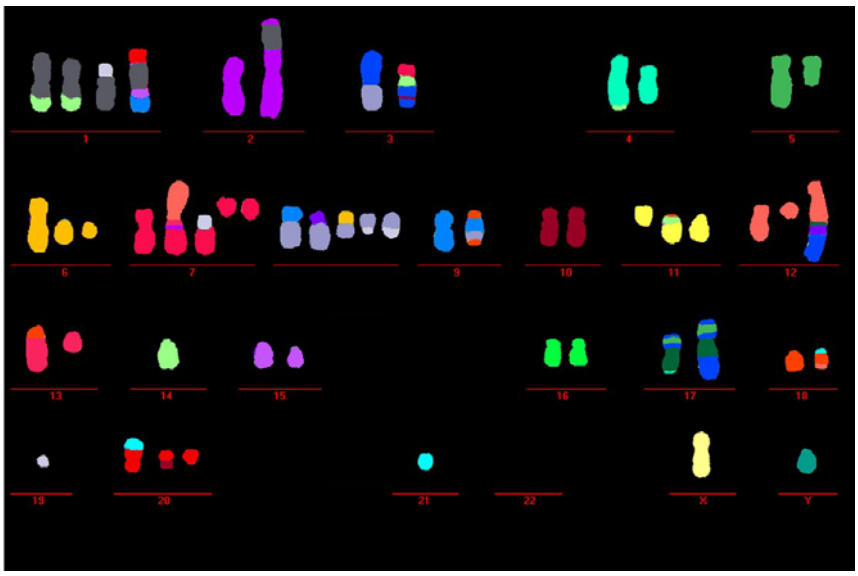


Figure 10: Multiplex *in situ* hybridization (M-FISH)
A) metaphase of osteosarcoma cell line Saos-2, intermediate ALT-type. B) Complex chromosomal rearrangements visualized by M-FISH. Shown are two marker chromosomes from a metaphase spread of osteosarcoma cell line Saos-2 with intermediate ALT-type. Using DAPI as a counterstain and 5 different fluorochrome pools, they are identified as chromosome 1 and 2 with complex rearrangements involving three and five different chromosomes. G-banding, as shown on the right hand of this picture, is not elusive for the composition of these two marker chromosomes.



A



B

Figure 11: Multiplex *in situ* hybridization (M-FISH)
Shown are typical metaphases from osteosarcoma cell lines A) MNNG (TA); B) ZK-58 (ALT)

