Telomere length and hTERT expression in patients with acute myeloid leukemia correlates with chromosomal abnormalities

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Background and Objectives. Acute myeloid leukemia (AML) is a malignant, genetically heterogeneous disorder characterized by uncontrolled growth of immature myeloid cells. The aim of this study was to analyze whether telomere length and/or hTERT expression are correlated with clonal chromosomal aberrations in AML.

Design and Methods. Telomere length in mononuclear cells derived from 137 previously untreated patients with ≥ 80% blasts was analyzed by flow fluorescent *in situ* hybridization. Results were expressed in telomere fluorescence Units (1 TFU=1 kb). The expression of hTERT, including its different splice variants, was studied by reverse transcription-polymerase chain reaction.

Results. Age-adjusted telomere length in AML patients was significantly reduced as compared to in matched controls, consisting of peripheral blood granulocytes from healthy individuals (0–90 years) (median: -2.5 TFU; $p<0.001$). Patients with an aberrant karyotype had significantly shorter telomeres than did patients with a normal karyotype (median -3.0 vs. -2.3 TFU; $p=0.03$). The shortest telomeres were found in patients with multiple aberrations (median -3.7 TFU; $p=0.03$). hTERT expression was found to be correlated with chromosomal abnormalities as well as with the detection of functional hTERT splicing variants.

Interpretation and Conclusions. These findings suggest an important role of intense telomere loss in the development of genetic instability during the pathogenesis of AML. It is assumed that critical telomere shortening in AML blasts could lead to telomerase activation and therefore prevent blasts from replicative senescence, one possible mechanism for clonal selection and disease progression. Therefore, telomere length might serve as a prognostic marker for AML patients. These findings need to be confirmed in large, prospective studies.

Key words: AML, genetic instability, telomere, hTERT, splicing.

Haematologica 2005; 90:307-316

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Telomeres are specialized structures composed of TTAGGG repeats[1] and associated proteins located at the end of eukaryotic chromosomes.[2,3] Due to the end-replication problem,[4,5] telomere repeats are lost with each cell division,[6,7] eventually leading to genetic instability[8] and cellular senescence when telomeres become critically short. The ribonucleoprotein enzyme telomerase is capable of synthesizing terminal TTAGGG telomeric repeats[9] onto the ends of the chromosomes and thus counteracts replicative telomere shortening. Telomerase consists of a catalytic subunit called telomerase reverse transcriptase (hTERT),[10,11] a RNA template (hTR)[12] and a number of associated proteins.[13,14] Reactivation of telomerase in malignant cells leads to stabilization or even re-elongation of their telomeres thereby allowing the cells to bypass senescence and crisis and providing for an unlimited proliferation potential.[15] Although hTERT function can be modulated at various steps at the post-transcriptional as well as the post-translational level, telomerase activity is mainly regulated via the transcriptional control of hTERT expression[16] in many cell types.[17] The transcriptional control of hTERT, e.g. by alternative splicing, results in truncated and partly dysfunctional protein products. These splicing variants of hTERT differ in their ability to generate telomerase activity. It has been suggested that only full-length transcripts (+α+β) translate into functionally active telomerase.[18] Therefore, the correlation between hTERT expression, including its different splicing patterns, and clinical outcome might be of potential clinical prognostic impact.[19,20] Acute myeloid leukemia (AML) is a malig-

nant, genetically heterogenous disorder.[21] Prognostic factors are urgently needed in order to be able to better predict treatment outcomes in defined subgroups of patients. Cytogenetic aberrations are among the most important independent prognostic factors.[22-24] However, about two thirds of AML patients display a normal karyotype according to standard cytogenetics. This group has intermediate disease-free and overall survival,[24] but the clinical outcome of individual patients within this group is still highly variable. Therefore, identification of parameters that allow the good risk patients to be separated from the bad risk ones within this cytogenetically defined group is crucial in order to improve risk-adapted treatment strategies in AML. Recently the analysis of FLT3-activating mutations[25] as well as gene expression profiling[26,27] have been shown to be options for identifying high-risk patients within the group of patients with a normal karyotype. A prognostic value of telomere shortening (and telomerase activity) has been suggested in various human hematopoietic malignancies,[28-31] and several studies have investigated telomerase activity and telomere length in mononuclear cells from patients with AML.[19,32-38] However, recent data have suggested that telomere length in subpopulations of peripheral blood leukocytes (i.e. lymphocytes including their naive and memory subsets, granulocytes and monocytes) is heterogeneous.[39] Therefore we decided to study telomere length and hTERT mRNA expression, including its various splice variants, in a cohort of 137 previously untreated AML patients with ≥80% blasts in the bone marrow or peripheral blood. The aim of the current study was to analyze whether telomere parameters in primary AML blasts are correlated with the degree of chromosomal abnormalities and might be of potential prognostic value for predicting treatment outcome particularly in AML patients.

