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Telomerase Activity and Expression and Telomere Analysis *in Situ* in the Course of Treatment of Childhood Leukemias

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Jan Maláska,[1,2,3] Marie Skleničková,[1,3] Kateřina Krejčí,[1,4] Lenka Fajkusová,[5] Milan Bajer,[2] Hana Hrstková,[2] and Jiří Fajkus[1,4]

ABSTRACT: Samples of blood and marrow from children with leukemia were assayed for telomerase activity and expression on the day of diagnosis and during the course of chemotherapy. A strong correlation between either variables and clinical response was observed in most patients. A unique case was observed in which telomerase activity was only moderately increased on diagnosis; it gradually increased in the course of therapy, and a subsequent decrease occurred only after application of intensified therapy. This patient did not respond to therapy, his disease progressed, and he finally died during intensified therapy. In another patient, analysis of telomere lengths using dideoxy-PRINS revealed a single telomere expansion on a long arm of chromosome 4, suggesting involvement of a telomerase-independent mechanism of telomere elongation.

Key Words: acute leukemias; telomerase; *in situ* analysis of telomeres; chemotherapy, predictive factors.

INTRODUCTION

Telomerase activity is regarded as an essential step for the immortalization of human cells, both *in vitro* and in tumor progression *in vivo.* Although altered telomerase activity is not a cause of malignancy, a strong association between the presence of telomerase activity and malignancy makes the enzyme one of the most common tumor markers (1). The knowledge of human telomerase subunit composition made it possible to combine the test for activity of telomerase [commonly performed as the telomere repeat amplification protocol, TRAP (2)] with the RT-PCR test for expression of its reverse transcriptase and RNA subunits. A further step in understanding telomere biology could be achieved through accumulating information on regulatory mechanisms controlling telomere lengths in the presence of telomerase activity, e.g., via folding of telomeric DNA into telomerase-inaccessible structures such as t-loops or quadruplexes promoted by specific proteins. Hopefully, the intriguing question of differential regulation of telomere lengths on individual chromosomes will also be answered. The method of terminal restriction fragments (TRF) has shown that telomeres gradually shorten during tumorigenesis and are stabilized only upon activation of telomerase, typically at their shortened sizes. Besides telomerase, rare cases of activation of alternative mechanism of telomere lengthening (ALT) were observed in both *in vitro* immortalized human cells and tumor tissue samples (3). The ALT mechanism, probably based on recombination events, results in generation of sequence-heterogeneous telomeres expanded to multiples of their original lengths. A commonly used TRF technique (Southern hybridization of terminal restric-

Correspondence and reprint requests to: Jiří Fajkus. Institute of Biophysics, Královopolská 135, CZ-61265 Brno, Czech Republic. Fax: +4205-41211293. E-mail: fajkus@ibp.cz.
[1] Department of Analysis of Biologically Important Molecular Complexes, Masaryk University Brno, Czech Republic.
[2] Faculty Hospital Brno, Jihlavská 20, CZ-639 00 Brno, Czech Republic.
[3] These two authors contributed equally.
[4] Institute of Biophysics, Academy of Sciences of the Czech Republic, Královopolská 135, CZ-61265 Brno, Czech Republic.
[5] Research Institute of Child Health, Černopolní 9, CZ-66262 Brno, Czech Republic.

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TABLE 1

Telomerase Activities in the Course of Therapy

Patient No.	Diagnosis	0 BM	0 PB	2 PB	4 PB	6 PB	8 PB	15 PB	34 PB	34 BM
1	AML	94.8	65.1	n	n	n	n	18.9	21.1	n
2	ALL	100.0	70.7	61.7	46.4	12.1	17.1	14.9	17.1	26.2
3	ALL	41.0	37.9	48.0	n	75.5	46.5	18.7	†	
4	ALL	n	98.0	n	25.3	21.7	16.2	21.0	10.4	n
5	ALL	n	52.7	36.2	30.0	31.5	19.9	11.1	n	n
6	AML	n	98.4	†						
7	ALL	n	80.5	n	16.3	13.5	11.3	9.2	n	n
8	ALL	n	120.9	†						
Control 1	—	n	18.2	n	n	n	n	11.8	14.6	n
Control 2	—	n	21.2	n	n	n	n	17.2	18.3	n