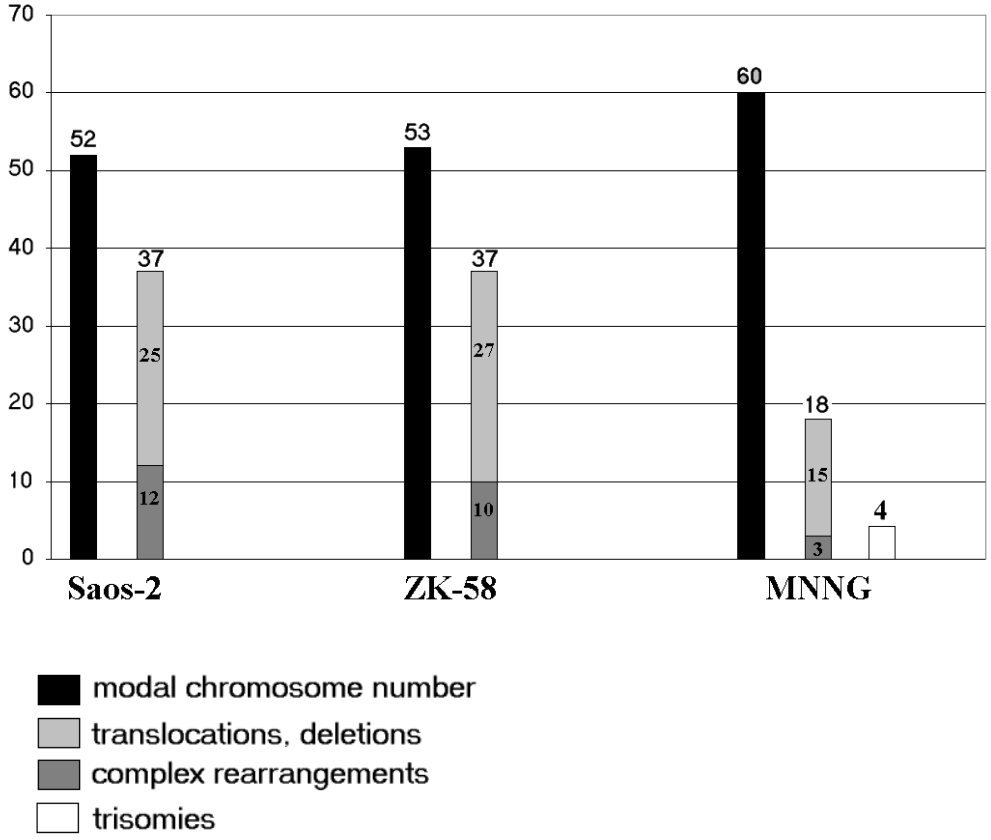


Figure 12: Multiplex *in situ* hybridization (M-FISH) diagram
Shown are the modal chromosome number, the total number of rearrangements subdivided into translocations and deletions, complex rearrangements (with three up to five different chromosomes involved) and trisomies for osteosarcoma cell lines Saos-2 (intermediate ALT-type), ZK-58 (ALT) and MNNG (TA).

3.6 p53 status

Osteosarcoma cell lines OST, SJSA-1 and ZK-58 harboured no point mutations in the p53 gene by sequencing using the GeneChip p53 Assay (Table 5).

Osteosarcoma cell line MNNG harbours a documented point mutation in codon 56 (Radig et al., 1998). Therefore, DNA of the cell line MNNG as a positive control for the GeneChip p53 Assay was included. Indeed, the described point mutation in codon 56 could be confirmed in this assay.

4. DISCUSSION

4.1. Frequency of telomerase activity in malignant bone tumors

Approximately 40% of the malignant bone tumors (21/52) included in the study displayed no TA, consistent with earlier investigations (Aue et al., 1998). When the malignant tumors were grouped according to their histologic subtype there were differences in the frequency of TA: 52% of the osteosarcoma tumor samples (15/29) were positive for TA, but 70% of the chondrosarcomas (16/23) displayed TA. The histologic composition of the osteosarcomas was different to that of the chondrosarcomas; 25 out of 29 osteosarcomas (86%) included in the study were high grade osteosarcomas. In contrast the collective of 23 chondrosarcomas comprised only 4 high grade chondrosarcomas, 6 low grade and 13 intermediate grade chondrosarcomas. All high grade chondrosarcomas were positive for TA. These results suggest that the frequency of TA may be generally lower in osteosarcomas.

Of the benign lesions 6/16 (38%), all of chondrogenic origin, were positive for TA. Levels were generally lower than in any of the examined malignant tumors. When examining the corresponding histologic slides, bone marrow stem cells and infiltration of lymphocytes in some regions of these lesions were found which are reported to display low TA (Harle and Boukamp, 1996; Counter et al., 1995).

Contamination with these cells in the corresponding samples could explain our findings of minimal TA.

4.2 Expression of the telomerase essential subunits hTERT and hTR

Expression of hTERT appears to be the rate-limiting step in telomerase activity (Bodnar et al., 1998; Meyerson et al., 1997). However TA did not correspond to hTERT expression in 5/22 tumors (23%) analyzed for gene expression. In many tumors little material was available, on one hand due to an excess of extracellular matrix, on the other hand due to the small size of the biopsies. One explanation for this discrepancy between TA and hTERT expression may be that the frozen tissue was first cut for protein extraction for the TRAP assay. In deeper sections, which were used for RNA extraction, cell content was less and there was a higher proportion of matrix. This hypothesis was proven by corresponding hematoxylin-eosin stained histological slides cut before and after the material for protein and RNA extraction.