Table 1. Patients' characteristics.

	Patients (n=137)
Age (years)	
Median (range)	58 (18-78)
≤60 years (n)	80
>60 years (n)	57
Disease status (n=137)	
De novo AML	125
Secondary AML	12
White blood cell count (×10^9/L)	
Median (range)	48.0 (1.2–372.0)
Bone marrow blasts	
Median % (range)	87% (80-99)
CD34 expression* (n=128)	
Median % (range)	22% (0-98)
≥20% (n)	65
<20% (n)	63
FLT3-ITD mutant/wild-type ratio (n=131)	
>0.8	21
≤0.8	110
FAB subtypes (n = 137)	
M0	11
M1	68
M2	7
M4	8
M4eo	4
M5	38
M7	1
Number of aberrations (n=137)	
Normal karyotype	78
One or two independent aberrations	46
Multiple (i.e. ≥ three) independent aberrations	13
Cytogenetic risk (n=137)	
Favorable	7
Intermediate	116
Unfavorable	14

**CD34 expression; positive cases reflect more than 20% positivity on the blast cells.*

Design and Methods

Patients and clinical parameters

Material was obtained from 137 previously untreated patients aged 18 to 78 years with *de novo* or secondary AML and ≥80% blasts in the bone marrow or peripheral blood at diagnosis. Patients were treated according to the protocol of the SHG-AML-96 study as published previously.[40] Patients diagnosed with the subtype FAB-M3 were excluded from the study. The study was approved by the ethics committee of the University of Dresden (EK210396) and was in accordance with the ethical standards of the Helsinki declaration of 1975 (and its revision in 2000). Informed consent was obtained from each patient. The patients' characteristics are listed in Table 1. A complete response (CR) was defined as the presence of <5% blasts in a standardized bone marrow puncture after the second course of induction therapy with a fully regenerated peripheral blood count.

Cytogenetics

A complete karyotype was obtained for all 137 examined patients. Analyses were performed on metaphases from direct preparations, as well as from 24 h and 48 h cultures of bone marrow and/or peripheral blood samples as described previously.[41] The cytogenetic preparation and G-banding were done according to routine laboratory procedures. Patients with one or two independent cytogenetic aberrations were regarded as having simple aberrations, whereas those

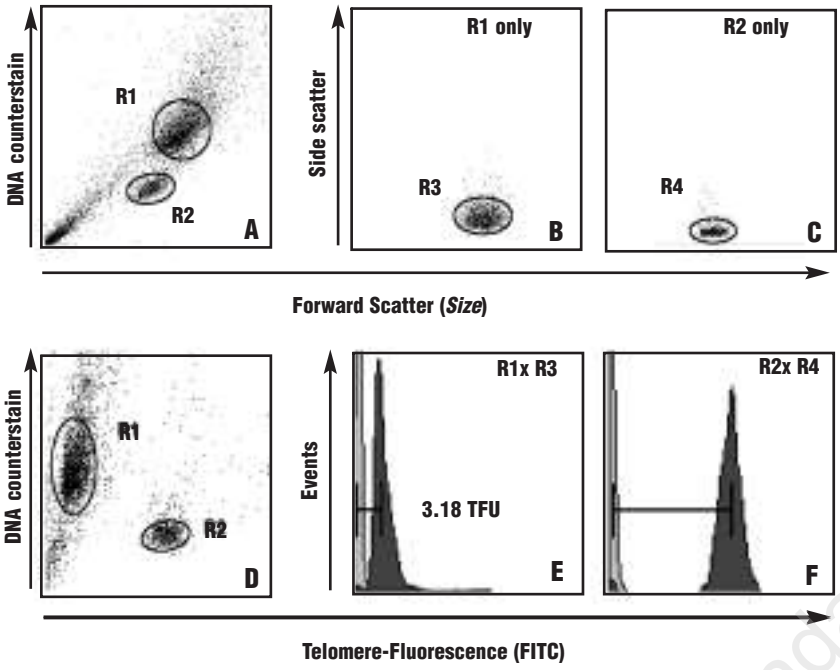

Figure 1. Experimental set-up for flow-FISH analyses of mononucleated cells (MNC) in AML patients. Gates were set on diploid cells (R1 as shown in A, B) and internal control cells (R2 as shown in A, C) on the basis of LDS fluorescence and forward scatter (FSC). The analysis was performed with and without a fluorescein isothiocyanate (FITC)-labeled telomere-specific PNA probe (D, dark and light gray peaks in E and F, respectively) to allow subtraction of autofluorescence of cells in the same light scatter window from telomere fluorescence (difference indicated by horizontal bars in E, F).

with three or more independent aberrations were regarded as having multiple aberrations.