Note. n, data not available; †, patient died; BM, bone marrow; controls 1, 2, healthy individuals (without treatment).

tion fragments) is able to show only the average size of telomeres, not their individual differences or even a complete loss or extreme expansion at certain chromosome arms. Instead of TRF, a quantitative analysis of telomeres *in situ* using a dideoxy-version of PRimed *IN Situ* labeling (dideoxy-PRINS) technique has become a tool of choice for detailed analysis of telomeres.

In this report we present results of our study on telomere dynamics (telomerase activity and expression, telomere analysis *in situ*) in the course of therapy of cases of childhood leukemia.

MATERIALS AND METHODS

Patients

Eight children with acute leukemia have been followed, six of them diagnosed as acute lymphoblastic leukemias (ALL) and two as acute myeloid

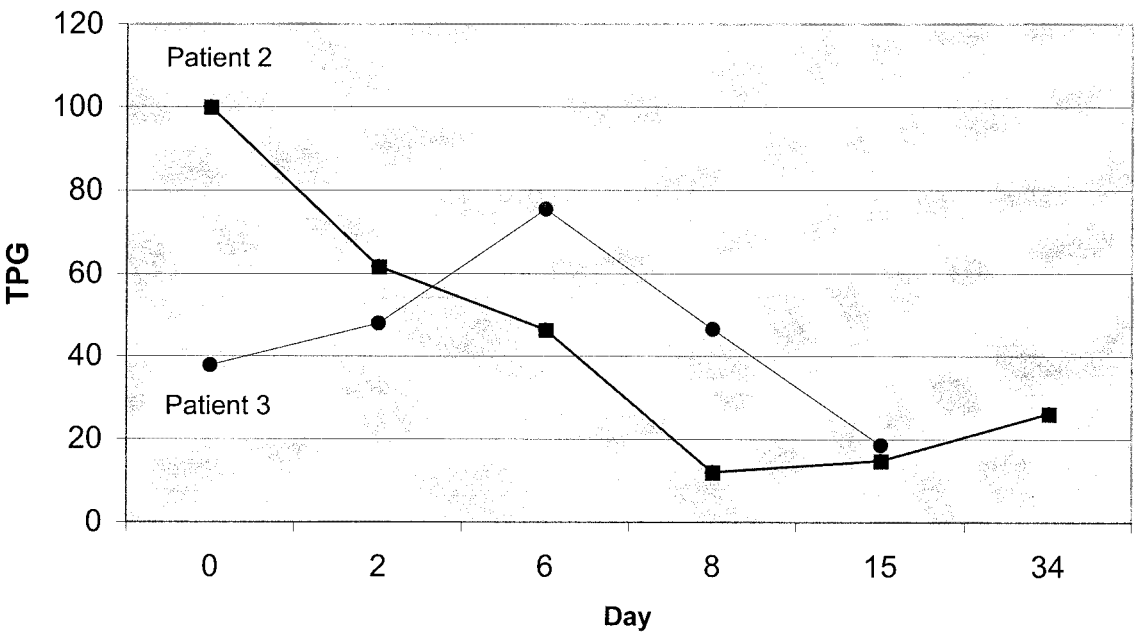

FIG. 1. Profiles of telomerase activities during treatment of childhood acute leukemias. Results in TPG units (total product generated, Intergen Manual) were calculated from the signals obtained from two parallel reactions (60 ng of total protein per assay) using the TRAPeze telomerase detection kit (Intergene). While the profile observed in patient 2 is typical for most patients (see Table 1), the increase in telomerase activity during the first 6 days of treatment in patient 3 is unique within our set of patients (see also Results and Discussion).