All tumors and cell lines except U2-OS did express hTR with a tendency to higher expression levels in telomerase positive cell lines which implies that lack of telomerase activity in these samples may not be due to abrogation of hTR. One could hypothesize that an atypical telomerase escaping detection by TRAP may be involved in ALT. However, some other ALT cells do not express any hTR, although the hTR sequence is wild-type (Bryan et al., 1997b). It has been found that the promoter of the telomerase RNA gene is methylated in ALT cell lines that

are characterized by a total absence of hTR expression (Hoare et al., 2001). Furthermore, telomere maintenance can occur in immortalized cell lines established from telomerase-deficient mice with a deleted telomerase RNA gene (Hande et al., 1999). Hence, the participation of an atypical telomerase in these cells can be excluded if there is not another RNA template that can be used. Furthermore, since ALT is a descriptive term refering to the presence of elongated telomeres in the absence of telomerase, it is possible that several mechanism account for the same phenomenon.

4.3 ALT in osteosarcomas and in other malignant human tumors

The equal frequency of high grade osteosarcomas with and lacking TA together with the finding of ALT in the osteosarcoma cell lines U2-OS and ZK-58, and in one primary tumor analyzed as an example, suggest that TA is not necessary for tumor progression in these lesions.

Notably, not all tumors without telomerase activity have been found to show ALT-like telomeres (Bryan et al., 1997a). Perhaps these findings reflect at least in part tumors that presented clinically before immortalization. Therefore, not all of the approximately 10% telomerase negative tumors (Shay and Bacchetti, 1997; Dhaene et al., 2000; Keith et al., 2001) may display the telomere morphology idiosyncratic for ALT. However, it cannot be excluded that other mechanisms exist that are independent from telomerase and do not exhibit the ALT pattern of telomere morphology.

So far, no extensive surveys for ALT have been carried out, but a variety of malignant human tumors may exhibit ALT (Mehle et al., 1996; Bryan et al., 1997a), Table 6. The rates of ALT activation are higher in *in vitro* immortalized cell lines than in tumor-derived cell lines (Table 7). Possibly, the incidence of ALT is higher in some cell types than others; most of the *in vitro* immortalized cell lines are from mesenchymal origin. Low-level telomerase activity is present in normal somatic cells such as basal keratinocytes (Harle and Boukamp, 1996) and mammary epithelial cells (Yasumoto et al., 1996), but not in fibroblasts (Hsiao et al., 1997). Epithelial cells may be simply more "competent" for telomerase activation. Since approximately 80% of solid tumors are carcinomas, this may account for the infrequent findings of ALT (also refer to Bryan et al., 1997a).

Tumor type	**Reference**
Adrenocortical carcinoma	(Bryan et al., 1997a)
Breast carcinoma, Li-Fraumeni syndrome	(Bryan et al., 1997a)
Malignant melanoma	(Bryan et al., 1997a)
Lung carcinoma	(Bryan et al., 1997a)
Ovarian carcinoma	(Bryan et al., 1997a)
Osteosarcoma	(Bryan et al., 1997a; Scheel et al., 2001)

Table 6: Primary tumors that were shown to exhibit the ALT phenotype.

	No. of samples	TA (TRAP)		Elongated and heterogeneous TRFs	
		+TA (%)	-TA (%)	-TA	+TA
In vitro immortalized cell lines	50	31 (62)	19 (38)	19	0
Tumor-derived cell lines	57	52 (91)	5 (9)	5	1
Total	107	83 (78)	24 (22)	24	1

Table 7: Frequency of the ALT phenotype in human cell lines (Bryan et al., 1995; Bryan et al., 1997a; Sprung et al., 1997; Scheel et al., 2001).