Cytogenetic risk groups were defined as follows: *unfavorable*: -5/del(5q), -7/del(7q), hypodiploid karyotypes (besides 45,X,-Y or –X), inv(3q), abnl 12p, abnl 11q, +11, +13, +21, +22, t(6;9); t(9;22); t(9;11); t(3;3), multiple aberrations; *intermediate*: patients without a low risk or high risk constellation; *favorable*: t(8;21) and t(8;21) combined with other aberrations.

FLT3-activating mutations

DNA was extracted by using either phenol/chloroform or a silica-based procedure according to the manufacturer's instructions (Qiagen DNA Blood Kit; Qiagen, Hilden, Germany).

The results of the FLT3 analysis have been published recently.[25] Briefly, FLT3 ITD mutations were screened by PCR for exons 14 and 15 with published primers 11F and 12R.[42] To identify the mutant/wild type FLT3 ratio, a Genescan analysis with 6-FAM-labeled FLT3 11F primer (TIB MOLBIOL, Berlin, Germany) was performed. The PCR for the Genescan analysis was run using 5 ng template DNA and AmpliTaq Gold DNA-polymerase. The PCR conditions were as follows: preincubation at 95°C for 11 min followed by 30 sec at 94°C, 30 sec at 57°C, and 60 sec at 72°C for 27 cycles, and a final elongation step at 60°C for 45 min to achieve quantitative addition of +A overhangs.[43] Since our previously published study[25] showed that a FLT3 mutant/wildtype ratio > 0.80 was a highly significant prognostic factor for treatment outcome in AML, we used this ratio and the threshold for further analyses.

Telomere length measurements by flow-FISH

The average telomere length in mononucleated cells (MNC) derived from AML patients, described below, was measured by flow-FISH as previously reported.[28,39,44-46] Samples were blinded and each sample included cow thymocytes as an internal control.[44] After correction with the internal control, results were expressed in TFU (telomere fluorescence units, 1 TFU = 1 kb). One representative example is shown in Figure 1. Average telomere length in individual experiments was measured on different days. The actual TFU value was calculated based on Southern blot analysis that had been performed on the cow thymocytes (R2 in Figure 1A, Figures 1C, F) used as internal controls.[47] Adjustment for age was made on the basis of peripheral blood granulocyte telomere length measurements in a control group of healthy individuals.[39]

RNA isolation and cDNA preparation

Total RNA was collected from 87 AML samples using the RNeasy Mini Kit (Qiagen GmbH, Germany) according to the manufacturer's protocol. RNA was quantified using spectrophotometric measurements. cDNA was synthesized by reverse transcription with Superscript™ First-Strand Synthesis Systems for RTQ-PCR (Life Technologies) according to the manufacturer's instructions.

Real-time quantitative PCR (RTQ-PCR)

Primers for RTQ-PCR were designed with Primer Express 2.0 software (Applied Biosystems, UK) using published sequence data from the NCBI database

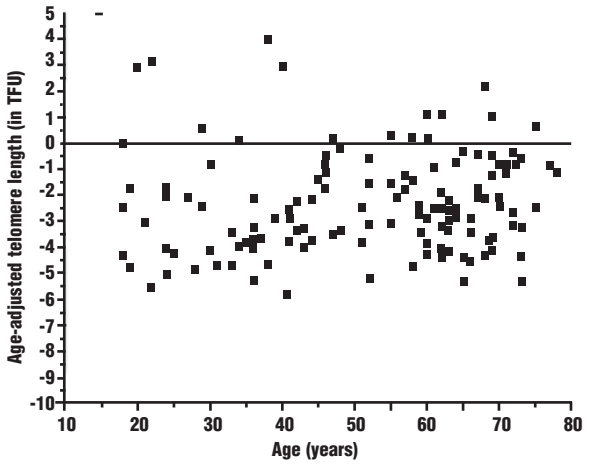

Figure 2. Distribution of age-adjusted telomere length in MNC derived from AML patients. Note that patients with AML had significantly shorter telomeres than did age-adjusted controls.

