ORIGINAL RESEARCH

# Variability within the human *TERT* gene, telomere length and predisposition to chronic lymphocytic leukemia

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**Background:** The human telomerase reverse transcriptase (*TERT*) gene encodes the catalytic subunit of telomerase that is essential for maintenance of telomere length. We aimed to find out whether variability within the *TERT* gene could be associated with telomere length and development of the disease in non-treated patients with chronic lymphocytic leukemia (CLL).

**Materials and methods:** Telomere length, rs2736100, rs2853690, rs33954691, rs35033501 single-nucleotide polymorphisms, and variable number of tandem repeats (VNTR-MNS16A) were assessed in patients at diagnosis. In addition, blood donors served as controls for the polymorphism studies.

**Results:** The minor rs35033501 *A* variant was more frequent among CLL patients than in healthy controls (OR=3.488, *p*=0.039). CLL patients over 60 years of age were characterized with lower disease stage at diagnosis (*p*=0.001 and *p*=0.008, for the Rai and Binet criteria, respectively). The MNS16A VNTR-243 short allele was more frequent in patients with a low disease stage (*p*=0.020 and *p*=0.028, for the Rai and Binet staging system) and also among older patients having longer telomeres (*p*=0.046). Patients with Rai 0–I stage were characterized with longer telomeres than those with more advanced disease (*p*=0.030). This relationship was especially pronounced in patients carrying the rs2736100 *C* allele, independently of the criteria used, ie, Binet (*p*=0.048) or Rai (*p*=0.001).

**Conclusion:** Our results showed that the genetic variation within the *TERT* gene seems to play a regulatory role in CLL and telomere length.

**Keywords:** telomere length, human telomerase reverse transcriptase, variable number of tandem repeats, single-nucleotide polymorphism, chronic lymphocytic leukemia

## Introduction

Chronic lymphocytic leukemia (CLL) is the most common leukemia among adults. It primarily affects the elderly and occurs twice as often in males than in females.[1] The disease is influenced by complex heterogeneous genetic and microenvironmental factors that can result in different clinical courses.[2,3] The pathogenesis of CLL varies depending on molecular heterogeneity background, mutational load and specific genomic aberrations.[4,5] Genome-wide association studies identified many susceptibility loci involved in B-cell biology and CLL development.[6–8] One study revealed that the telomere/telomerase system may be impaired in the early stages of CLL.[9] Also, several studies focused on the potential prognostic significance of telomere length and human telomerase reverse transcriptase (hTERT) activity in CLL.[10–13]

Human telomerase activity is regulated by expression of the telomerase reverse transcriptase (*TERT*) gene that encodes the catalytic subunit of telomerase.[14] The *TERT* gene is located on chromosome 5p15.33 and consists of 16 exons and 15 introns spanning over 40 kb, and is essential for maintenance of telomere length by protecting chromosome ends. It has been documented that single-nucleotide polymorphisms (SNPs) within the *TERT* gene may affect the length of telomeres and telomerase activity, thus contributing to CLL disease susceptibility.[7,8,15–17] Main processes such as transcription, alternative mRNA splicing, phosphorylation and many other changes, including mutations and gene variants of *TERT*, have been shown to play pivotal roles in the regulation of telomerase activity and cancer risk.[18,19]

Alternative splicing of hTERT mRNA is considered to be one of the most precise regulators of telomerase activity in human cells. Induction of an hTERT splicing variant was associated with increased expression of the apoptotic endonuclease EndoG, a splicing regulator. It was demonstrated that EndoG (an apoptotic endonuclease capable of destroying both DNA and RNA) induced alternative splicing of the telomerase catalytic subunit hTERT and inhibited telomerase activity in normal human $CD4^+$ T lymphocytes.[20,21]

The promoter region and sequences upstream interact with both positive and negative regulators of the *TERT* gene through many transcriptional binding sites.[22,23] It has been demonstrated that *TERT* promoter activity depends on variable numbers of tandem repeats (VNTRs), such as MNS16A, which constitutes a binding site for a transcription factor GATA binding protein 1 (GATA-1).[24] Research on the functional significance of this genetic polymorphism showed that shorter MNS16A is related to higher *TERT* promoter activity.[24,25] This functional polymorphism may play an important role in human longevity, disease progression and response to therapy of patients with non-Hodgkin's B-cell lymphomas and the development of other cancers.[26–29]

Mechanisms underlying telomere maintenance and telomerase reactivation in leukemogenesis are currently being investigated. Increasing data on the CLL genetic background and landscape, including the role of many gene variants in disease initiation and progression in the context of telomere length, may be crucial for understanding CLL pathogenesis.[30,31] This prompted us to investigate the role of MNS16A VNTR and selected SNPs located within the *TERT* gene in relation to telomere length and stage of disease in non-treated patients with CLL.