Even if only a small fraction of primary tumors express ALT, its role as a possible backup-mechanism when telomerase activity is abolished and the investigation of the particular molecular circumstances under which ALT is being induced or repressed, are important aspects in telomere signaling, particularly in the light of new treatment strategies targeting telomere maintenance mechanisms (reviewed in Bearss et al., 2000)

4.4 ALT, TA and telomere morphology and function

4.4.1 Telomere morphology in oseosarcoma cell lines with TA

In telomere length analysis the telomerase positive cell lines MNNG, HOS, OST and SJSA-1 exhibited short telomeres of homogeneous length consistent with

most malignant immortalized cells examined to date (Shay and Wright, 1996). Semiquantitative analysis of telomere morphology of OST by T-FISH reflected these findings by showing four fluorescent spots of similar intensity at each telomeric position. A similar morphology was also found in the telomerase positive neuroblastoma cell line Lan-5 and a normal human lymphocyte culture. These findings reflect the *modus operandi* of telomerase in telomere lengthening: although there are differences in telomere lengths in cell populations with telomerase, those are relatively subtle, because telomerase creates a dynamic balance between elongating and shortening and stabilizes telomeres around an optimized length by elongating the shortest telomere (Pommier et al., 1995; Hemann et al., 2001).

4.4.2 Saos-2: an osteosarcoma cell line with intermediate ALT-type

Osteosarcoma cell line Saos-2 proved to have low telomerase activity and elongated telomeres typical for the ALT phenotype. It is possible that the low telomerase activity found in Saos-2 is not sufficient to prevent telomere shortening which in turn could give rise to ALT. Saos-2 was determined as an ALT cell line of intermediate type.

Perrem et al (Perrem et al., 1999) demonstrated that the ALT telomere phenotype was abrogated in cell hybrids between ALT and telomerase-positive cells, implying that co-existence of ALT and telomerase activity is rather unlikely. On the other hand, some telomerase positive tumors were shown to have elongated and heterogeneous telomeres which could indicate a coexistence of ALT and

telomerase activity (Bryan et al., 1997a; Reddel et al., 2001). Furthermore, coexistence of ALT and telomerase appears to be biologically possible and has been described in three recent studies on *in vitro*-immortalized ALT cell lines by ectopic expression of telomerase (Cerone et al., 2001; Perrem et al., 2001; Grobelny et al., 2001).

4.4.3 Telomere morphology in oseosarcoma cell lines displaying ALT

Fluorescence intensity corresponds directly to telomere length (Lansdorp et al., 1996). Saos-2 and ALT cell lines U2-OS and ZK-58 revealed intracellular and interchromosomal variations in telomere morphology, implying that the heterogeneity of telomere length found by Southern blotting is not an intercellular phenomenon due to a mixed population composed of cells with long telomeres and cells with short telomeres. These long telomeres are reminiscent of telomerase-null "type-II" survivor cells in the yeast *Saccharomyces cerevisiae* (Lundblad and Blackburn, 1993; Teng and Zakian, 1999). Type-I survivors arise by recombination events in the telomere-adjacent Y' sequence elements, have very short terminal telomere repeats and grow slowly. In contrast, type-II survivors have long hypervariable tracts of telomere repeats, possibly due to recombination initiated within the telomere-repeat sequences. These type-II survivor yeast cells grow at a rate similar to wild-type cells with stability and growth rate suggesting that the addition of telomeric repeats by recombination is sufficient for genome maintenance (Teng et al., 2000)

Furthermore, Dunham et al. demonstrated that ALT in human cells can indeed occur by means of homologous recombination and copy-switching (Dunham et al., 2000)

4.4.4 ALT is associated with chromosomal instability in osteosarcomas

However**,** the ALT cell lines we analysed presented not only intracellular polymorphism in telomere length, but also a spectrum of unusual chromosomal loci and configurations of telomeric repeats with the occurrence of long, dicentric marker chromosomes. Telomeres are important for chromosomal stability (Counter et al., 1992; Autexier and Greider, 1996). A peak of dicentric chromosomes resulting mostly from telomeric associations occurs in SV40 transformed fibroblasts prior to crisis when telomere shortening reaches a critical point (Ducray et al., 1999). Activated telomerase homogenizes telomeres in a dynamic balance between elongation and shortening towards a stabilized length which in turn promotes chromosomal stability.

The results of this study suggest that ALT cells do not expand their replicative potential under telomere stabilizing conditions (Figure 13, Lansdorp et al., 1997).

