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Research Article

TELOMERASE – BASED INHIBITOR LIMITING HUMAN CANCER CELL PROLIFERATION

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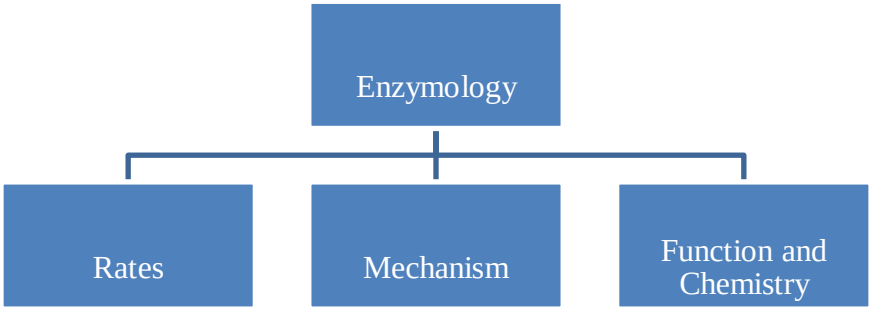
ABSTRACT
Enzymes are proteins that catalyze increase or decrease the rates of chemical reactions. The molecules at the beginning of this process, substrates, will convert into products. An enzyme inhibitor is a molecule that binds to enzymes and decreases their activity. Many drug molecules are enzyme inhibitors, so their discovery and improvement is an active area of research in biochemistry and pharmacology. A medicinal enzyme inhibitor is judged by its specificity and its potency. A high specificity and potency ensure few side effects and low toxicity. Enzymes in a metabolic pathway can be inhibited by downstream products. This is an important way to maintain homeostasis in a cell. Other cellular are proteins that specifically bind to and inhibit an enzyme target. This can control enzymes that may be damaging to a cell, such as proteases or nucleases . Natural enzyme inhibitors can also be poisons and are used as defenses against predators or as ways of killing prey. Recent advances in the structural biology of telomerase, including high resolution structures of the catalytic subunit of a beetle telomerase and two domains of a ciliate telomerase catalytic subunit, provide new insights into the roles of telomerase in human biology.

Keywords: *Telomerase–Based Inhibitors, Human Cancer Cell, Enzyme Inhibitors, Proliferation*

INTRODUCTION
Enzymology refers to Chemistry and activity of enzymes, which is the kinetic analysis to study enzyme mechanisms.



The ends of the chromosomes in eukaryotic cells are called telomeres, and consist of repeated DNA sequences in a complex with many proteins. Normal cells replicate only a limited number of times before they permanently stop dividing (senescence). Ends of linear DNA molecules cannot be replicated fully by the cell's DNA synthesis machinery and thus in many human cells telomeres shorten with every cell division. There is now good evidence for the hypothesis that telomere shortening is the 'molecular clock' that leads to senescence.
Cells derived from most human tumors do not senesce when grown in culture, and are referred to as being "immortal". Immortal cells all possess ways to overcome telomere shortening through the action of an enzyme called telomerase, a complex of protein and RNA (large molecule found in all cells and similar in structure to DNA, molecule that plays a major role in protein synthesis and cellular chemical processes) that adds DNA sequences to the ends of chromosomes. Telomerase is a broadly applicable and critical tumor target. It is expressed in a broad array of malignant tumors, essential for malignant cell growth and absent or expressed transiently, typically at low levels, Telomerase are a specialized DNA–protein

complex at the ends of linear chromosomes. They are essential for the proper maintenance of chromosomes and may play a role in aging and cancer. Telomerase are specialized reverse transcriptase involved in the synthesis of telomeres in most organisms. They are very interesting DNA polymerases in that they carry RNA template within them. Biochemical and genetic studies have established an association between telomere maintenance and cellular transformation. The loss or shortening of telomeres in telomerase negative somatic cells has been linked with genomic instability and carcinogenesis.

Telomerase continues to generate substantial attention both because of its pivotal roles in cellular proliferation and aging and because of its unusual structure and mechanism. By replenishing telomeric DNA lost during the cell cycle, telomerase overcomes one of the many hurdles facing cellular immortalization. Functionally, telomerase is a reverse transcriptase, and it shares structural and mechanistic features with this class of nucleotide polymerases. Telomerase is a very unusual reverse transcriptase because it remains stably associated with its template and because it reverses transcribes multiple copies of its template onto a single primer in one reaction cycle.

Active telomerase has been detected in 85 - 90% of human tumors, and is not detected in most normal human cells. Recent experiments have shown that blocking the action of telomerase causes tumor cells to die, at least in culture. This raises the exciting possibility that inhibitors of telomerase will be a very specific and non-toxic treatment for human cancer.

