Amplification of *hTERT* and *hTERC* genes in leukemic cells with high expression and activity of telomerase

TOMASZ NOWAK[1], DANUTA JANUSZKIEWICZ[1,2,3], MARIOLA ZAWADA[1], MONIKA PERNAK[1], KRZYSZTOF LEWANDOWSKI[1], JOLANTA REMBOWSKA[1], KARINA NOWICKA[1], PRZEMYSLAW MANKOWSKI[2] and JERZY NOWAK[1]

[1]Institute of Human Genetics PAN; [2]Academy of Medical Sciences; [3]Department of Medical Diagnostics, Poznan, Poland

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Abstract. Reactivation of telomerase plays an important role in carcinogenesis. Malignant cells almost always possess high activity and expression of telomerase. The aim of this study was to see whether there is any relationship between telomerase activity and expression and *hTERT* and *hTERC* gene amplification in acute lymphoblastic leukemia (ALL) and non-lymphoblastic leukemia (ANLL) cells. In addition telomere length was tested in leukemic cells at the time of diagnosis and during remission. Expression of the three components of telomerase (*hTERT*, *hTERC* and *TP1*) as well as telomerase activity was found both in ALL and ANLL cells. Telomerase activity was diminished in patients in remission. The leukemic cells showed considerable heterogeneity of terminal restriction fragments, that is telomere length. ALL cells showed a variable pattern of telomere length in contrast to ANLL cells which produced a predominantly short telomere pattern. Telomere length in the lymphocytes of leukemia patients was shorter in remission as compared to the time of diagnosis. FISH analysis revealed amplification of *hTERT* and *hTERC* genes in ALL and ANLL cells. Quantitative analysis showed that leukemic cells possess higher number of *hTERT* and *hTERC* copies than the normal PBL. Our results suggest that the activation of telomerase in leukemic cells is connected with amplification of *hTERT* and *hTERC* genes. The high expression and activity of telomerase found in leukemic cells may be partially explained by amplified *hTERT* and *hTERC* genes. Amplification of the telomerase genes seems to be a common event in carcinogenesis and may play a role in telomerase reactivation leading to cell immortalization.

Correspondence to: Dr Jerzy Nowak, Institute of Human Genetics, Polish Academy of Sciences, Strzeszynska 32, 60-479 Poznan, Poland
E-mail: nowakjs@man.poznan.pl

Abbreviations: GAPDH, human glyceraldehyde-3-phosphate dehydrogenase; *hTERT*, human telomerase reverse transcriptase; hTR, human telomerase RNA; RT-PCR, reverse transcriptase polymerase chain reaction; *TP1*, telomerase associated protein; TRAP, telomere repeat amplification protocol; TRF, terminal restriction fragments

Key words: telomerase, telomere length, human telomerase reverse transcriptase and telomerase RNA amplification, leukemia

Introduction

Telomeres, the end of chromosomes, shorten during each cell division and the general belief states that the length of telomeres serves as a clock determining the replicative capacity of the cell. Due to the absence telomerase activity, proliferating normal cells become senescent (1). Cancer cells overcome the senescence pathway and become immortalized. Immortalization is almost always accompanied by expression of telomerase, which stabilizes the telomere length and is most likely necessary for the continued growth of cancer cells. Telomerase is activated in most malignant tumours but is usually inactive in normal somatic cells (1,2). Telomerase is a ribonucleoprotein complex consisting of reverse transcriptase (*hTERT*), proteins (*hTP1*) and RNA template for telomeric DNA synthesis (*hTERTC*) (3,4).

Despite the questionable role of telomerase reactivation in cell immortalization and carcinogenesis, telomerase itself may serve as diagnostic marker for tumour development (5-7). Several studies have demonstrated that the presence of telomerase activity can be used to distinguish malignant from normal tissue of various organs (1,8). However, the majority of reports are devoted either to telomerase activity or expression (8-10). In this study we analyzed both telomerase activity and expression of the three components of telomerase complex (*hTERT*, *hTP1* and *hTERC*) along with telomere length of leukemic cells. Taking into account gene amplification found in some cancers we questioned whether high expression and activity of telomerase can be explained by *hTERT* and *hTERC* amplification. The objective of this study was to investigate whether there is any relationship between telomerase activity and expression and *hTERT* and *hTERC* amplification in leukemic cells. In addition, we studied the possible alternations in telomerase activity and expression and telomere length in children with acute leukemia at the time of diagnosis and during remission.