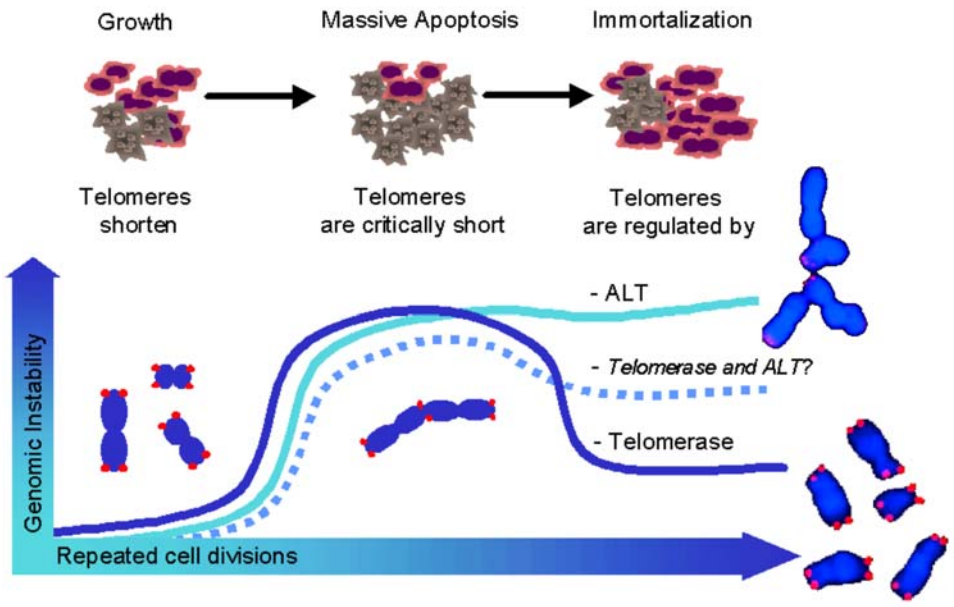


Figure 13: Growth and telomere dynamics in cells during immortalization. With progressive telomere shortening, genomic instability and the apoptosis rate increase, reaching a peak at crisis. Dependent on precedent mutations, immortalized survivor cells regulate telomere length either by telomerase activity or ALT. A coexistence of both mechanisms may be possible as well. Dependent on the telomere elongation mechanism, cells may continue to proliferate with different tolerances and levels of genomic instability.

ALT cells demonstrate a great heterogeneity in telomere length; signal-free ends and interchromosomal telomeric signals occur within single metaphases and even between sister chromatids of the same chromosome (Lansdorp et al., 1997; Perrem et al., 2001; Scheel et al., 2001; Ford et al., 2001). The occurence of elongated and shortened telomeres and signal-free chromosome ends could give rise to chromosomal end-to-end associations and breakage-fusion-bridge cycles resulting in an increased number of complex non-reciprocal chromosomal rearrangements (McClintock, 1941; Autexier and Greider, 1996; Bouffler et al., 1996). Indeed, M-FISH analysis provides evidence for this hypothesis by showing

a significant higher proportion of complex rearranged chromosomes (with 3-6 chromosomes involved) as well as a higher overall number of rearrangements in the two osteosarcoma with the ALT phenotype. As a corollary it can be assumed that ALT reflects a compromised state of telomere function.

Furthermore increasing data suggests that rescue of telomeric function is not only determined by net lengthening of telomeres. Zhu et al. proposed a requirement for capping of telomeres by telomerase(Zhu et al., 1996), another function that might be abrogated in ALT cells. Moreover, telomerase is capable to heal broken chromosome ends (Flint et al., 1994; Melek and Shippen, 1996; Slijepcevic and Bryant, 1997) and inhibit chromosomal instability (Hackett et al., 2001). Possibly, active telomerase itself prevents recombination in telomeric regions. In telomerase-deleted mutants of the yeast species *Kluyveromyces lactis* recombination events between the telomeric tracts increase in frequency even in cells where telomeres are still relatively long (McEachern and Blackburn, 1996). It appears that in many ways, introduction of telomerase is a tool in dividing cells to carefully monitor complete replication of telomeric DNA in a cell-cycle dependent manner in order to guarantee telomere function and therefore chromosomal integrity (reviewed in Blackburn, 2001; Gasser, 2000). Consequently, T-loop formation and therefore capping of the telomere are at least in part dependent on telomere length. Shortening telomeres may continually undergo regulated degrees of temporary unfolding and uncapping, providing access for telomerase to elongate the shortest telomeres in a telomere population (Hemann et al., 2001; Blackburn, 2001). Cooperation of telomerase with components of the DNA damage machinery in gaining access to the telomere possibly allows cell cycle checkpoint control of telomere replication (Gasser,