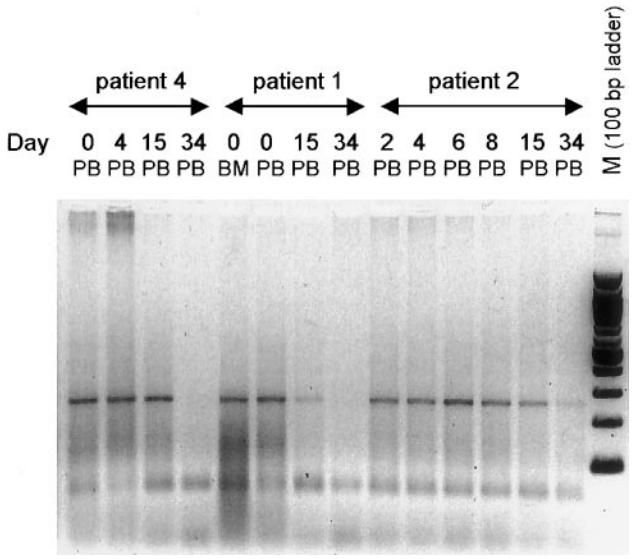

FIG. 2. Expression of the catalytic subunit of telomerase (hTERT) during the treatment period of three different patients. RNA samples were first analyzed for G3PDH as a reference transcript, and then used for RT-PCR of hTERT. PB, peripheral blood; BM, bone marrow.

leukemia (AML). Written informed consent was obtained from each patient or their parents. On the day of diagnosis (day 0), bone marrow (BM) and peripheral blood (PB) was collected. Further collection of blood was performed on days 2, 4, 6, 8, 15, and 34 of therapy.

Patients with ALL were treated according to the BFM 95 protocol, and those with AML according to the BFM 98 protocol.

Telomerase Activity and Expression Assays

Nucleated cells were isolated from blood or marrow samples using HISTOPAQUE-1.077 (Sigma Diagnostics) according to the manufacturer's instructions. The resulting cell pellet was divided into aliquots for preparation of cell extracts for TRAP assay, total RNA preparation and cell counting. A TRAPeze telomerase detection kit (Intergene) was used for preparation of cell extracts and for semiquantitative TRAP assay according to the supplier's instructions. A non-isotopic detection method using SYBR Green (Molecular Probes) staining of reaction products after separation by polyacrylamide electrophoresis has been used throughout. The protein concentration in cell extracts was determined by the Bradford procedure (4).

Total RNA was isolated from blood or marrow cells using a RNeasy Mini kit (QIAGEN) and the quality of the product was checked on an agarose gel. RT-PCRs were performed using an Enhanced Avian RT-PCR kit with primers specific for the telomerase catalytic subunit (5′-CCTCTGTGCTGGGCCTGGACGATA-3′ and 5′-ACGGCTGGAGGTCTGTCAAGGTAG-3′), the RNA subunit (5′-CCTAACTGAGA-AGGGCGTAGG and 5′-GACTCGCTCCGTTC-CTCTTC-3′) and for G3PDH mRNA (5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′) as a reference transcript. After reverse transcription (46°C/45 min) and initial denaturation (94°C/3 min), PCR proceeded for 30 cycles (94°C/45 s, 55°C/30 s, 68°C/45 s) followed by a final extension step (68°C/5 min).

In Situ Analysis of Telomeres

Cell suspensions obtained on the day of diagnosis from blood of eight patients were analyzed by dual color PRINS (PRimed *IN Situ* labeling). In the first step, telomeres were labeled by dideoxy-primed *in situ* labeling (dideoxy PRINS) using the probe Telo2 $(CCCTAA)_7$ (5). PRINS utilizes an unlabeled probe which after hybridization to its target sequence serves as a primer for chain elongation using Tth DNA polymerase to incorporate labeled nucleotides. Dideoxy-PRINS is more specific for labeling sequences which lack one or more of the four bases of DNA; the base(s) not needed for chain elongation can be added in the form of dideoxynucleotide(s) to reduce non-relevant priming from random breaks in chromosomal DNA. In the case of the human C-rich telomeric strand, the dideoxynucleotide used is ddGTP. Telo2-primed synthesis was visualized through incorporation of fluorescein–dUTP (Roche). After the chain elongation, the 3′-ends of products were blocked by ddNTP before the next PRINS reaction (6).