Materials and methods

The cells of acute lymphoblastic leukemia (ALL) (n=29), acute non-lymphoblastic leukemia (ANLL) (n=16), myeloblastic cell line K562, normal bone marrow cells (n=5) and normal and PHA stimulated peripheral blood lymphocytes from healthy individuals (n=15) were used in this study. Acute leukemia was diagnosed according to the FAB criteria. The ALL cases were of L1 morphology and had precursor B-cell phenotype. The ANNL cases were of M1 morphology. The leukemic blasts exceeded 90% of nucleated cells either in peripheral blood or bone marrow at the time of diagnosis. Peripheral blood lymphocytes were analysed in some ALL patients in remission after six-month therapy.

Ficoll-Hypaque isolated normal and leukemic cells were washed twice with PBS, counted, and suspended in a final concentration of 2 millions/ml. Telomerase expression was evaluated by RT-PCR technique using specific primers for the human telomerase reverse transcriptase gene (*hTERT*), telomerase associated protein gene (*TP1*) and telomerase RNA gene (*hTERC*). RNA was isolated from 2 millions either leukemic or normal cells. RNA was transcribed into cDNA using random primers, ribonuclease inhibitor and MMLV reverse transcriptase (Promega). The PCR conditions were: initial incubation at 94˚C for 5 min, followed by 40 cycles of denaturation at 94˚C for 1 min, annealing at 62˚C for 1 min (for *hTERT* and *TP1*) or at 54˚C (for *hTERC* and *GAPDH*), elongation at 72˚C for 2 min, and 7 min final extension at 72˚C. Each 50 μl PCR reaction contained Red Taq-polymerase (Sigma), PCR buffer (Sigma), d-NTPs (TIB-MOLBIOL), and primers (TIB-MOLBIOL).

Telomerase activity was analyzed with TRAP-eze-Telomerase detection kit assay (Oncor) (11-13). PCR products were stained in polyacrylamide gel with SYBR-Green (Sigma) and visualized by EDAS 290 system (Kodak Eastman). Telomerase activity was also determined semi-quantitatively by photometric enzyme immunoassay using Telo TAGGG Telomerase PCR ELISA Plus (Roche) (13).

For the determination of telomere length non-radioactive chemiluminescent assay was used (Telo TAGGG Telomere length assay, Roche). Possible *hTERT* and *hTERC* gene amplification was analysed by the FISH technique. Commercially available labeled probes for *hTERT*, *hTERC* as well as 3 alpha satellite reference probe of chromosome 3 and D5S89 of chromosome 5q31 (QBiogene) were used.

The Ethics Committee of the Academy of Medical Sciences in Poznan, Poland approved the study.

Results

Both ALL and ANLL cells expressed *hTERT*, *TP1* and *hTERC*. The expression of the three components of telomerase complex was observed also in K562 cells and PHA-stimulated peripheral blood lymphocytes (PBL). The amplification signals produced by leukemic cells were usually stronger than those observed in normal PHA-stimulated PBL. The majority of normal non-stimulated PBL from healthy individuals did not show any expression of telomerase components. As a control expression of human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was detected in leukemic cells and peripheral blood lymphocytes and always gave a strong positive signal.