2000). In a conceivable scenario, when telomeres shorten and there is neither telomerase nor certain inhibitory mechanisms acting on the unfolding telomeres, recombination as in ALT may take place. Thus, all factors contributing to form the DNA-protein complex at the telomere are candidates to be altered in order to allow ALT to proceed (Figure 13).

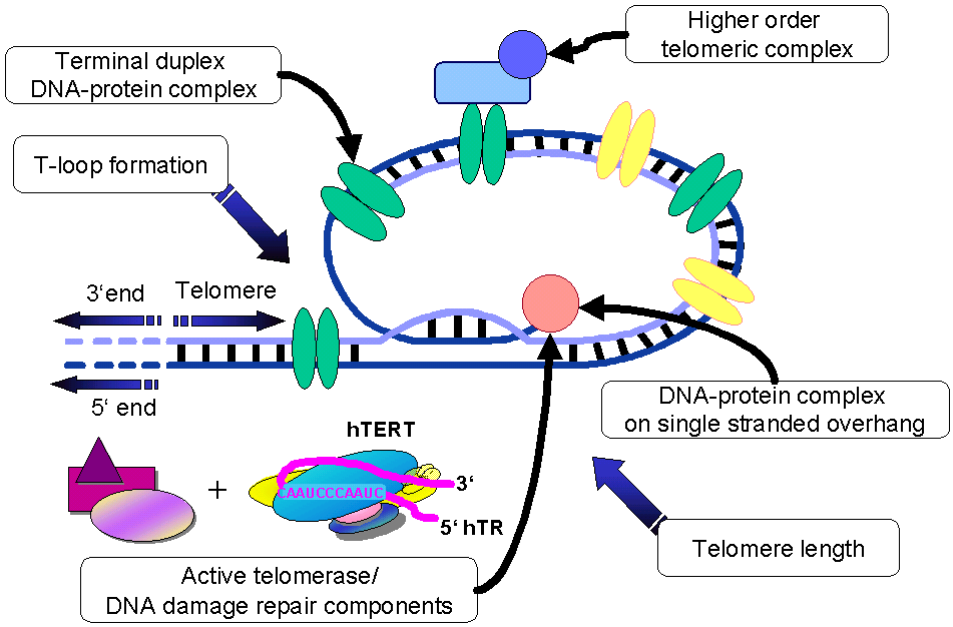


Figure 13: Telomere maintenance: a complex picture
Some factors influencing telomere maintenance in human cells that are candidates to be altered in order to let ALT take place.

4.5 Regulation of ALT

Results from Perrem et al. employing somatic cell fusion (Perrem et al., 1999) imply that one or more repressors of ALT exist in normal, telomerase negative and tumor-derived telomerase positive cells and that repression of ALT in hybrid cells may not be mediated by telomerase. Somatic fusion of an ALT cell line with

a normal fibroblast cell line led to repression of ALT, evidenced by a rapid attrition of telomere length and induction of senescence after approximately 30 population doublings (Perrem et al., 1999). Rare escapees from senescence reverted to ALT as demonstrated by TRAP and TRF analysis. Interestingly, during repression of ALT, telomeres shortened at a rate that is approximately 10-fold higher (Perrem et al., 1999) than observed in normal somatic cells (50 – 200 base pairs, reviewed in Broccoli and Cooke, 1993). This rapid attrition was not observed in hybrid cells resulting from fusion of an *in vitro* immortalized ALT cell with tumor derived cell lines displaying telomerase activity: in these hybrids ALT was repressed with telomeres shortening at a rate comparable to normal cells. Further studies are required to determine whether ALT occurs due to a dominant activating mutation or due to loss of repressors which are present in normal and telomerase positive cells.