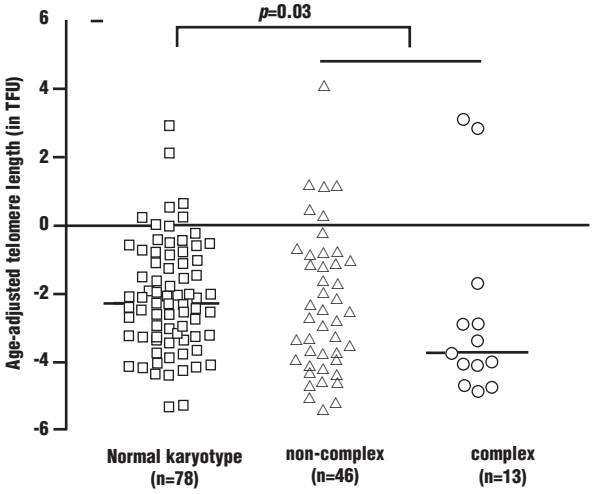

Figure 3. Average age-adjusted telomere length in MNC derived from patients with AML in correlation with karyotype. Age-adjusted telomere length (TFU) in patients with normal karyotypes was significantly shorter than telomeres from patients with aberrations (p=0.03). Differences between patients with non-complex and complex karyotypes were significant (p=0.39).

and synthesized by MWG-BIOTECH AG (Ebersberg, Germany) for hTERT: (forward primer: 5'-TGA CAC CTC ACC TCA CCC AC-3', reverse primer: 5'-CAC TGT CTT CCG CAA GTT CAC-3', probe: 5'-FAM-ACC CTG GTC CGA GGT GTC CCT GAG-TAMRA-3'). The primers and the probe were designed to detect only full-length hTERT transcripts (i.e. +α+β). For *Homo sapiens* GAPDH, we used a Pre-Developed TaqMan Assay Reagent Control Kit (Applied Biosystems) as described in the manufacturer's instructions.

Amplification reactions contained 7 μL cDNA, 10 μL of the Universal Taqman 2X PCR mastermix (Applied Biosystems) and 1.0 μL of each of the specific primers and probe. The primer and probe concentrations in the final volume of 20 μL were 200 nM and 100 nM, respectively. All reactions were performed in duplicate in a Sequence Detection System 7000 (Applied Biosystems, ABI PRISM, UK) and the thermal cycling conditions were: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min. GAPDH expression in AML cells was used as endogenous reference to normalize hTERT expression for sample-to-sample differences in RNA input, RNA quality and reverse transcriptase efficiency. The number of PCR cycles to reach the fluorescence threshold was the cycle threshold (Ct). Relative expression levels were calculated using the comparative cycles to threshold (Ct) method. Therefore, the target PCR Ct values are normalized to the Ct-value of the reference gene (*GAPDH*) by subtracting the GAPDH Ct-value from the target Ct-value. The relative expression level for each target PCR was calculated using the equation:

$$\text{relative expression} = 2^{-[\,Ct\,(\text{target}) - Ct\,(\text{GAPDH})\,]} \times 100$$

Splicing patterns of hTERT

The distribution of hTERT mRNA alternate splicing patterns was determined by RT-PCR. For RT-PCR we used primers designed to generate a 457 bp product containing the A and B reverse transcriptase motifs, and therefore able to detect the α and β deletions when present (Figures 4 A,B). Splice variant products were amplified from 3.5 μL cDNA using the hT2164F (5'-GCC TGA GCT GTA CTT TGT CAA-3') and hT2620R (5'-CGC AAA CAG CTT GTT CTC CAT GTC-3') primers, synthesized by MWG-BIOTECH AG (Ebersberg, Germany) and the Red Taq™ Ready Mix™ PCR Reaction Mix (Sigma). We used the following cycles: 94°C for 15 min, followed by 40 cycles of 95°C for 30 sec, 64°C for 45 sec, 72°C for 45 sec, and finally 72°C for 5 min. The integrity of generated cDNA was assessed by performing PCR for *GAPDH* as a housekeeping gene with the Red Taq™ Ready Mix™ PCR Reaction Mix and the primers: 5'-TGA AGG TCG GAG TCA ACG GAT TTG GT-3' (forward) and 5'-CAT GTG GGC CAT GAG GTC CAC CAC-3' (reverse) to generate a 983 bp product, also synthesized by MWG-BIOTECH AG. The PCR settings were: 94°C for 45 sec, 61°C for 45 sec, 72°C for 2 min (all repeated for 30 cycles) then 72°C for 10 min. GAPDH-PCR products were separated in 1.5% agarose, hTERT splicing patterns in