The projects currently ongoing in the lab are directed at understanding telomerase and telomere function, and include:

1. Multimerisation status of Tetrahymena telomerase
2. Domain structure and function of human and Tetrahymena TERT proteins
3. Identification of DNA-binding regions of human and Tetrahymena TERT
4. Identification of protein binding partners of human TERT
5. Recruitment of human telomerase to telomeres
6. Telomerase interactions with telomeres of different structures

Telomerase, which maintains the ends of chromosomes, consists of two core components, the catalytic subunit (TERT) and the telomerase RNA component (TERC). The 5p- or Cri du chat syndrome is caused by a partial deletion of the short arm of chromosome 5. The majority of deletions are spontaneous de novo deletions, though some patients inherit the deletion from a parent with a balanced translocation. 50 families with at least one family member with 5p- were investigated during the Annual Meetings of the 5p- Society in St. Louis, 2005 and in San Jose 2006. All individuals with 5p- had clinical features characteristic of the syndrome. Clinical manifestations in 5p vary with the size and location of the chromosomal deletion.

Human telomeres function as a protective structure capping both ends of the chromosome. They are composed of long, repetitive sequences of TTAGGG, associated with a variety of telomere-binding proteins. Telomeres protect the chromosomes from end-to-end fusion, recombination, and degradation, all events that can lead to cell death. At cell replication, telomeres cannot be completely replicated. They are gradually shortened, and when the telomeres reach a critical threshold, cell replication is arrested in what is called "replicative senescence." Thus, telomeres act as an intrinsic "counting" mechanism of the cell's aging process. Telomerase is an enzymatic ribonucleoprotein complex that acts as a reverse transcriptase in the elongation of telomeres. Telomerase activity is almost absent in somatic cells, but it is detected in embryonic stem cells and in the vast majority of tumor cells. Tumor cells, in fact, may contain short and stable telomeres that confer immortality to the cancer cells, which are thus able to replicate indefinitely. The deregulation of telomeres thus plays an important role in the relationship between premature aging syndrome and cancer. This review describes the recent advances in the molecular characterization of telomeres, the regulation of telomerase activity in cancer pathogenesis, and the potential of targeting telomerase for cancer therapy.

Here, we review recent findings that illuminate our understanding of telomerase. The specific emphasis is on structure and mechanism.

In the early 1930s, Hermann J. Muller and Barbara McClintock described the telomere (from the Greek word "telos," meaning end, and "meros," meaning part) as a protective structure at the terminal end of the chromosome. When this structure is absent, end-to-end fusion of the chromosome may occur, with ensuing cell death. In the 1970s, James D. Watson described what he called "end-replication problems." During DNA replication, DNA-dependent DNA polymerase does not completely replicate the extreme 5' terminal end of the chromosome, leaving a small region of telomere uncopied. He noted that a compensatory mechanism was needed to fill this terminal gap in the chromosome, unless the telomere was shortened with each successive cell division.

Meanwhile, in the 1960s, Hayflick described a biological view of aging. He found that human diploid cells proliferate a limited number of times in a cell culture. The "Hayflick limit" is the maximal number of divisions that a cell can achieve in vitro. When cells reach this limit, they undergo morphologic and biochemical changes that eventually lead to arrest of cell proliferation, a process called "cell senescence.

Then in the 1970s, Olovnikov connected cell senescence with end-replication problems in his "Theory of Marginotomy," in which telomere shortening was proposed as an intrinsic clocklike mechanism of aging that tracks the number of cell divisions before the arrest of cell growth or replicative senescence sets in. Greider and colleagues, in 1988, corroborated this theory when they observed a progressive loss in telomere length in dividing cells cultured in vitro.

In 1978, Elizabeth Blackburn found that the molecular structure of telomeres in *Tetrahymena pyriformis* contains long repeating units rich in thymine (T) and guanine (G) residues. In 1984, she and her colleagues isolated telomerase, the enzyme responsible for the maintenance and elongation of telomere length. In 1989, Gregg reported the existence of telomerase activity in human cancer cell lines, which was thought to contribute to the immortality of tumor cells. At about the same time, Greider and associates found that telomerase was nearly always absent in normal somatic cells.

In the 1990s, Shay and Harley detected telomerase in 90 of 101 human tumor cell samples (from 12 different tumor types), but found no activity in 50 normal somatic cell samples (from 4 different tissue types). Since then, more than 2600 human tumor samples have been examined and telomerase activity detected in about 90% of all tumor cells. The obvious implication is that telomerase may play a major role in the pathogenesis of cancer.