However, our CGH analysis could not elucidate if ALT cells are deficient for functional telomerase, or if a pattern of aberrations the tumor cells have acquired allow them to progress under conditions of chromosomal instability.

Furthermore, we detected no point mutations in p53 in ALT cell lines U2-OS and ZK-58, which however does not exclude non-functioning of the p53 pathway. Genetic changes known to be permissive to ALT activation include transformation of cells with SV40 or human papillomavirus (HPV) oncogenes and spontaneous loss of the tumor suppressors p53 and $p16^{INK4a}$; however, to a greater proportion these changes result in activation of telomerase (Rogan et al., 1995; Vogt et al., 1998; Bryan et al., 1995). Transformation by both SV40 and HPV are dependent on disruption of p53 and the retinoblastoma protein (pRb, reviewed by

Whitaker et al., 1995). The key-role of p53 (Wynford-Thomas, 1996) makes it difficult to dissociate specific executors of immortalization: pRb, $p16^{INK4a}$ and other proteins such as cyclin D1 are all linked in a common pathway that is disrupted in the majority of cancers. Active pRb inhibits cell cycle progression, active cyclin D1 is part of the complex inhibiting pRb and, finally, $p16^{INK4a}$ inhibits cyclin D1-action. Proliferative potential can be increased by disruption of either pRb or $p16^{INK4a}$ (Shapiro et al., 2000). Recently, it was demonstrated that cyclin D1 overexpression indeed has a similar effect (Opitz et al., 2001). Oral-esophageal carcinomas are a model-system to investigate the molecular mechanisms underlying squamous carcinogenesis and frequently overexpress cyclin D1 while the p53 tumor suppressor is inactivated. Opitz. et al. demonstrated that cyclin D1 overexpression alone extended the replicative lifespan of normal oral keratinocytes. A combination of cyclin D1 overexpression and inactivation of p53 led to their immortalization by activation of ALT. Likely, these changes contribute to a permissive environment for mutations resulting in activation of ALT, for example by fostering genetic instability. An interesting strategy to further delineate the pathways contributing to telomere maintenance via ALT or TA could be the use of small interering RNAs (RNAi).

4.6 Perspectives

4.6.1 Screening for ALT

Recently, a potentially useful morphological marker of ALT has been described: ALT-associated PML bodies (APBs, Yeager et al., 1999). PML protein was so

named for its involvment in fusion events in promyelocytic leukemia (de The et al., 1991). PML bodies can be found in normal interphase nuclei and are donut-shaped structures containing PML protein (Hodges et al., 1998). APBs are PML bodies with distinctive contents. They contain PML protein, telomeric DNA and the doublestrand telomere binding proteins human telomere repeat factor (TRF) 1 and 2. Furthermore, they contain replication factor A, RAD51 and RAD52: proteins involved in DNA synthesis and recombination. In one *in vitro* immortalized fibroblast cell line, APBs could be detected at the time of immortalization, concomitant with the appearance of ALT (Yeager et al., 1999). Xenografted ALT-tumors in nude mice were positive for APBs (Yeager et al., 1999) and in all ALT cell lines examined to date APBs could be readily found.

Currently, to test cells for ALT, it is necessary to collect fresh frozen tissue samples for whole cell lysates to be used in the TRAP assay, genomic DNA for TRF analysis and/or to prepare metaphases for t-FISH. APBs can be found in 5% of interphase nuclei and therefore appear feasible to be detected by immunohistochemistry in paraffin-embedded tumor tissue; evidence of APBs can be provided by colocalization of PML with either TRF1 and/or TRF2 (Yeager et al., 1999). These findings should render it possible to analyse a wide panel of archival tumor tissue for the presence of ALT.

4.6.2. Mismatch repair and telomere maintenance

ALT does not appear to be an avenue for telomere replication in normal cells in higher eukaryotes; probably, it would represent an illegitimate recombinational event.