*TERT* SNPs were chosen using the SNP Function Prediction tool of the National Institute of Environmental Health Sciences website and other auxiliary databases (https://snpinfo.niehs.nih.gov/snpinfo/snpfunc.html; https://www.ncbi.nlm.nih.gov/snp/; https://www.ensembl.org/index.html). The following criteria were used: minor allele frequency in Europeans >0.01, change in the amino acid chain, potential splicing site and/or miRNA binding site (Figure 1). The rs35033501 polymorphism is one of the most commonly studied *TERT* SNPs. It represents a change of one proline codon (CCG) into another (CCA), without altering the amino acid sequence in the hTERT protein. This silent, synonymous mutation may potentially cause changes of splicing patterns or efficiency by disrupting splice sites. Similarly to rs35033501, the rs33954691 SNP also alters a splice site, potentially leading to changes in the splicing pattern. Another polymorphic variation investigated, rs2853690, is a common SNP in the 3′ untranslated region of the *TERT* gene. This cytosine-to-thymine substitution occurs in a locus responsible for miRNA binding, and may possibly affect miRNA-dependent mRNA stability and translation efficiency. The fourth SNP, rs2736100, was found to be a susceptibility factor for a variety of cancers and myeloproliferative neoplasms. The rs2736100 polymorphism is located in intron 2 of the *TERT* gene. Although its exact mechanism of action is unknown, several
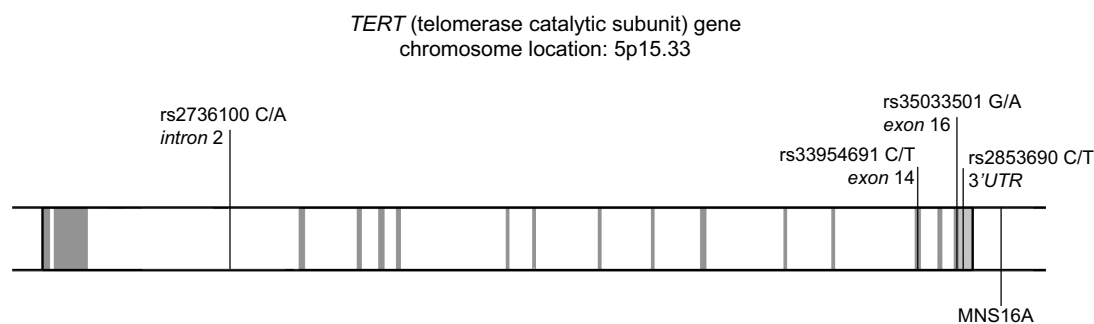


**Figure 1** Genomic structure of the human telomerase gene. Exons are marked in gray while intronic regions are in white.

lines of evidence suggest that the *C* allele is associated with longer telomere length.[32]

## Materials and methods
### Patients and controls
This retrospective study was conducted on a group of 68 treatment-naïve CLL patients (41 men and 27 women; aged 39–85 years, average 65.8 years) recruited at the Department of Haematology, Blood Neoplasms and Bone Marrow Transplantation, Wroclaw Medical University. Whole blood taken on EDTA from CLL patients and healthy individuals was used for genetic analysis and to assess telomere length.

Written informed consent was provided by the patients. The study was approved by the Wroclaw Medical University Ethics Committee and all the procedures were in accordance with the ethical standards of the Helsinki Declaration, as revised in 2013.

Stage of the disease was graded according to the Rai and Binet staging systems, based on clinical and laboratory parameters.[30,31] Patients with CLL who were characterized with lower stages of disease (0–I in the Rai, and A in the Binet criteria) constituted approximately 70% of patients. The remaining patients were characterized with II–IV and B or C stage of disease at diagnosis. Patients' characteristics are shown in Table 1. Genetic variants of the *TERT* gene and telomere length were assessed in the patients. In addition, blood donors served as controls for the polymorphism studies.

### Human cancer and leukemia cell lines
The following human cancer and leukemia cell lines (all commercially available) were used in the study: A549 (Sigma-Aldrich, Steinheim, Germany); HT-29, MV-4-11, HL-60, MDA-MB-231, Hs294T (American Type Culture Collection, Rockville, MD, USA); and KG1a, K562 (Leibniz-Institut DSMZ – German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). They were maintained in liquid nitrogen at the Cell Culture Collection of the Hirszfeld Institute of Immunology and Experimental Therapy (Wroclaw, Poland). Cell line characteristics and culture conditions are presented in Table S1.

### DNA isolation
DNA was isolated from $10^6$ cultured cells of cancer and leukemia cell lines or from 5 mL of whole blood from CLL patients taken on EDTA using the Qiagen DNA Isolation Kit (Qiagen, Hilden, Germany) and following the recommendations of the manufacturer.