Because of their role in physiologic aging, cancer pathogenesis, and premature aging syndromes (eg, progeria), telomeres and telomerase are currently under intensive investigation. This review focuses on the molecular structure of telomeres, telomerase and associating proteins, the role of telomere shortening, the activation of telomerase in cancer pathogenesis, and the potential of targeting telomerase for cancer therapy.

Telomerase is a cellular RNA-dependent DNA polymerase that serves to maintain the tandem arrays of telomeric TTAGGG repeats at eukaryotic chromosome ends (Morin, 1989; Blackburn and Greider, 1995). In human cells, the enzyme comprises a high molecular weight complex with a template-containing RNA subunit (Feng *et al*., 1995) and protein components including the catalytic subunit human telomerase reverse transcriptase, hTERT (Harrington *et al*., 1997; Meyerson *et al*., 1997; Nakamura *et al*., 1997). Telomerase activity has been demonstrated in immortalized cell lines and in 80–90% of human cancer specimens representing a range of cancer types (Counter *et al*., 1994; Kim *et al*., 1994; Shay and Bacchetti, 1997) and recently, human telomerase has been directly implicated in cellular immortalization and tumorigenesis (Bodnar *et al*., 1998; Hahn *et al*., 1999a). In most normal human cells, telomerase activity is low or not detectable, and telomeric DNA is progressively lost at a rate of 30–120 bp with each replication cycle (Harley *et al*., 1990; Hastie *et al*., 1990; Counter *et al*., 1992). Eventually, telomeres shorten to a critical length and lose their ability to protect the ends of chromosomal DNA (Counter *et al*., 1992; Blasco *et al*., 1997).

Uncapped chromosomes are sensitive to degradation and fusion and can activate DNA damage checkpoints, thus potentially contributing to the replicative senescence and growth arrest observed in aged primary cultured cells (Hayflick and Moorhead, 1961). Indeed, it has been proposed that telomere length specifies the number of cell divisions a cell can undergo prior to senescence (Cooke and Smith, 1986;

Harley, 1991). In cancer cells, the reactivation of telomerase is thought to stabilize telomere length, thereby compensating for the cell division-related telomere erosion and providing unlimited proliferative capacity to malignant cells (Counter *et al*., 1992; Kim *et al*., 1994). As a corollary to this hypothesis, the inhibition of telomerase in tumour cells should disrupt telomere maintenance and return malignant cells to proliferative crisis followed by senescence or cell death (Harley *et al*., 1990; Counter *et al*., 1992). Genetic experiments using a dominant-negative form of human telomerase demonstrated that telomerase inhibition can result in telomere shortening followed by proliferation arrest and cell death by apoptosis (Hahn *et al*., 1999b; Zhang *et al*., 1999).

A challenge for the development of pharmaceutically useful telomerase inhibitors is the long lag period required to observe telomere attrition. Cellular growth arrest that depends on telomere shortening will require a series of cell division cycles to become apparent, and treatment may have to be given continuously for weeks to months, potentially in conjunction with other treatment modalities. Therefore, potency of action, selectivity, tolerability and suitable pharmaceutical formulations are formidable tasks to be met in telomerase drug design. Here we describe a novel structural class of non-peptidic, non-nucleosidic inhibitors of human telomerase that are highly potent and selective in vitro and pharmacologically active in the control of human Cancer cell proliferation.

A number of genetic validation experiments indicate that telomere maintenance by the enzyme telomerase is a key event in the immortalization process and the continuous proliferation of a large proportion of human cancers (Bodnar *et al.*, 1998; Vaziri and Benchimol, 1998; Hahn *et al.*, 1999a). The pharmacological results presented here demonstrate that extended propagation of human tumor cell lines in the presence of compounds from a novel class of selective, non-nucleosidic small molecule telomerase inhibitors results in progressive telomere shortening followed by the induction of a senescence phenotype and profound anti-proliferative effects in vitro and in vivo.

Several strategies to inhibit telomerase activity have been reported. These include peptide nucleic acids and 2′-O-MeRNA oligonucleotides directed towards the telomerase RNA template (Herbert *et al.*, 1999), compounds that target telomeric DNA such as cationic porphyrins or anthraquinones (Sun *et al.*, 1997) and nucleosidic reverse transcriptase inhibitors (Strahl and Blackburn, 1996). So far, these pharmacological strategies have had only limited success in vivo due to moderate efficacy or the inability of test compounds to penetrate cellular membranes under physiological conditions. Another approach described recently made use of dominant-negative alleles of hTERT, expression of which resulted in cell death of telomerase-positive cancer cell lines (Hahn *et al.*, 1999b; Zhang *et al.*, 1999). Although very effective and selective in vitro, this gene therapy approach may not be readily applicable to the clinical setting.