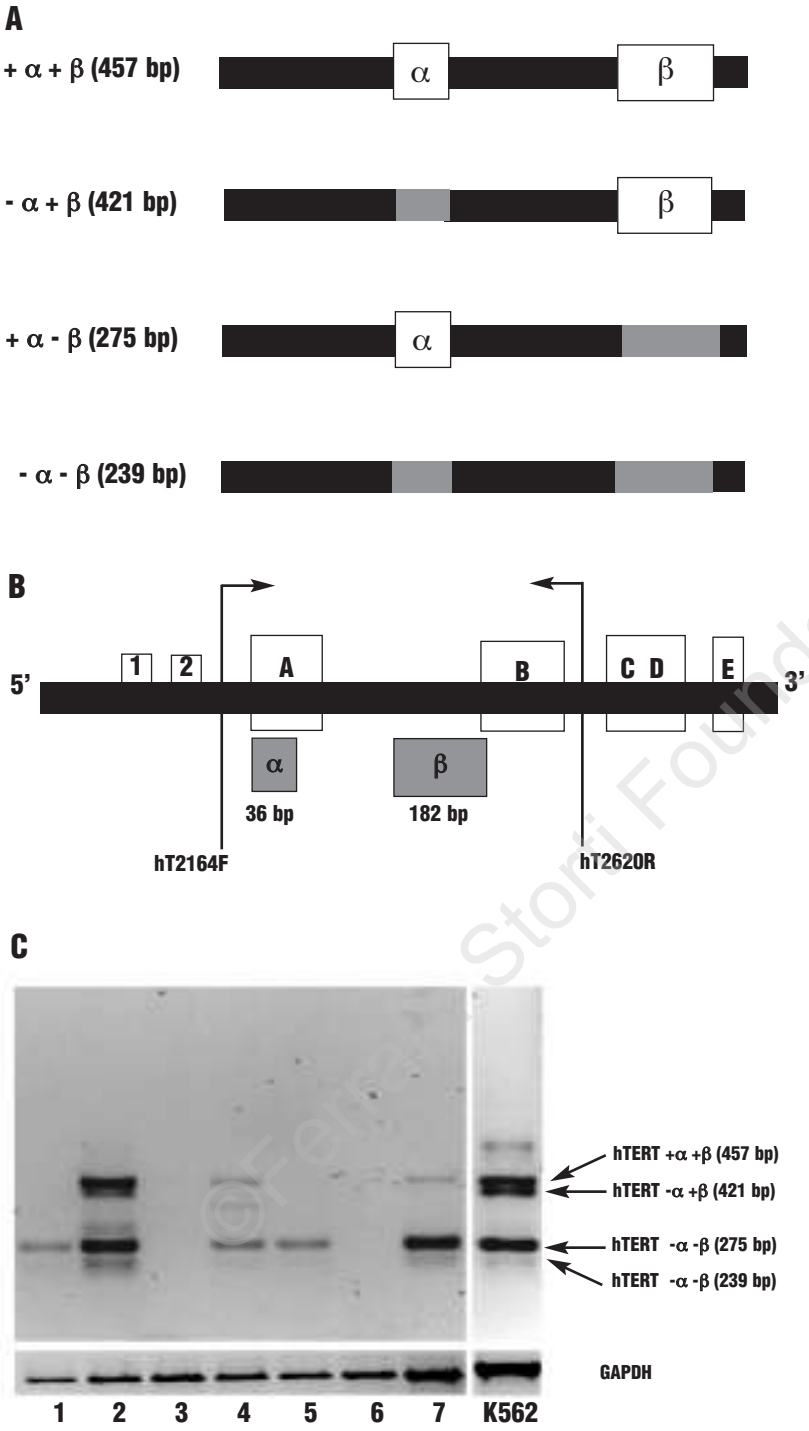

Figure 4. The reverse transcriptase domain of hTERT mRNA with alternative splicing sites and respective PCR products. A. The potentially active hTERT mRNA (+α+β) and three inactive alternative splicing variants of hTERT. B. Genomic organization of the hTERT gene. RT motifs and alternative splicing sites (deletions α and β) flanked by primers hT2164F and hT2620R as described above. C. An example of different hTERT splice variants in AML patients (lane 1-7) and K562 as the positive control.

2% agarose, both containing ethidium bromide (0.8 μg/mL, Sigma) in 1×TBE buffer. PCR products were directly visualized under UV illumination (Figure 4C).

Statistical analysis

Basic statistical data such as mean values, standard deviations and frequencies were obtained using the SPSS software package. Differences in telomere length or quantitative hTERT expression between the analyzed subgroups were evaluated by the two-tailed Mann-Whitney U test, and differences in binary parameters by a two-tailed Fisher's exact test. Correlations between quantitative parameters were tested by the method of Pearson. Differences in median survival were compared using Wilcoxon's

test. For survival analyses, patients who received an allogeneic peripheral blood stem cell transplantation within the therapy course were censored at the time of transplantation.