**Table 1** *TERT* SNP genotype and allele distribution in CLL patients and controls

| SNP genotype and alleles | CLL patients | Controls |
|---|---|---|
| rs2736100 | N=67 | N=238 |
| CC | 14 (21%) | 50 (21%) |
| CA | 40 (58%) | 117 (49%) |
| AA | 14 (21%) | 70 (30%) |
| C | 68 (50%) | 217 (46%) |
| A | 68 (50%) | 257 (54%) |
| rs2853690 | N=67 | N=100 |
| CC | 52 (78%) | 68 (68%) |
| CT | 15 (22%) | 32 (32%) |
| TT | – | – |
| C | 119 (89%) | 168 (84%) |
| T | 15 (11%) | 32 (16%) |
| rs33954691 | N=68 | N=99 |
| CC | 50 (74%) | 84 (85%) |
| CT | 16 (24%) | 15 (15%) |
| TT | 2 (2%) | - |
| C | 116 (85%) | 183 (92%) |
| T | 20 (15%) | 15 (8%) |
| rs35033501 | N=68 | N=99 |
| GG | 59 (87%) | 95 (96%) |
| GA | 9 (13%)[a] | 4 (4%)[a] |
| AA | – | – |
| G | 127 (93%) | 194 (98%) |
| A | 9 (7%) | 4 (2%) |

**Notes:** [a]OR=3.488, 95% CI 2.702–4.501, *p*=0.039.
**Abbreviations:** SNP, single-nucleotide polymorphism; CLL, chronic lymphocytic leukemia.

## SNP genotyping of the *TERT* gene
The *TERT* polymorphic variants (rs2736100, rs2853690, rs33954691, rs35033501) in patients and controls were detected with the use of LightSNiP typing assays (TIB MOLBIOL, Berlin, Germany), employing real-time polymerase chain reaction (PCR) amplifications with melting curve analysis. The reactions were performed on a LightCycler 480 II Real-Time PCR system (Roche Diagnostics International, Rotkreuz, Switzerland), following the recommendations of the manufacturer.

## VNTR MNS16A genotyping of the *TERT* gene
Presence of the MNS16A *TERT* gene polymorphism was assessed in all CLL patients and in healthy individuals. PCR was carried out in a 2720 Thermal Cycler instrument (Applied Biosystems, Foster City, CA, USA) using forward and reverse

primer sequences (5′-AGGATTCTGATCTCTGAAGGGTG -3′ and 5′-TAMRA-TCTGCCTGAGGAAGGACGTATG-3′) prepared by Genomed (Warsaw, Poland), based on Wang et al.[24] The amplification procedure consisted of an initial denaturing step for 5 minutes at 95°C, followed by 35 cycles of: 30 seconds at 95°C, 45 seconds at 60°C and 1 minute at 72°C, as well as a final extension step for 10 minutes at 72°C. PCR products were diluted with formamide and GeneScan-500 ROX size standard (Applied Biosystems). Samples were denatured at 95°C for 5 minutes and quickly transferred to ice before analysis on the 3500 Genetic Analyzer (Applied Biosystems) with an eight-capillary system filled with the POP7 polymer. Alleles were identified with GeneMapper version 4.2 software (Applied Biosystems).

## Telomere length analysis in CLL patients and human cancer cell lines

The average telomere length of target genomic DNA samples from human cell lines cultured *in vitro* and from whole blood of CLL patients was assessed by real-time quantitative polymerase chain reaction (qPCR) in a LightCycler480 II Real-Time PCR system (Roche Diagnostics International, Rotkreuz, Switzerland) using SYBR® Green assay kits (ScienCell's Absolute Human Telomere Length Quantification qPCR Assay Kit [AHTLQ], Carlsbad, CA, USA), following the recommendations of the manufacturers. For each DNA sample, two consecutive reactions were performed: the first to amplify a single-copy reference (SCR) gene and the second for the telomere sequence. The SCR primer set recognizes and amplifies a 100 bp-long region on human chromosome 17 and serves as a reference for data normalization. Both PCRs were performed in a final volume of 20 μL using 1 μL of reference/genomic DNA samples from patients and controls (5 ng), 2 μL of primer stock solution (telomere or SCR), 10 μL of 2×qPCR FastStart Essential DNA Green Master Mix (Roche Diagnostics International) and 7 μL of nuclease-free water. The PCR conditions were as follows: 95°C for 10 minutes followed by 32 cycles of: 95°C for 20 seconds, 52°C for 20 seconds and 72°C for 45 seconds. All reactions were run in duplicate.

## Statistical analysis

Genotype and allele frequencies were compared between the study groups by the chi-squared test with Yates' correction or Fisher's exact test when necessary, using online tools (available online: http://www.socscistatistics.com/tests/

Default.aspx). Linkage disequilibrium between the *TERT* SNPs and the MNS16A VNTR was analyzed with Haploview 4.2 software (VassarStats: Website for Statistical Computation; available online: http://vassarstats. net/tab2x2.html). Probability (*p*) values <0.05 were considered statistically significant, while those between 0.05 and 0.10 were considered as indicative of a trend.

## Results

### *TERT* rs35033501 *A* variant as a potential genetic factor affecting CLL development

Minor allele frequencies in the healthy control group of the current study did not differ from those reported previously for Europeans, taken from the NCBI website (https://www.ncbi.nlm.nih.gov/snp) (*C*=0.46 vs 0.50, *T*=0.16 vs 0.17; *T*=0.08 vs 0.10; *A*=0.02 vs 0.03; for minor allele counts of rs2736100, rs2853690, rs33954691 and rs35033501 polymorphisms, respectively).

Comparison of the SNP genotypes and allele frequencies (rs2736100, rs2853690 and rs33954691) between our CLL patients and healthy subjects did not show significant differences, suggesting no association with disease predisposition. However, it was observed that CLL patients were more often characterized by the presence of the rs35033501 *A* allele than healthy individuals (9/68 vs 4/99, OR=3.488, 95% CI 2.702–4.501, *p*=0.039), implying that the rs35033501 SNP may affect disease susceptibility (Table 1).