The non-nucleosidic small molecule telomerase inhibitors described here overcome several of the past obstacles. Telomerase inhibition by BIBR1532 or BIBR1591 results in a continuous erosion of the telomeres in human cancer cell lines derived from fibro sarcoma, lung, breast and prostate carcinoma. No other cellular changes as assessed by morphological or gene expression parameters appear to be triggered by these selective drugs until the telomeres erode to a critically short length and the cells slow their growth. The phenotypic signs of senescence were observed not only in the p53-positive NCI-H460 cells but also in the p53-deficient HT1080, DU145 and MDA-MB231 cell lines, consistent with broad therapeutic utility. In contrast to previous results employing anti-sense oligonucleotides or the expression of dominant-negative hTERT alleles (Hahn *et al*., 1999b; Herbert *et al*., 1999; Zhang *et al*., 1999) we saw no evidence for an increased rate of apoptosis in the inhibitor-treated cells. This could be attributed to the different means employed to inhibit telomerase or to differences in the apoptotic potential of the non-overlapping set of cell lines used in these studies.

Changes that define age-related senescence of normal cells include enlarged and flattened morphology, increased granularity, expression of SA-β-GAL and multiple nuclei (Hayflick and Moorhead, 1961; Smith and Pereira-Smith, 1996). The senescence phenotype may be refined at the molecular level by comparing our list of regulated genes with that of a recent microarray study focusing on gene expression levels in explicative senescence of normal cells and age-related diseases (Chang *et al*., 2000; Ly *et al*.,

2000). Many of the genes described in the physiological or premature ageing process, such as the down-regulation of mRNAs coding for cell cycle control proteins, proteins required for mitosis, DNA synthesis, replication and repair, are also found in the telomerase-inhibitor-treated, senescent NCI-H460 cancer cells and they may obey a programmed switch for mitotic events in senescent cells. A related feature of the inhibitor-treated NCI-H460 cells may be up-regulation of $p21^{Waf1}$, a cycling-dependent kinas inhibitor whose induction triggers growth arrest associated with senescence and damage response.

The similarities to the gene expression changes observed in recent array studies of p21-induced senescence (Chang *et al*., 2000) suggest that the senescent phenotype we observed upon telomerase inhibition may result, at least in part, from effects mediated by p21.

The average TRF lengths in all inhibitor treated, senescent cells reach a size of only 1–2 kb at the onset of growth inhibition. A significant portion of these TRFs likely consists of subtelomeric sequences rather than TTAGGG repeats; suggesting that the true length of telomeric TTAGGG repeats has been reduced to only several hundred base pairs. Cytogenetic analysis indicates that some telomeres may even have lost all measurable TTAGGG repeats. Defective telomeres are unable to cap chromosomes effectively and the marked disarray in the genome of the inhibitor-treated cancer cell lines, including telomere loss and chromosomal end-to-end fusions, resembles the karyotypic changes in $mTERC^{-/-}$ mouse cells (Blasco *et al*., 1997). The role of telomerase in the immortalization of rodent cells may not be identical to human cells in all respects, but this mutant mouse model is extremely valuable for evaluating the biological consequences of telomerase inhibition. The lack of phenotypic effects in the early generation $mTERC^{-/-}$ mice suggests that general telomerase inhibition may be well tolerated with no severe toxic effects (Blasco *et al*., 1997). The ability of $mTERC^{-/-}$ mice to develop certain cancer types in later generations has been attributed to chromosome end-to-end fusions or the activation of telomerase-independent telomere maintenance mechanisms (Blasco *et al*., 1997; Greenberg *et al*., 1999; Rudolph *et al*., 1999). A similar ALT mechanism has been described for virus-transformed human cells and a small proportion of established human cancer cell lines that lack telomerase activity (Bryan *et al*., 1995). We have never observed induction of the ALT phenotype in any of the cell lines used in our studies. Induction of ALT has also never been reported in previous publications using other means of telomerase inhibition (Hahn *et al*., 1999b; Herbert *et al*., 1999; Zhang *et al*., 1999).

A recent analysis of telomeric recombination mechanisms, which are apparently the mechanistic basis for ALT, suggests that telomere-positive cells may not possess a telomere maintenance mechanism other than telomerase (Dunham *et al*., 2000).

Recent data suggest that in $mTERC^{-/-}$ mouse cells telomere dysfunction is a prominent trait that impairs DNA repair and enhances sensitivity to ionizing radiation (Wong *et al*., 2000). This link between telomerase inhibition and radio sensitivity may provide a basis for further studies employing a combination of telomerase inhibitors and radio therapeutic strategies for cancer treatment. In a clinical setting, the most likely use of telomerase inhibitors would be as an adjuvant treatment in combination with surgery, radiation treatment and conventional chemotherapy. Another potential application would be post-remission therapy in order to eliminate minimal residual disease.