Results

Age-adjusted telomere length in AML patients

Flow-FISH measurements of telomere fluorescence were obtained from a total of 137 AML patients as described above. Telomere fluorescence in peripheral blood granulocytes from patients with AML was compared with measurements made previously in peripheral blood granulocytes in a population of 436 normal healthy controls (0-90 years of age) as described elsewhere.[39] As shown in Figure 2, age-adjusted telomere length in AML patients was significantly shorter than the telomere length of matched healthy controls (median: -2.5, range: -5.5 to 4.0 TFU; $p<0.001$). The patients' characteristics are shown in Table 1. Telomere length was not correlated with disease status (*de novo* versus secondary AML) or with the percentage of CD34 positive cells in AML patients (Table 2). Interestingly, within the FAB classification system, patients with the monocytic subtype M5 had the shortest telomeres (-3.8 TFU); for comparison telomere length in patients with M1 was -1.9 TFU ($p<0.001$). Furthermore, patients with a high FLT3 ratio had shorter telomeres than did patients with a low FLT3 ratio ($p=0.04$). This influence of FLT3 ratio on telomere length was even more pronounced for patients with a normal karyotype (median: -2.9 vs. –2.0 TFU; $p=0.01$). Surprisingly, when we compared telomere length of AML patients of different ages, patients younger than 60 years had significantly shorter age-adjusted telomere length than did patients older than 60 years (median: -3.4 vs. -1.7 TFU; $p<0.001$).

Telomere shortening correlates with the degree of chromosomal abnormalities

In order to analyze whether telomere length was linked to the degree of chromosomal abnormalities in AML patients, we grouped the samples based on conventional cytogenetics (Table 1). Seven patients were characterized as having a favorable cytogenetic risk and 14 patients an unfavorable cytogenetic risk with 13 of the latter displaying multiple aberrations. Age-adjusted telomere length was found to be significantly shorter in patients displaying an aberrant karyotype than in patients with a normal karyotype (median -3.0 vs. -2.3 TFU; $p=0.03$) despite the fact that patients with normal karyotypes more frequently had a high FLT3 ratio, were older and had a higher leukocyte count at diagnosis than patients with an aberrant karyotype. No differences in FAB subtype distribution or stage of disease were seen between patients with normal and aberrant karyotypes. Interestingly, the shortest telomeres were found in patients with multiple cytogenetic aberrations (median -3.7 TFU; $p=0.03$, Table 2). For the entire group of patients, telomere length did not have a significant influence on treatment response, overall survival or disease-free survival (*data not shown*). However, looking at AML patients with a normal karyotype (n=78), telomere length had a significant influence on treatment outcome. Surprisingly only 41% of patients with an age-adjusted telomere length longer than the median of -2.3 TFU reached a complete response, compared to 67% of patients with a age-adjusted telomere length shorter than the median ($p=0.04$).

Table 2. Correlations between age-adjusted telomere length and different parameters.

	n	*Telomere length [TFU]* Median	Range	*p**
Karyotype				
Normal	78	-2.3	-5.3–2.9	
Aberrant	59	-3.0	-5.5–4.0	0.03°
One or two aberrations	46	-2.9	-5.5–4.0	0.10
≥ Three aberrations	13	-3.7	-4.9–3.1	0.03°
FLT3-ITD mut/wt ratio				
> 0.8	21	-3.2	-5.3–4.0	0.04
≤ 0.8	110	-2.4	-5.5–3.1	
FAB				
M0	11	-2.1	-5.2–2.9	0.71@
M1	68	-1.9	-5.3–4.0	
M2	7	-1.0	-2.9–2.9	0.21
M4	8	-3.3	-4.7– -0.6	0.03°@
M4eo	4	-3.2	-4.6– -1.3	0.13@
M5	38	-3.8	-5.5–0.3	< 0.001°@
M7	1	-2.4		0.69@
Disease status				
de novo	125	-2.5	-5.5–4.0	
Secondary	12	-1.3	-5.0–0.2	0.35
CD34+ expression				
Negative	63	-2.5	-5.5 – 2.9	
Positive	65	-2.5	-5.3 – 4.0	0.94
Age				
≤ 60 years	80	-3.4	-5.5 – 2.2	
> 60 years	57	-1.7	-4.0 – 4.0	< 0.001°

**Statistical analysis was done for FAB by logistic regression, for the other parameters by two-tailed Mann-Whitney-U-test; °significant correlations are indicated by p-values less than 0.05; @telomere length of different FAB subtypes was correlated with FAB M1.*

Expression of hTERT mRNA and splicing patterns in AML

For 87 of the 137 patients included in the study, we were able to examine hTERT mRNA expression as a surrogate marker for telomerase expression as well as

Table 3. Distribution of hTERT splicing variants according to different karyotype.