Telomerase activity both in leukemic and normal cells was positive in all samples tested provided the lysate was from at least 10,000 cells. The difference between leukemic and normal cells was observed when a lower number of cell-lysates were used. At 300-cell lysates a clear-cut difference between normal and leukemic cells resulted. At 300 normal peripheral blood lymphocytes the telomerase activity was negative, whereas 300 leukemic cells and PHA-stimulated lymphocytes were positive. In dose-dependent experiments telomerase activity was found in 30-cell lysates in ALL and ANLL cells. The highest telomerase activity was observed in ANLL and K562 cells - the signals were produced by 30-cell lysate and even by 10-cell lysates. The above-mentioned differences were also observed when telomerase activity was assessed by the semiquantitative immunosorbance PCR ELISA test. The results of PCR ELISA test showed the highest telomerase activity produced by ANLL cells. Telomerase activity in K562 and ALL cells was slightly lower than that showed by ANLL cells. PBL possessed only a very low telomerase activity in PCR ELISA test (Fig. 1). Telomerase activity studied in ALL patients in remission was at the level comparable to that found in normal PBL (Fig. 1). It should be added, that in a standard blood smear all patients in remission had exclusively normal leukocytes.

Telomere length was determined by chemiluminescent detection of terminal restriction fragments (TRF) from DNA isolated from leukemic and normal cells. The leukemic cells showed considerable heterogeneity of terminal restriction fragments, i.e. telomere length. The majority of ALL cells had long telomere lengths (range 6.1-16.5 kbp), in contrast to the ANLL cells which produced both short and long telomere patterns of TRF (range 2.6-13.7 kbp). Normal peripheral blood lymphocytes produced rather uniform patterns of TRF lengths ranging from 9.1 to 11.4 kbp as well as mononuclear cells isolated from bone marrow (range 5.9-7 kbp). Peripheral blood lymphocytes isolated from ALL patients in remission had a telomere pattern of TRF in a range of 8.5-10.7 kbp.

FISH analysis revealed amplification of *hTERT* and *hTERC* genes in ALL and ANLL cells. Quantitative analysis showed that leukemic cells possess variable number of *hTERT* and *hTERC* copies. Usually leukemic cells have a variable number of *hTERT* copies in a wide range from 2 up to 60 (Fig. 2). *hTERC* gene was present in leukemic cells in a range of 2 to 12 copies (Fig. 3). In contrast to leukemic cells the majority of resting PBL have two copies of *hTERT* and *hTERC*. Representative results of FISH analysis are presented in Fig. 4.

Discussion

Telomerase activity has been reported in most tumour types as well as in leukemic cells (14-16). It is assumed that over 80% of various cancers have telomerase activity (2,11). There are fewer reports on the expression of telomerase complex. However, the majority of reports are devoted either to telomerase activity or expression (9,10). In this study we analyzed both telomerase activity and expression of the three

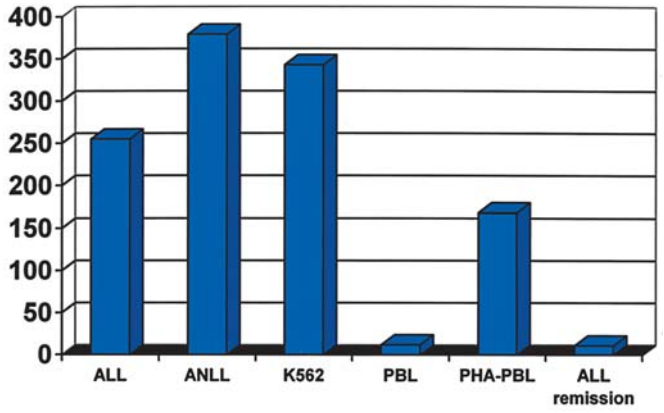

Figure 1. The telomerase activity of leukemic and normal cells as assessed semiquantitatively by the immunosorbance PCR ELISA test.

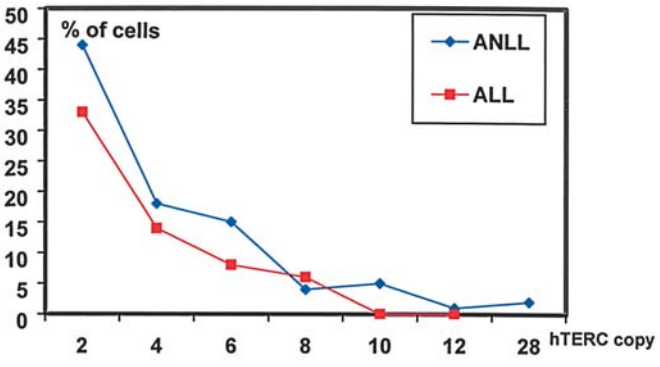

Figure 3. Amplification of *hTERC* in leukemic cells studied by the FISH technique.