Although an analysis of telomere lengths in primary tumors suggests that tumor telomeres are usually short, predicting that the phenotypic lag may be limited, we nevertheless expect that oral treatment may have to be administered continuously for weeks to months. Therefore, the success of a telomerase inhibitor therapy requires that compounds be sufficiently well tolerated, have a low toxicity profile and are easy to administer. The compounds described here fulfill many of the required criteria and further studies with continued treatment in vivo are needed to determine the best candidates to study for clinical efficacy. At the very least, our discovery of a highly potent and selective class of telomerase inhibitors highlights the potential of targeting this enzyme in a mechanism-based approach to the development of new treatment modalities in cancer.

This stud investigated the consequences of a TERT gene deletion in patients with 5p- syndrome. In this study population clinical manifestations varied from severely affected to almost normal. Individuals were examined for clinical signs of premature aging.

MATERIALS AND METHODS

Methodology

A thorough description of the participants, procedure, and instrumentation carried out for this study are presented in this section.

Participants

Patients with 5p- syndrome whose clinical manifestations varied from severely affected to almost normal, were participated in this study. Individuals were examined for clinical signs of premature aging. Individual probands had ridged fingernails, atrophic skin on palms and early graying.

Instrumentation

The first instrument was PCR-based protocol followed by a TCA precipitation step (Schnapp *et al.*, 1998) to determine the IC_{50} curves.

The second instrument used in this study was Liquid scintillation counting to measure the total amount of incorporated [^{33}P] dCMP.

Procedure

The following procedures were carried out to conduct the research:

First, the Telomerase activity assays to determine the IC_{50} curves were performed and quantified using a PCR-based protocol followed by a TCA precipitation step (Schnapp *et al.*, 1998).

Second, The total amount of incorporated [^{33}P]dCMP was measured by liquid scintillation counting and normalized to the control. As a source for telomerase, nuclear extracts derived from HeLa cells were used.

Third, for the direct telomerase assay, telomerase was reconstituted with insect cell expressed hTERT and in vitro transcribed hTR as described (Wenz *et al.*, 2001).

Fourth, Taq polymerase activity was determined as described by the manufacturer (Promega). Also, the activity of DNA polymerases present in HeLa nuclear extracts (40 µg of total protein) was assayed under the following conditions: 20 mM Tris–HCl pH 7.9, 8 mM $MgCl_2$, 50 mM KCl, 0.5 mg/ml bovine serum albumin, 5 mM dithiothreitol, 2 mM spermidine, 80 µM each of dGTP, dCTP and TTP, 5 µM [α-^{32}P]dATP (3.6 Ci/mmol) and either 4 µg activated calf thymus DNA (Pharmacia) or 2 µg of poly A(dT) (Biotech) as template.

After that, Reactions were incubated for 30 min at 37°C and the radioactivity present in trichloroacetic acid precipitates collected on class-fibre filters was determined by liquid scintillation counting. Then, Purified pure calf thymus DNA polymerase was obtained from Professor N.C.Brown (University of Massachusetts Medical School, Worcester) and Cambio (Cambridge, UK) was assayed in the presence of 4 µg of activated calf thymus DNA as described above. Human RNA polymerase I was purified from HeLa nuclear extracts and assayed as described (MonoQ fraction, Schnapp and Grummt 1996). The activity of human RNA polymerases I, II and III was also assayed in HeLa nuclear extracts in the presence of 200 µg/ml α-amanitin using the same conditions. The activity of RNA polymerase II and III was calculated from the difference of the non-inhibited versus the α-amanitin-inhibited extract.

Finally, In vitro translation assays were performed in the rabbit reticulocyte system as described by the manufacturer (Promega). Escherichia coli helicase I (Amersham) and pcTA Helicase from Bacillus stearothermophilus (Cambio) were assayed in the helicase ^{3}H SPA enzyme assay system as described by the manufacturer (Amersham). HIV1 reverse transcriptase was tested in the reverse transcriptase SPA enzyme assay (Amersham).

RESULTS AND DISCUSSION

The current stud investigated the consequences of a TERT gene deletion in patients with 5p- syndrome. In this study population clinical manifestations varied from severely affected to almost normal. Individuals were examined for clinical signs of premature aging. Individual probands had ridged fingernails, atrophic skin on palms and early graying. With the exception of 2 individuals in all participants the chromosomal deletion included one copy of TERT located at 5p15.33 as determined by Q-PCR and confirmed by FISH analysis. Peripheral blood cell analysis showed normal values in all participants, though colony assays

showed a lower number of progenitors in 5p- individuals. Telomere length measured in peripheral blood cells using flow-FISH showed no significant differences in telomere lengths in 5pindividuals compared to age matched controls. There was no correlation between telomere length, blood cell counts or clinical signs suggestive for premature aging. Our results demonstrate that the majority of individuals with 5p- including a TERT gene deletion have telomere lengths similar to healthy controls, indicating that a TERT gene deletion does not lead to excessively short telomeres within one generation and that premature shortening of telomeres is unlikely to contribute to the clinical manifestations of the 5p- syndrome.
The following parts will explain the findings through the research procedure.