Finally, centromeres of chromosome 4 were labeled with the common PRINS reaction using the probe Alpha 4/12 (5′-CTG AGA ATG CTT

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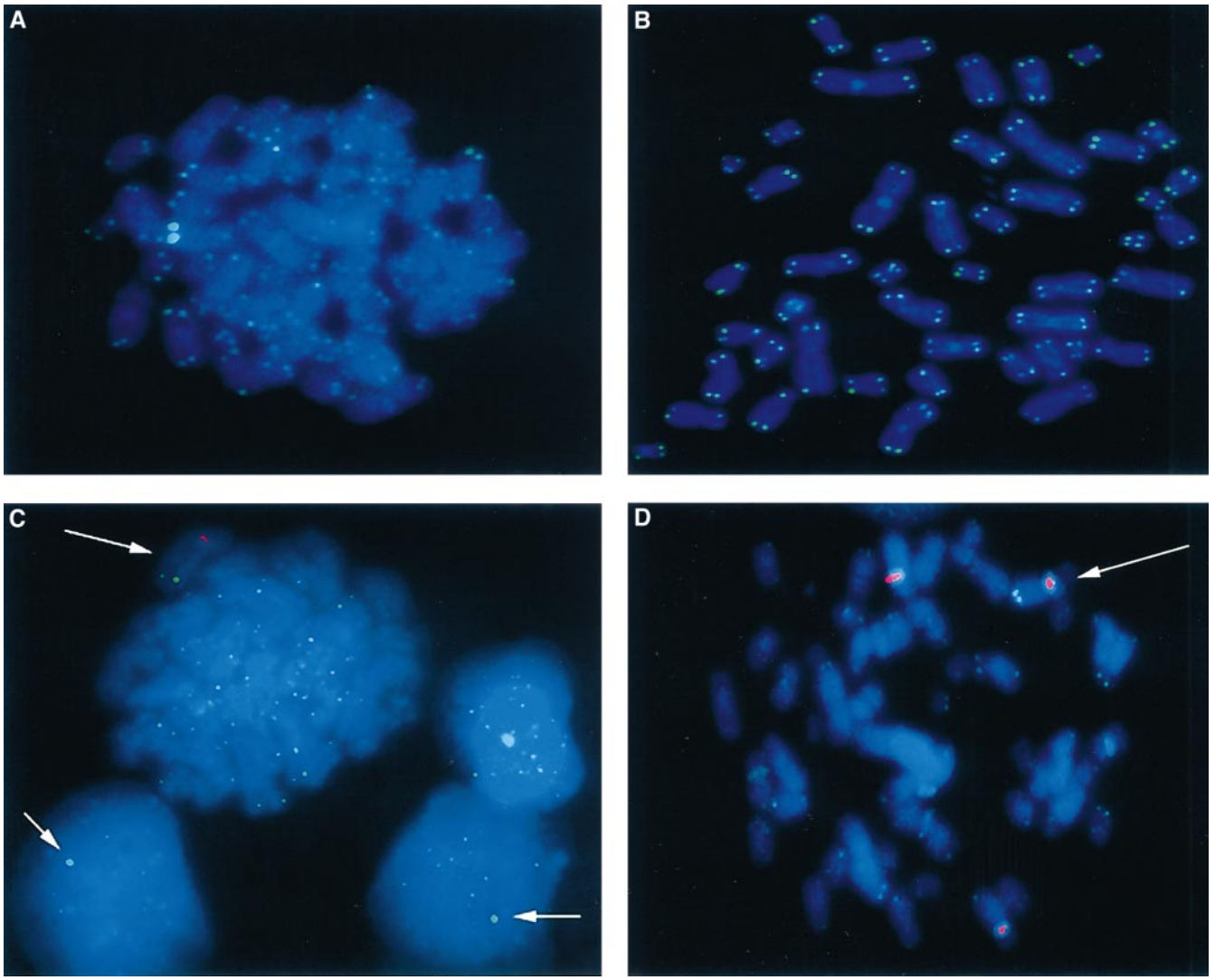