	Normal	*Non-Complex*	*Complex*
Total number of samples (n=87)	49	27	11
Total number of splicing variants (n=37)	16	14	7
Distribution of splicing variants			
+α+β	5	10	3
-α	8	4	2
+α+β including -α	3	2	2
-β	9	11	4
-α -β	9	11	5

hTERT splice variants (Figure 4 A-C). hTERT mRNA was detectable in 18/87 AML-patients (21%). Consistent with these findings, full length active hTERT (+α+β) was found in 18 (21%) patients. However, two hTERT positive samples did not express the active splicing variant of hTERT (+α+β), whereas no hTERT mRNA could be identified in two samples with full length hTERT (+α+β). Seven AML patients expressed full length hTERT (+α+β) in combination with the inactive variant -α which has been demonstrated to be a dominant negative inhibitor of hTERT.[48] Nineteen patients (22%) had inactive splicing variants only (-α, -β, and -α-β), whereas 50 (58%) had no detectable hTERT variant at all. There was a strong correlation between expression of hTERT mRNA, detected by RTQ-PCR, and full length hTERT (+α+β), detected by RT-PCR ($p<0.001$). When we compared samples from patients with functional hTERT without the dominant negative splicing variant -α we found significantly higher hTERT expression levels than in patients with -α. No significant expression of hTERT mRNA was detected in patients without functional full length hTERT. Surprisingly, age-adjusted telomere length did not differ significantly between patients without hTERT expression (n=69; mean TFU±S.E.; –2.5±0.2) and patients with expression of functional hTERT (n=18; –2.0±0.6 TFU). In order to confirm these results, we analyzed age-adjusted telomere length in different splicing patterns. Telomere length was also not significantly different between patients without functional hTERT (n=69; –2.4±0.2 TFU) and patients with functional hTERT (+α+β) (n=18; –2.3±0.5 TFU).

The presence or absence of active hTERT correlates with the karyotype of AML patients

Next we investigated whether alternative splicing and/or increased hTERT mRNA levels are correlated with chromosomal abnormalities in AML patients (Table 3). As shown in Figure 5, hTERT expression was detected in 4/11 (= 36%) samples from patients

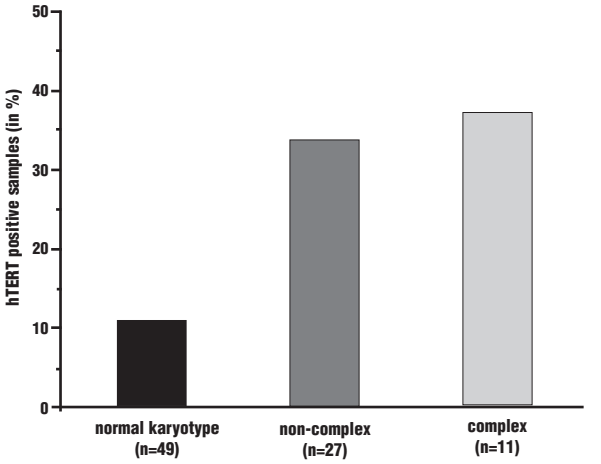

Figure 5. Distribution of hTERT expression in samples with normal, non-complex or complex karyotypes. The boxes represent median expression of hTERT mRNA, detected by quantitative RT-PCR (Taqman® PCR). The dark gray box represents samples with a normal karyotype, the medium gray box represents non-complex karyotypes, whereas the light gray box represents samples with complex karyotypes (> 3 aberrations).

with a complex karyotype, in 9/27 (= 33%) samples with a non-complex karyotype and in 5/49 (= 10%) samples with a normal karyotype. In line with these findings, aberrant karyotypes were more frequently found in patients with functional hTERT (+α+β) than in patients without (72% vs. 36%, $p=0.008$).

Discussion

In this study, we analyzed telomere length and hTERT expression in a large and homogeneous (i.e. > 80% blasts in bone marrow or blood) cohort of well-characterized AML patients at diagnosis. The results of this study showed pronounced and highly significant telomere shortening in patients with AML as compared to age-matched healthy controls, consistent with observations made by others.[49-51] When the degree of telomere shortening was correlated with clinical parameters, most pronounced telomere shortening was found in the group of patients with FAB subtype M5. The difference between degree of telomere shortening was greatest between this subtype and subtype M1. The other FAB subtypes were too rare to be able to draw final conclusions. This is possibly due to the selection bias of including only patients with 80% blasts or more. However, these results reflect an increased fraction of FLT3-activating mutations in patients with AML FAB M5,[25] which could account for an increased proliferation rate.[52] Indeed, in our series, patients with a high FLT3 mutant/wildtype ratio had shorter telomeres than did patients with a low ratio.