Cell Lines

The lung cancer cell line NCI-H460, the fibrosarcoma cell line HT1080, the breast cancer cell line MDA-MB231 and the prostate cancer cell line DU145 were maintained in RPMI supplemented with 5 or 10% fetal calf serum in 5% CO_2 at 37°C. The cells were grown in 24-well tissue culture plates and replated every 2–3 days to ensure log-phase growth. The culture medium contained 10 µM of BIBR1532 or 50 µM of BIBR1591 dissolved in 0.1% DMSO and was replenished at every replating step or every 2–3 days for slower growing cells. The compounds were stable under these conditions. Control cells were untreated or treated with corresponding solvent concentrations. Cell growth and viability in the 7 day cytotoxicity study was determined using the tetrazolium dye assay.

Isolation of Cellular DNA and Telomere Length Analysis

Cell samples (2×10^6 cells) were harvested, washed and resuspended in DNazol (Life Technologies). Total cellular DNA was extracted according to the manufacturer's protocol. To measure telomere length, genomic DNA was digested with restriction enzymes HinfI and RsaI, fractionated on 0.6% agarose gel and transferred onto a nylon membrane. Telomere sequences were detected by hybridization with a synthetic oligonucleotide probe $(CCCTAA)_3$ end-labelled with fluorescein-dUTP. Detection relies on an anti-fluorescein-antibody conjugated to alkaline phosphatase (Amersham).

Cellular Assays

SA-β-galactosidase activity was detected as described (Dimri *et al.,* 1995). For cell cycle analysis, cells were fixed with2% paraformaldehyde for 20 min at room temperature and permeabilized with 0.25% Triton X-100 in phosphate-buffered saline by incubation for 5 min on ice. Cells were pelleted by centrifugation (1000 g, 5 min, 4°C), resuspended in propidium iodine staining buffer (0.1% RNase, 10 µg/ml propidium iodine in PBS) and incubated for 20 min at room temperature. The DNA content was analysed using a FACS Calibur (Becton Dickinson, Heidelberg, Germany). TUNEL assay was performed according to the manufacturer's protocol (Pharmingen, Heidelberg, Germany).

Fluorescence in Situ Hybridization and Quantitative Image Analysis

Individual telomere length was analyzed by quantitative fluorescence in situ hybridization (Q-FISH) as described previously (Martens *et al.,* 1998, 2000). Digital images of metaphase spreads were recorded with a digital camera (Sensys, Photometrics) on a Zeiss Axioplan II fluorescence microscope using the Vysis workstation QUIPS. Telomere profiles were analysed by the TFL-TELO software (Poon *et al.,* 1999). Telomere fluorescence intensity values were expressed in arbitrary units.

Analysis of Gene Expression using Oligonucleotide Arrays

Total RNA was extracted from frozen cell pellets by using Trizol reagent (Life Technologies). RNA was purified on RNeasy Mini columns (Qiagen) for RNA cleanup and DNase treatment (RNase-Free DNase Set Protocol, Qiagen). RNA was converted into double-stranded cDNA by using the Superscript Choice System (Life Technologies). Biotin-labelled cRNA synthesis was carried out using 10 µg of total RNA according to the Affymetrix technical manual (Lockhart *et al.,* 1996; Fambrough *et al.,* 1999). Hybridization, washing, staining, and scanning of Affymetrix Genechip HuGeneFL oligonucleotide arrays (Affymetrix) was carried out according to the Affymetrix technical manual (Lockhart *et al*., 1996; Fambrough *et al*., 1999) in an Affymetrix hybridization oven and fluidics station and a Hewlett-Packard GeneArray Scanner. Data analysis was performed using Affymetrix software. Expression levels of untreated or DMSO-treated control cells were compared with expression levels of cells treated with the telomerase inhibitor BIBR1591. Four pairwise comparisons were calculated by comparing two unrelated

sets of cells grown with BIBR1591 to either untreated NCI-H460 cells or to cells treated with DMSO as a solvent control. Selected genes were required to show at least a 2-fold regulation in at least three out of four pairwise comparisons.