### Distribution of various MNS16A VNTR genotypes and alleles: association with CLL stage at diagnosis

Six different alleles and 11 genotypes of MNS16A were identified and classified as either long (L) or short (S) according to the length of PCR fragments.[24,38] VNTR-364, VNTR-333 and VNTR-302 were marked as long (L) and VNTR-212, VNTR-243 and VNTR-274 as short (S).[24] Based on this classification, various MNS16A genotypes were assigned to the three genotype groups: SS, SL or LL.[38,39] Five different VNTRs of MNS16A were detected in our CLL patients (VNTR-243, VNTR-274, VNTR-302, VNTR-333 and VNTR-364) and four in healthy controls (VNTR-243, VNTR-274, VNTR-302, VNTR-333). Both groups harbored nine different genotypes. The MNS16A genotype and allele distributions in healthy controls are similar to other European populations, as reported by Andersson et al[40] and Carpentier et al.[41] The CLL patients and healthy

**Table 2** *TERT* MNS16A genotype and allele distribution in CLL patients and controls

| MNS16A VNTR | | CLL patients | Controls |
|---|---|---|---|
| Genotypes | | N=68 | N=126 |
| LL | | 27 (40%) | 53 (42%) |
| | 302/302 | 24 (36%) | 51 (40%) |
| | 302/333 | 2 (3%) | 2 (2%) |
| | 302/364 | 1 (1%) | – |
| SL | | 36 (53%) | 54 (43%) |
| | 243/302 | 29 (43%) | 51 (40%) |
| | 243/333 | 1 (1%) | 2 (2%) |
| | 274/302 | 6 (9%) | 1 (1%) |
| SS | | 5 (7%) | 19 (15%) |
| | 243/243 | 4 (6%) | 17 (13%) |
| | 274/274 | – | 1 (1%) |
| | 243/274 | 1 (1%) | 1 (1%) |
| Alleles | | | |
| L | | 90 (66%) | 160 (63%) |
| | 302 | 86 (63%) | 156 (62%) |
| | 333 | 3 (2%) | 4 (2%) |
| | 364 | 1 (1%) | – |
| S | | 46 (34%) | 92 (37%) |
| | 243 | 39 (29%) | 88 (34%) |
| | 274 | 7 (5%) | 4 (2%) |

**Abbreviations:** CLL, chronic lymphocytic leukemia; VNTR, variable number of tandem repeats; L, long allele; S, short allele.

subjects did not show significant differences in the MNS16A genotypes and allele frequencies. Genotype and allele distributions among CLL cases and healthy controls are presented in Table 2.

Some differences were noticed when stage of the disease at diagnosis was considered. According to the Rai and Binet criteria,[34,35] CLL patients older than 60 years were more frequently characterized by lower disease stage at diagnosis compared to patients aged 60 or younger (36/45 vs 9/23, $p=0.001$; 38/45 vs 12/23, $p=0.008$, for patients >60 years vs ≤60 years for the Rai and Binet staging systems, respectively) (Figure 2).

Moreover, in CLL patients who had a low disease stage at diagnosis (0–I and A), the MNS16A VNTR-243 (short allele) occurred more frequently than in patients with advanced stage (II–IV, B–C), according to the Rai (28/45 vs 7/23, $p=0.020$) and Binet systems (30/50 vs 5/18, $p=0.028$) (Figure 3). Thus, presence of the MNS16A VNTR-243 allele was more common in older patients with less advanced disease.

## Relationships between VNTR MNS16A alleles and *TERT* SNP variants

The distribution of all genetic variants was compared between rs2736100, rs2853690, rs33954691 and rs35033501 polymorphisms and MNS16A tandem repeats. The *TERT* SNPs and MNS16A VNTR were not found to be in linkage disequilibrium (analyzed with Haploview 4.2 software: http://vassar stats.net/tab2x2.html) (Figure 4). None of the studied SNP alleles was correlated with S or L VNTR alleles. We further examined whether there were any relationships between the most common MNS16A alleles (either 243 or 302 allele) and each SNP. However, no association between the presence of different genetic variants was observed.

## Telomere length in CLL patients and in vitro cultured cell lines

Telomere length was analyzed in whole blood samples of 67 CLL patients at diagnosis (before treatment) and in eight cancer and leukemic cell lines cultured *in vitro*. The median of telomere length in patients was 5.71 kb. The median of telomere length in cell lines was even shorter and equaled 1.09 kb (Table S1). Both in CLL patients and in *in vitro* cultured cell lines, telomere length was shorter than the median telomere length of 7.54 kb and 7.26 kb reported for healthy donors by Jebaraj et al[36] and Dos Santos et al,[37] respectively.