FIG. 3. Analysis of telomeres of patient 2 by dideoxy-PRINS with the probe Telo2. On one chromosome, an increased telomeric signal was observed (arrows) corresponding to a (8.6 ± 3.5)-fold telomere expansion with respect to the average of the other telomeric signals (A). Healthy control is shown for comparison (B). The chromosome possessing the expanded telomere was identified as chromosome 4 using PRINS with the centromeric probe Alpha 4/12 (C, D). The amplified telomeric signal can also be seen in interphase nuclei (C).

CTC TCT AGA TCT AGT TAT TAT ATG TCA TCC C-3′) (7) and digoxygenin–dUTP label (Roche). Rhodamine-conjugated anti-digoxygenin antibody (Roche) was used for visualization.

RESULTS AND DISCUSSION

Telomerase Activity Profiles in the Course of Therapy

All eight acute leukemia patients were telomerase-positive on the day of diagnosis and showed several fold increase of activity compared to healthy donors (Table 1). In further samples collected in the course of therapy, a tight correlation of telomerase activity with the clinical response was observed: most patients showed a complete morphological remission and a corresponding decrease of telomerase activity almost to the levels of healthy donors within several days after diagnosis (a typical profile is shown in Fig. 1, patient 2). A unique case was observed in the case of ALL patient 3 whose telomerase activity on the day of diagnosis (day 0) was only moderately increased above the normal; in the course of ther-

apy the activity gradually increased until day 6, and a subsequent decrease occurred only after application of intensified therapy (Fig. 1, patient 3). This patient did not respond to therapy, his disease progressed and he finally died during intensified therapy.

Expression of Telomerase RNA and Catalytic Subunit

RT-PCR examination of expression of the RNA subunit (hTR) revealed its ubiquitous occurrence in all samples collected. Telomerase catalytic subunit (hTERT) mRNA was present in all samples until day 15, and disappeared or was substantially reduced on day 34 (Fig. 2). Expression of hTERT, was also detected in RNA samples from healthy donors, corresponding to the low levels of telomerase activity found in their cell extracts.

Dideoxy-PRINS Analysis of Telomeres

The telomere-specific probe Telo2 showed a striking expansion of only a single telomere in one of eight patients (patient 2), while the normal telomere length distribution was found in 7 cases as well as in healthy donors. As chromosomes of this patient formed clusters (Fig. 3), it was difficult to obtain a karyotype to identify the chromosome bearing the expanded telomere. Therefore, a secondary PRINS reaction with a centromere-specific probe was applied which proved that the signal was localized on the q arm of chromosome 4. The telomeric signals from eight metaphases were evaluated using software Telomere Quantifier (DAKO, Denmark), and that on the expanded telomere corresponded to a (8.6 $\pm$ 3.5)-fold elongation with respect to the average of lengths of all the other telomeres on a given metaphase.

Due to the low number of cases analyzed here, it is not possible to deduce a direct connection between this single telomere expansion and the diagnosis. Nevertheless, the occurrence of this phenomenon and the extent of expansion suggests the presence of a telomerase-independent mechanism of telomere elongation and corresponds with the previous observation (8) in a Ph-positive, B-ALL patient. In contrast to previously described mechanism of alternative lengthening of telomeres (ALT) (3, 9), the single telomere expansion observed here occurs in telomerase-positive cells, while ALT and telomerase activity seem to be mutually exclusive and apply to all telomeres within a cell (10). Our data thus suggest that a certain level of telomere length regulation is chromosome- or even telomere-specific.

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