Quantitative RT–PCR

We used the same RNA preparations for both microarray and quantitative RT–PCR analyses. mRNA quantitation was performed using the TaqMan EZ RT–PCR kit (PE Applied Biosystems) and all samples were analysed in triplicates on the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems). Gene-specific oligonucleotide probes with 5′-fluorescent and 3′-rhodamine (quench) tags were designed for hTERT, Wnt5a, IGFBP4, $p21^{Waf1}$, cyclin A, and GAPDH as an internal standard. PCR conditions as well as sequences of RT–PCR primers and probes will be provided on request.

Conclusion

This study aimed to explore the consequences of a TERT gene deletion in patients with 5p- syndrome.

Tumorigenicity Assays

The ability of untreated and inhibitor treated HT1080 fibrosarcoma cells to form tumours was determined by injecting 1.5×10^6 cells subcutaneously in immunodeficient nu/nu NMRI mice. BIBR1532 was prepared in a vehicle of 20% cremophore RH40, 80% water with equimolar amounts of NaOH. Treatment was administered by gavage. Growth of the tumours was recorded by calliper measurements determining length and width of the palpatable, subcutaneous tumour mass three times per week. Progressive shortening of telomeres was associated with continuous cell division in normal somatic cells. Telomerase was activated in most cancer cells and immortal germ cells to maintain their telomeric lengths. The occurrence and clinical pathological significance of telomerase activation was evaluated in various types of human cancer. Telomerase activation is a common event in human cancers and may be as a useful marker for malignant cells. Telomerase may also be a therapeutic target for cancer treatment.

REFERENCES

Blackburn E and Greider C (1995). *Telomeres* (Cold Spring Harbor Laboratory Press) Cold Spring Harbor, New York, NY.

Blasco MA, Lee HW, Hande MP, Samper E, Lansdorp PM, DePinho RA and Greider CW (1997). Telomere shortening and tumour formation by mouse cells lacking telomerase RNA. *Cell* **91** 25–34.

Bodnar AG *et al.*, (1998). Extension of life-span by introduction of telomerase into normal human cells. *Science* **279** 349–352.

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

Chang BD, Watanabe K, Broude EV, Fang J, Poole JC, Kalinichenko TV and Roninson IB (2000). Effects of $p21^{Waf1/Cip1/Sdi1}$ on cellular gene expression: implications for carcinogenesis, senescence, and age-related diseases. *Proceedings of the National Academy of Sciences, USA* **97** 4291–4296.

Cooke HJ and Smith BA (1986). Variability at the telomeres of the human X/Y pseudoautosomal region. *Cold Spring Harbor Symposia on Quantitative Biology* **51** 213–219.

Counter CM, Avilion AA, LeFeuvre CE, Stewart NG, Greider CW, Harley CB and Bacchetti S (1992). Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. *EMBO Journal* **11** 1921–1929.

Counter CM, Botelho FM, Wang P, Harley CB and Bacchetti S (1994). Stabilization of short telomeres and telomerase activity accompany immortalization of Epstein–Barr virus-transformed human B lymphocytes. *Journal of Virology* **68** 3410–3414.

Dimri GP *et al.*, (1995). A biomarker that identifies senescent human cells in culture and in ageing skin *in vivo*. *Proceedings of the National Academy of Sciences USA* **92** 9363–9367.

Dunham MA, Neumann AA, Fasching CL and Reddel RR (2000). Telomere maintenance by recombination in human cells. *Nature Genetics* **26** 447–450.

Fambrough D, McClure K, Kazlauskas A and Lander ES (1999). Diverse signaling pathways activated by growth factor receptors induce broadly overlapping, rather than independent, sets of genes. *Cell* **97** 727–741.