No difference was observed between telomere length of female and malepatients. Similarly, patients below and over 60 years of age did notshow significant differences in telomere length. The latter observation is in line with the previous studies reporting a lack of correlation between telomere length and age of CLL patients.[9,10,42]

## Relationship between telomere length and VNTR and SNP genetic variants in CLL patients

It appeared that telomeres were longer in CLL patients (N=44) with less advanced stage of disease (0–I) compared to CLL patients (N=23) with more advanced disease (II–IV stage), according to the Rai criteria (7.95 vs 5.99 kb, $p=0.030$). Moreover, among CLL patients carrying the *TERT* rs2736100 C allele, telomeres were longer in patients (N=40) in a less advanced stage of disease (Binet A) in comparison to those (N=13) at Binet stage B or C (7.50 vs
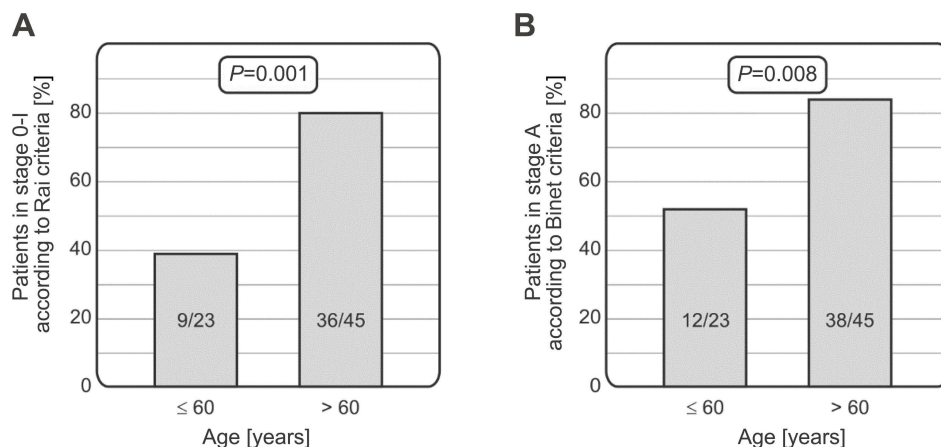
**Figure 2** Relationship between age of patients and CLL stage at diagnosis. Patients older than 60 years had lower stage of the disease at diagnosis than those aged 60 or younger, using both the Rai (**A**) and Binet (**B**) staging systems.
**Abbreviation:** CLL, chronic lymphocytic leukemia.
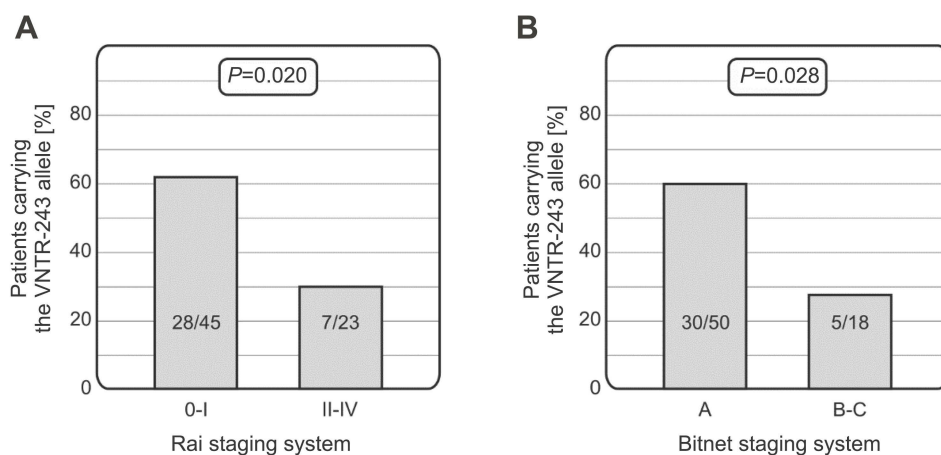


**Figure 3** Relationships between the *TERT* VNTR polymorphism and CLL stage at diagnosis. Patients carrying the MNS16A-243 allele were characterized with lower stage of the disease according to both the Rai (**A**) and Binet (**B**) criteria.
**Abbreviations:** VNTR, variable number of tandem repeats; CLL, chronic lymphocytic leukemia.

4.55 kb, $p=0.048$) (Figure 5A). A similar relationship was observed when the Rai criteria were considered. In patients in a less advanced stage (0–I) possessing the *TERT* rs2736100 *C* allele (N=36), significantly longer telomeres were detected than in those carrying the *C* allele but diagnosed with stages II–IV (N=17) (7.86 vs 4.48 kb, $p=0.010$) (Figure 5B).

It was also observed that among CLL patients over 60 years of age, those carrying the VNTR-234 allele (N=27) had longer telomeres than patients lacking this allele (N=17) (with an average telomere length of 9.01 vs 6.03 kb, $p=0.046$) (Figure 6). For the other SNPs studied, no significant associations with telomere length were observed.