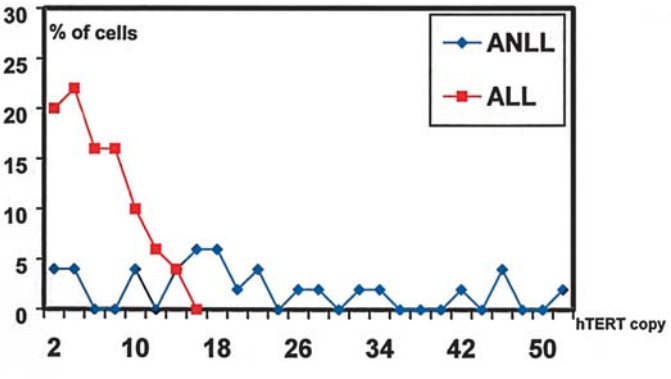

Figure 2. Amplification of *hTERT* in leukemic cells studied by FISH technique.

components of the telomerase complex (*hTERT*, *hTP1* and *hTERC*) along with telomere length of leukemic cells and *hTERT* and *hTERC* gene amplification.

We have shown that both leukemic and normal cells have telomerase activity. Compared to lymphocytes of healthy individuals telomerase activity was elevated both in ALL and ANLL children. However, the major difference between normal and leukemic cells was seen when quantitative analysis was performed. We have observed that telomerase activity is a function of the cell number. At 300-1000 normal lymphocyte lysates consistently gave negative results. The cell-number dependency of telomerase activity can explain some conflicting results comparing neoplastic and normal tissue (17-20). Due to the sensitivity of the TRAP-eze test it is possible that quantitative differences in cell number may give conflicting data. The results indicated the presence of

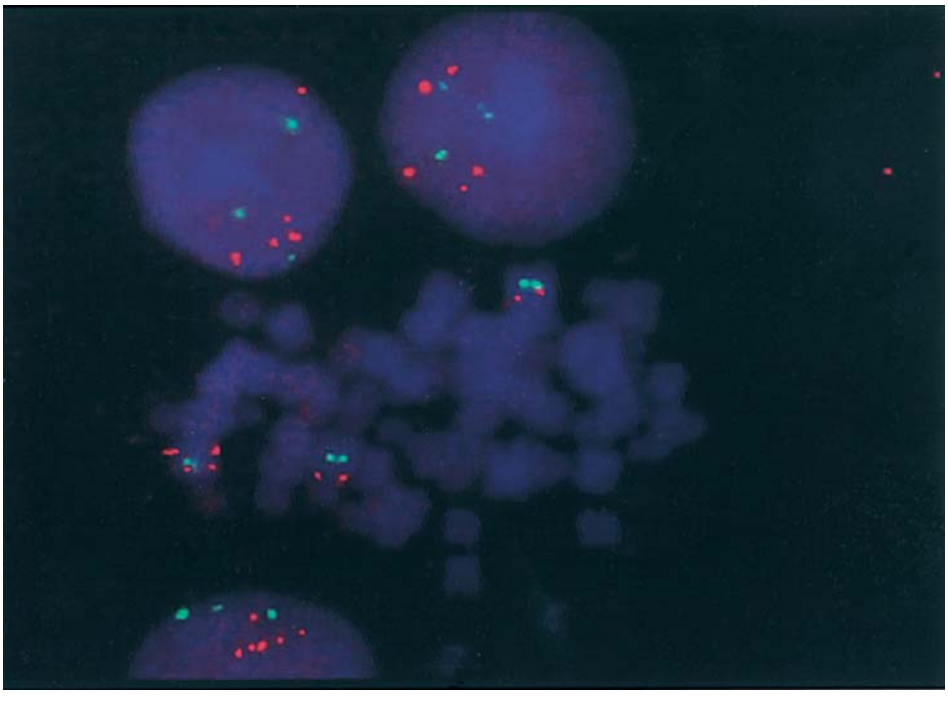

Figure 4. *hTERC* gene amplification in ALL cells by the FISH technique. Specific molecular probe for *hTERC* labeled with rhodamine, the chromosome 3 alpha satellite reference probe labeled with fluorescein.