Feng JL *et al.*, (1995). The RNA component of human telomerase. *Science* **269** 1236–1241.
Greenberg RA, Chin L, Chin L, Femino A, Lee KH, Gottlieb GJ, Singer RH, Greider CW and DePinho RA (1999). Short dysfunctional telomeres impair tumorigenesis in the INK4aΔ2/3 cancer-prone mouse. *Cell* **97** 515–525.
Hahn WC, Counter CM, Lundberg AS, Beijersbergen RL, Brooks MW and Weinberg RA (1999a). Creation of human tumour cells with defined genetic elements. *Nature* **400** 464–468.
Hahn WC *et al.*, (1999b). Inhibition of telomerase limits the growth of human cancer cells. *Nature Medicine* **5** 1164–1170.
Harley CB (1991). Telomere loss: mitotic clock or genetic time bomb? *Mutation Research* **256** 271–282.
Harley CB, Futcher AB. and Greider CW (1990). Telomeres shorten during ageing of human fibroblasts. *Nature* **345** 458–460.
Harrington L, Zhou W, McPhail T, Oulton R, Yeung DSK, Mar V, Bass MB and Robinson MO (1997). Human telomerase contains evolutionarily conserved catalytic and structural subunits. *Genes Development* **11** 3109–3115.
Hastie ND, Dempster M, Dunlop MG, Thompson AM, Green DK and Allshire RC (1990). Telomere reduction in human colorectal carcinoma and with ageing. *Nature* **346** 866–868.
Hayflick L and Moorhead P (1961). The serial cultivation of human diploid cell strains. *Experimental Cell Research* **25** 585–621.
Herbert BS, Pitts AE, Baker SI, Hamilton SE, Wright WE, Shay JW and Corey DR (1999). Inhibition of human telomerase in immortal human cells leads to progressive telomere shortening and cell death. *Proceedings of the National Academy of Sciences USA* **96** 14276–14281.
Kim NW *et al.*, (1994). Specific association of human telomerase activity with immortal cells and cancer. *Science* **266** 2011–2015.
Lee CK, Klopp RG, Weindruch R and Prolla TA (1999). Gene expression profile of aging and its retardation by caloric restriction. *Science* **285** 1390–1393.
Lee CK, Weindruch R and Prolla TA (2000). Gene-expression profile of the ageing brain in mice. *Nature Genetics* **25** 294–297.
Lockhart DJ *et al.*, (1996). Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nature Biotechnology* **14** 1675–1680.
Ly DH, Lockhart DJ, Lerner RA and Schultz PG (2000). Mitotic misregulation and human ageing. *Science* **287** 2486–2492.
Martens UM, Zijlmans JM, Poon SS, Dragowska W, Yui J, Chavez EA, Chavez EA, Ward RK and Lansdorp PM (1998). Short telomeres on human chromosome 17. *Nature Genetics* **18** 76–80.
Martens UM, Chavez EA, Poon SS, Schmoor C and Lansdorp PM (2000). Accumulation of short telomeres in human fibroblasts prior to replicative senescence. *Experimental Cell Research* **256** 291–299.
Meyerson M *et al.*, (1997). hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumour cells and during immortalization. *Cell* **90** 785–795.
Morin GB (1989). The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. *Cell* **59** 521–529.
Nakamura TM, Nakamura TM, Morin GB, Chapman KB, Weinrich SL, Andrews WH, Lingner J, Harley CB and Cech TR (1997). Telomerase catalytic subunit homologs from fission yeast and human. *Science* **277** 955–959.
Poon SS, Martens UM, Ward RK and Lansdorp PM (1999). Telo mere length measurements using digital fluorescence microscopy. *Cytometry* **36** 267–278.
Rudolph KL, Rudolph KL, Chang S, Lee HW, Blasco M, Gottlieb GJ, Greider C and DePinho RA (1999). Longevity, stress response, and cancer in aging telomerase-deficient mice. *Cell* **96** 701–712.
Schnapp A and Grummt I (1996). Purification, assay, and properties of RNA polymerase I and class I-specific transcription factors in mouse. *Methods in Enzymology* **273** 233–248.
Schnapp G, Rodi HP, Rettig WJ, Schnapp A and Damm K (1998). One-step affinity purification protocol for human telomerase. *Nucleic Acids Research* **26** 3311–3313.

Shay JW and Bacchetti S (1997). A survey of telomerase activity in human cancer. *European Journal of Cancer* **33** 787–791.
Shelton DN, Chang E, Whittier PS, Choi D and Funk WD (1999). Microarray analysis of replicative senescence. *Current Biology* **9** 939–945.
Smith JR and Pereira-Smith OM (1996). Replicative senescence: implications for *in vivo* ageing and tumour suppression. *Science* **273** 63–67.
Strahl C and Blackburn EH (1996). Effects of reverse transcriptase inhibitors on telomere length and telomerase activity in two immortalised human cell lines. *Molecular and Cellular Biology* **16** 53–65.
Sun DY, Thompson B, Cathers BE, Salazar M, Kerwin SM, Trent JO, Jenkins TC, Neidle S and Hurley LH (1997). Inhibition of human telomerase by a G-quadruplex-interactive compound. *Journal of Medicinal Chemistry* **40** 2113–2116.
Vaziri H and Benchimol S (1998). Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. *Current Biology* **8** 279–282.
Wenz C, Enenkel B, Amacker M, Kelleher C, Damm K and Lingner J (2001). Human telomerase contains two cooperating telomerase RNA molecules. *EMBO Journal* **20** 3526–3534.
Wong KK *et al.*, (2000). Telomere dysfunction impairs DNA repair and enhances sensitivity to ionising radiation. *Nature Genetics* **26** 85–88.
Zhang XL, Mar V, Zhou W, Harrington L and Robinson MO (1999). Telomere shortening and apoptosis in telomerase-inhibited human tumour cells. *Genes & Development* **13** 2388–2399.