## Discussion

Genetic variability within many genes can modulate telomere length and thus such genetic variants may constitute risk factors for the development of cancer and non-neoplastic diseases.[43,44] Several studies suggest that genetically determined longer telomere length in peripheral leukocytes, also related with the *TERT* gene variants, could be associated with an increased risk of CLL.[16] Also, a positive relationship between telomere length and multiple non-Hodgkin's lymphoma (NHL) subtypes, particularly for CLL/SLL (small lymphocytic lymphoma), was reported.[15]

The present retrospective study was conducted on a group of newly diagnosed untreated CLL patients. Assessment of telomere length, genetic factors related
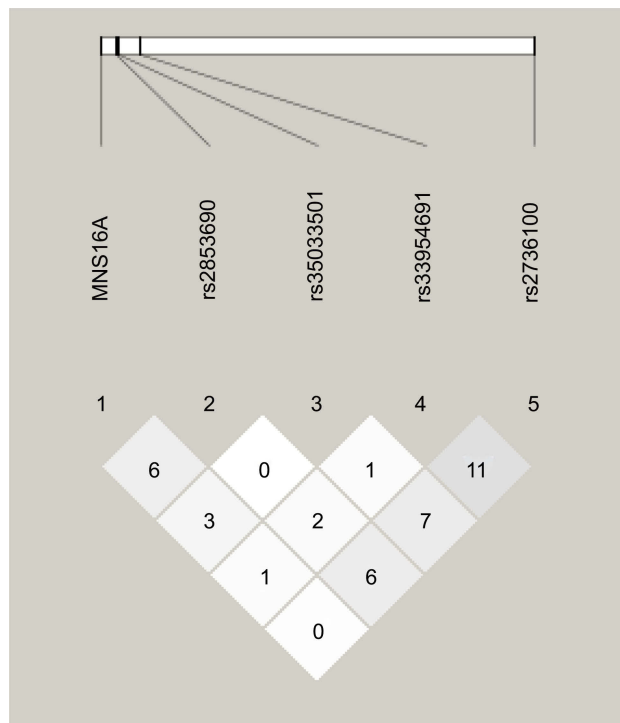
**Figure 4** Lack of LD between polymorphic variants under study. MNS16A VNTR is shown to the left, and *TERT* SNPs to the right. Darker color shows higher $r^2$ values, while the value shown inside the squares is $r^2 \times 10^2$. LD was considered medium for $r^2 > 20$ and strong for $r^2 > 80$. The graph was created using Haploview 4.2 software (http://vassarstats.net/tab2x2.html).
**Abbreviations:** LD, linkage disequilibrium; VNTR, variable number of tandem repeats; SNP, single-nucleotide polymorphism.

to *TERT* gene polymorphism, and identification of their relationships with susceptibility and clinical course of the disease were the key goals of this work. Therefore, four SNPs (rs2736100, rs2853690, rs33954691 and rs35033501) and the VNTR MNS16A gene polymorphism, all located within the *TERT* gene, were analyzed.

Results of a previous work by Ojha et al suggested some associations between CLL risk and the rs2736100 *C* allele that were not observed in the present study.[16] Comparison of rs2736100, rs2853690, rs33954691, rs35033501 and VNTR MNS16A genotypes and allele frequencies between our CLL patients and healthy individuals did not indicate significant differences, except for the rs35033501 minor *A* variant. This variant was more frequently detected in CLL patients than in healthy controls, and was associated with a greater than three-fold increase in risk for CLL development. The rs35033501 polymorphism is a synonymous substitution that does not affect the amino acid sequence. However, such silent variants may potentially cause changes of splicing patterns.

Many recent studies have focused on the role of various factors affecting splicing in patients with CLL. The study by Palma et al aimed to characterize hTERT splice variants in CLL cells, as well as to examine the expression of hTERT splice variants and telomere length in relation to disease activity and clinical stage. The authors described two splicing sites that generate shorter transcripts and one full-length transcript that is translated into a functional protein. They observed that all transcripts were more frequently expressed in progressive than non-progressive patients and showed that average full-length transcript expression was 5.5-fold higher in immunoglobulin heavy chain variable region (IGHV)-unmutated CLL patients than in IGHV-mutated patients.[44]

A mutation within the splicing factor *SF3B1* (splicing factor 3b subunit 1) gene in CLL was identified.[45] *SF3B1* encodes a protein involved in binding of the spliceosomal U2 snRNP to the branch point of the 3′ splicing site. Truncation of the protein by the introduction of premature stop codons is the most common outcome of splicing aberrations induced by *SF3B1* mutations, affecting 90% of cases. In CLL, *SF3B1* mutations are more frequent in later stages of the disease; they are associated with markers of poor clinical outcome and predict poor prognosis.[45]

In addition, genetic variability within other genes was reported to affect splicing in CLL patients. Puente et al identified novel recurrent mutations in non-coding regions, including the 39th region of notch receptor 1 (NOTCH1), which cause aberrant splicing events, increase NOTCH1 activity and result in a more aggressive disease.[46]

The aforementioned results show that various factors could affect splicing in patients with CLL, including genetic variability within non-coding regions also observed for the *TERT* rs35033501 SNP in the present study. Furthermore, some of these factors could be related to clinical parameters of the disease.