telomerase activity both in neoplastic and normal cells. Telomerase activity in normal lymphocytes may be due to some activated cells, presented in peripheral blood (1). A variable number of activated lymphocytes in healthy individuals may produce either positive or negative results when 1000-cell lysates are used. The high number of PHA-activated lymphocytes resulted in much higher telomerase activity than seen in non-stimulated PBL (Fig. 1). When comparing leukemic cells and PHA-stimulated lymphocytes the question arises whether the observed difference in telomerase activity is related to the number of proliferating cells. Assuming that the ANLL cells are activated and PHA-PBL are ~70% activated, the ELISA results can be partially explained by the various number of activated cells with telomerase activity. In our opinion telomerase activity is indeed present in activated normal lymphocytes. Various tissues may have different telomerase activity depending on the proliferation rate (21,22). The observed sharp decrease of telomerase activity in ALL patients during remission can be explained by the removal of leukemic cells by chemotherapy. Therefore telomerase activity may be one of the markers for monitoring the effectiveness of chemotherapy in ALL patients.

Telomere length is supposed to be a marker of somatic cell aging (1). It is generally believed that telomeres shorten after each cell division. The presented results indicate that telomere length among leukemic cells varied, especially in ANLL cases. We conclude that high telomerase activity and expression in leukemic cells is not correlated with telomere length (TRF pattern). The same TFR pattern observed in normal individuals and in ALL patients during remission is due to the absence of leukemic cells after chemotherapy.

Zhang *et al* found amplification of the *hTERT* gene both in tumour cell lines and in primary tumours including lung and cervical cancer, breast carcinoma and neuroblastoma (23,24). The question arrises whether *hTERT* gene amplification is correlated with the activity of telomerase. It can be postulated, that *hTERT* gene amplification may indeed contribute to the expression of activity and the expression of telomerase. Authors who documented *hTERT* gene amplification in certain neoplasms came to conflicting conclusions. Fan *et al* documented *hTERT* gene amplification in embryonal brain tumours. These authors stated that *hTERT* gene amplification is relatively common in several tumours and is correlated with high expression and activity of telomerase (25). Takuma *et al* found the amplified *hTERT* gene in hepatocellular carcinoma (26). However, they concluded that expression of *hTERT* (mRNA) did not correlate with the number of *hTERT* genes and that overexpression of *hTERT* gene was rarely due to an increased *hTERT* gene copy number in hepatocellular carcinoma. In our opinion *hTERT* gene amplification is most likely responsible for the upregulation of the *hTERT* expression and activation of telomerase in leukemic cells. However, there is no evident correlation between the degree of *hTERT* gene amplification and multiplication of telomerase expression and activity. ALL blasts possessing more than ten copies of the *hTERT* gene have approximately 30-50% higher telomerase activity as compared to PHA-stimulated lymphocytes with only two copies of the *hTERT* gene (Fig. 1). Therefore, it may be postulated that *hTERT* gene amplification may partially contribute to the increased telomerase expression and activity in leukemic cells.

Our study showed that all leukemic cells tested have higher telomerase expression and activity, as compared to normal cells. Quantitative analysis of telomerase activity and expression may distinguish normal from malignant cells. Our results suggest that the activation of telomerase in neoplastic cells is connected with the amplification of *hTERT* and *hTERC* genes. Amplification of the telomerase genes seems to be a common event in carcinogenesis and may play a role in telomerase reactivation leading to cell immortalization.

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