Interestingly, Rizki and Lundblad demonstrated that defects in the DNA mismatch repair machinery (MMR) promote telomerase-independent proliferation in telomerase-defective budding yeast (Rizki and Lundblad, 2001), indicating that the anti-recombination activity of the MMR machinery (Modrich and Lahue, 1996) might have an inhibitory effect on the ability of telomerase-defective cells to proliferate. A barrier to recombination has been proposed to preserve chromosomal integrity by preventing undesirable exchanges between chromosomes (Rayssiguier C. et al., 1989). Rizki and Lundblad showed that removal of this barrier may, under certain conditions, promote cell proliferation and thereby eventually alleviating the requirement for telomerase activity. It might be of interest, whether defects in the MMR can be linked to the occurrence of ALT in human tumors.

4.6.3 Possible implications of ALT for cancer diagnosis and therapy

A strong diagnostic or prognostic impact of the presence of telomerase activity does not automatically imply that inhibition of telomerase will prove to be a succesful therapeutic strategy. ALT has been shown to occur alone and in coexistence with telomerase in *in vitro* immortalized cell lines, tumor derived cell lines and primary tumors. In the light of new telomere maintenance targeted cancer therapy approaches, the existence of ALT has to be carefully evaluated. Telomerase inhibitors will be likely less effective or even ineffective for tumors containing ALT cells. As a corollary, inhibition of telomerase may increase the selection pressure for the activation of ALT in previously telomerase-positive tumors. The association of ALT with chromosomal instability and telomere dysfunction may have major implications for tumor biology. The requirement of a

somewhat higher degree of telomere stabilization in tumor progression may be dependent on preceding mutations during tumorigenesis that render tumor cells more chromosomally instable. Elucidation of the molecular circumstances under which ALT is being repressed and induced will be important not only for a more complete understanding of signaling at the telomere, but also for pushing forward the newest approaches in cancer therapy strategies that aim at generally disrupting telomere maintenance and function

5. SUMMARY

Telomeres act as crucial maintainers of chromosomal stability and cell viability in a variety of different species. Activation of telomerase, a specialized reverse transcriptase, is a predominant mechanism from yeast to human cells in maintaining telomere length and function. Telomere maintenance is regarded as a key mechanism in cellular immortalization and thereby oncogenesis. Although more than 90% of all malignant tumors display telomerase activity, under certain circumstances, telomerase appears not to be essential in this process. Alternative lengthening of telomeres (ALT) in the absence of telomerase has been described in different organisms and has recently started to be elucidated in immortalized and transformed human cells. ALT is a mechanistic model to describe the presence of modified telomeres in the absence of telomerase activity (TA).

In this study, the prevalence of TA, gene expression of telomerase subunits and ALT was evaluated in matrix producing bone tumors and in osteosarcoma cell lines in relation to telomere morphology and function. This thesis presents evidence for a direct association of ALT with telomere dysfunction and chromosomal instability.

Terminal restriction fragment analysis (TRF) and telomere fluorescence *in situ* hybridization (T-FISH) in ALT cells revealed elongated and shortened telomeres. Furthermore, T-FISH in ALT cells revealed telomeres in unusual configurations

and loci, dicentric marker chromosomes and signal-free chromosome ends. Free ends give rise to end-to-end associations and may induce breakage-fusion-bridge cycles resulting in an increased number of complex chromosomal rearrangements, as was detected by multiplex-FISH (M-FISH). Using comparative genomic hybridization (CGH) no specific aberrations were found in ALT cells compared to TA cells.

This study suggests that ALT cannot be regarded as an equivalent to telomerase activity in telomere maintenance. Its association with telomere dysfunction and chromosomal instability may have major implications for tumor progression.

Linking mechanisms whereby ALT may act in immortalized human cells to the growing understanding about telomere structure, capping of telomeres and signaling at the telomere has only been started and will continue to provide fascinating insights into telomere biology. Recent advances in the understanding of induction and repression of ALT will be associated with the implications of the observation of presence of ALT in cancer cells in the light of new treatment strategies targeted against telomere maintenance mechanisms.

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