The polymorphic number of MNS16A tandem repeats was reported to be associated with the risk of several malignancies, including lung[24,47] or colorectal and prostate cancer,[38,39] malignant gliomas,[40,41] as well as Alzheimer's disease.[48] So far, the MNS16A VNTR polymorphism of the hTERT catalytic subunit has been described in two lymphoproliferative disorders: first in our study on NHL,[27] and later in diffuse large B-cell lymphoma (DLBCL).[49] Our previous work identified some relationships between the VNTR-243 variant with more aggressive disease and with less favorable response to therapy.[27] The study on DLBCL revealed that Egyptian carriers of the S allele or the SS genotype of MNS16A were at higher
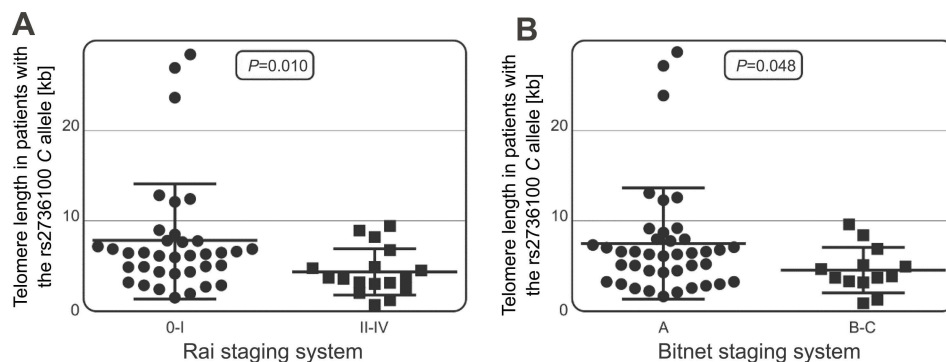
**Figure 5** Telomere length in patients with varying severity of the disease. In patients carrying the *TERT* rs2736100 *C* allele, longer telomeres were associated with less advanced disease according to the Rai staging system (**A**) and the Binet criteria (**B**).
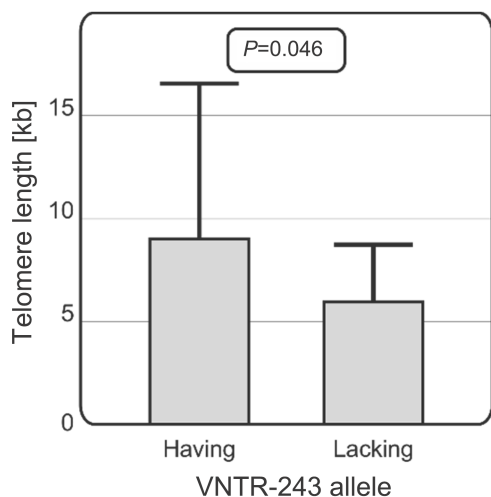


**Figure 6** Relationship between telomere length and the presence of VNTR alleles in older patients. Among CLL patients over 60 years of age, those carrying the VNTR-234 allele had longer telomeres than patients lacking this allele (with an average telomere length of 9.01 vs 6.03 kb, *p*=0.046).
**Abbreviations:** VNTR, variable number of tandem repeats; CLL, chronic lymphocytic leukemia.

risk of disease development.[49] Wang et al investigated promoter activity of MNS16A VNTRs in lung cancer cells and showed that the shorter VNTR (S) variant correlated with lower promoter activity, while the longer VNTR (L) was associated with increased risk of lung cancer.[24] Hofer et al investigated promoter activity of all six known MNS16A VNTRs in different cell lines and showed the distribution of relative promoter activities of different MNS16A VNTRs determined by luciferase reporter assays. In all investigated cell lines, promoter activity of shorter constructs (also VNTRs-234) was higher than promoter activity of longer constructs, reflecting an indirect correlation between VNTR length and promoter activity.[25] In addition, Zhang et al observed that carriers of the SL

genotype had lower *TERT* expression compared to LL carriers when analyzing nasopharyngeal carcinoma tissue by immunohistochemical staining.[26]

The current results suggest a lack of association between the MNS16A *TERT* genetic polymorphism and predisposition to CLL. However, there were some interesting observations in regard to the MNS16A VNTR-243 short allele, which was more frequently detected in patients with less advanced disease (Rai 0–I and Binet A) than in patients with Rai II–IV and Binet B or C. These results are consistent with previous data showing a correlation between the presence of short MNS16A genotypes or alleles and advanced age at diagnosis of patients with prostate cancer or nasopharyngeal carcinoma and breast cancer.[38,50] They imply that the presence of the MNS16A VNTR-243 short allele may affect the course of the disease. Indeed, in our previous study the presence of the MNS16A VNTR-243 short allele was found to play a role in progression and response to therapy of NHL patients.[27]

In various cancer scenarios, a dual role of telomere length has been observed. In general, in most patients who develop cancer the presence of long telomeres in tumor-initiating cells was detected. However, it is also well documented that critically short telomeres may lead to chromosomal instability, which can cause tumorigenesis. As for CLL, telomere dysfunction was observed in advanced stages of the disease, when the presence of critically short telomeres correlated with the occurrence of many genome rearrangements.[11,12,51–53] Moreover, several studies showed that early-stage CLL patients exhibited extensive telomere erosion and fusion, indicating that telomere shortening and dysfunction can precede clinical progression.[9,54] Also, no significant difference was observed between telomere length measured in tumor

cells and in healthy cells of these CLL patients (Binet stage A and Rai stage 0). The study by Hoxha et al indicated the association between telomere status and genomic instability in CLL and found a significant correlation between telomere shortening and DNA hypomethylation in an early phase of disease.[9] These results suggested that impairment of the telomere/telomerase system may represent an early event in CLL pathogenesis.[9]

We observed that CLL patients at a less advanced stage (Rai 0–I) at diagnosis had significantly longer telomeres than those with Rai stage II–IV, although their median telomere length equaled 5.71 kb and was shorter than median telomere length of 7.54 kb and 7.26 kb reported for healthy donors by Jebaraj et al[36] and Dos Santos et al,[37] respectively.