Secondly, when we compared age-adjusted telomere length in patients older than 60 years to that in younger patients (< 60 years), to our surprise, we found significantly shorter telomere length in the younger group of patients. Nevertheless, it is of note, that Ohyashiki *et al*.[53] also found no significant increase in telomere length reduction in relation to age in patients with myelodysplastic syndrome or secondary AML. However, a higher proportion of karyotypic abnormalities in the younger patients (50% with aberrant karyotypes) than in the older patients (34% aberrant karyotypes) in our population of patients most likely contributed to these results. Approximately 43% of the patients in our study had karyotypic abnormalities and 9.5% of them had complex karyotypes. Interestingly, telomere shortening was significantly more pronounced in patients with cytogenetic alterations than in patients with normal karyotypes. The shortest median telomere length was found in the group with complex karyotypes, although it should be noted that relatively few patients were included in this group. A larger and possibly prospective trial needs to be set up to confirm our results. Furthermore, within patients with normal karyotypes the FLT3 mutant/wild type ratio was able to discriminate between patients with shorter and longer telomeres indicating that not only cytogenetic aberrations but also mutations within cell cycle relevant genes may lead to telomere shortening.

Surprisingly, hTERT expression levels by RT-PCR were found to increase with the degree of karyotypic changes in AML blasts. We found that hTERT was most frequently expressed in patients with complex karyotypes, then in patients with non-complex karyotypes and least in patients without karyotypic changes. This might suggest that with increasing karyotypic abnormalities telomere attrition either by replication-dependent or independent mechanisms becomes more pronounced and, as a consequence, telomerase upregulation becomes essential to prevent replicative senescence of the malignant clone.

Several investigators have observed detectable telomerase activity in most human AML blast samples with a very heterogenous distribution in the level of activity.[19,38,54] A rate-limiting step in activation of telomerase is the expression of human telomerase reverse transcriptase subunit (hTERT).[17] As opposed to the finding of elevated telomerase activity in the majority of AML patients,[19,35,38] we found, in line with other studies, that hTERT mRNA was expressed in only 21% of new diagnosed AML patients.[55] These observations are supported by a study of Xu *et al*.[38] who showed that hTERT expression was only detectable in AML samples with intermediate or high levels of telomerase activity, as detected by the TRAP assay. Recently, it has been proposed that alternative splicing of hTERT is involved in regulation of telomerase activity.[56] Therefore, in addition to hTERT mRNA expression, we also analyzed its different splicing patterns in AML samples. hTERT expression correlated very well with the expression of the active hTERT splicing variant +α+β.[18] However, unlike in other disease entities,[57,58] our data suggest that alternative splicing does not seem to be the crucial mechanism by which telomerase is regulated in AML patients.

In analogy to the data obtained from patients with chronic myelogeneous leukemia in late chronic phase,[28,59] genetic instability due to progressive telomere shortening could be linked to up-regulation of telomerase activity and disease evolution in AML. Due to the uneven distribution of telomere length on individual chromosome arms,[60] critical shortening of telomeres on selected chromosome arms could promote the formation of chromosomal aberrations and thus be of importance for clonal evolution even if the average telomere length in an individual cell remains well above the critical level of shortening.

However, it remains unclear whether telomere length as a single parameter may be of prognostic relevance in AML. As shown in this study other parameters, such as FLT3 mutations, influence telomere length. In the future multi-parameter assays, such as gene profiling,[26] might be broadly applicable and might give light on the multiple pathways influencing pathogenesis and prognosis in AML.

Appendix

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UH, THB, SB, CT, TI and MS contributed to the conception and design of the study and the interpretation of the data; UH performed most of the experiments regarding telomere length, hTERT expression and the splicing variants; CT performed the FLT3 experiments, TI and MS collected the patients' data and performed the statistical analyses.

We would like to thank Iris Schäfer and Bettina Kirchner for excellent technical assistance, Silke Soucek for expert assistance with the statistical analysis and the members and physicians of the German SHG AML96 study group (see "Appendix") for entering their patients into the trial. The authors declare that they have no potential conflict of interest.

The study was supported by grants from the Deutsche Krebshilfe to THB and GE.

Manuscript accepted October 14, 2004. Accepted January 22, 2005.

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