Moreover, significantly longer telomeres were observed among patients with less advanced disease (Rai 0–I or Binet A) possessing the *TERT* rs2736100 *C* allele, compared to patients with the *TERT* rs2736100 *C* allele, but exhibiting more advanced CLL stage (Rai III–IV or Binet B–C) (7.50 vs 4.55 kb and 7.86 vs 4.48 kb, for the Rai and Binet criteria, respectively). The observations above are in line with data from the Ojha et al study, which described the rs2736100 *C* allele as being associated with long leukocyte telomere length in a group of CLL patients.[16]

A common genetic variant in the *TERT* gene, rs2736100 *C/A*, is associated with both telomere length and risk in various diseases. This effect, however, was not directly visible in our CLL patients. A large meta-analysis performed by Snetselaar et al showed various effects of the *TERT* rs2736100 polymorphism in different disease association studies.[55] Cancer cases were more often characterized by the presence of the *TERT* rs2736100 *C* allele, while for non-malignant diseases positive associations of the *TERT* rs2736100 *A* allele with disease risk were observed.[55] Thus, the results of Snetselaar et al reflect a fundamentally different role of telomere biology in malignancies as opposed to non-malignant diseases, and illustrate the duality of telomere biology in different disease predisposition.[55] In addition, the strength of the effect of rs2736100 polymorphism may vary between populations, as shown, for example, for Swedish and Chinese males with myeloproliferative neoplasms by Dahlström et al.[56]

In conclusion, the current results focusing on SNPs and the MNS16A polymorphism of the *TERT* gene and telomere length in CLL patients may add new valuable information to the knowledge regarding potential significance

of telomere length/telomerase activity and expression in hematological malignancies. We showed that older CLL patients, who carried the MNS16A VNTR-243 short allele, were characterized by lower disease stage at diagnosis. That may be a result of a higher telomerase activity than that observed in patients with the long/long genotype and the long allele. Carriers of the long allele were previously shown to exhibit stronger *TERT* promoter activity and to carry the highest number of GATA-1 transcription factor binding sites, which leads to increased expression of antisense *TERT* mRNA and silencing of the sense telomerase transcript.

As SNPs may affect mRNA expression via splicing sites and/or miRNA binding site modifications, their detection within the *TERT* gene offers new prognostic opportunities. The current results also show an association between the rs35033501 *A* allele and disease susceptibility. Moreover, especially among patients possessing the *TERT* rs2736100 *C* allele, an association between telomere length and severity of the disease at diagnosis was observed.

Our results may help researchers to understand CLL development and identify new genetic biomarkers involved in this disease. The present study was particularly novel because it was the first to address the issue of *TERT* genetic variants (a VNTR and SNPs) in relation to telomere length in a hematological disorder. However, we are aware that our results should be further supported by functional tests of *TERT* expression in large groups of patients. Nevertheless, understanding the *TERT* polymorphism and expression in the context of CLL progression is an important step toward finding new ways to improve clinical care.

## Abbreviations

CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; hTERT, human telomerase reverse transcriptase; IGHV, immunoglobulin heavy chain variable; L, long VNTR allele; qPCR, quantitative polymerase chain reaction; S, short VNTR allele; SCR, single-copy reference; SNP, single-nucleotide polymorphism; VNTR, variable number of tandem repeats.

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## Author contributions

## Disclosure

The authors report no conflicts of interest in this work.

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# Supplementary material

**Table S1** Telomere length in cancer and leukemia cell lines, cell line characteristics and culture conditions

| Cell line (available from) | | Telomere length (kb) | Culture conditions |
|---|---|---|---|
| **A549** (Sigma-Aldrich)[a] | Caucasian lung adenocarcinoma | 3.38 | RPMI 1640 + HEPES medium and Opti-MEM medium (1:1; IIET, Wroclaw, Poland) with the addition of 5% FBS (HyClone; GE Healthcare, Amersham, UK), 2.0 mM L-glutamine and 1.0 mM sodium pyruvate (both Sigma-Aldrich, Steinheim, Germany) |
| **HT-29** (ATCC)[b] | Caucasian colon adenocarcinoma grade II | 2.76 | |
| **MV-4-11** (ATCC)[b] | Biphenotypic B myelomonocytic leukemia | 0.78 | RPMI 1640 medium with GlutaMAX (Gibco, Paisley, UK) with addition of 10% FBS (Sigma-Aldrich, Steinheim, Germany) and 1 mM sodium pyruvate (Sigma-Aldrich) |
| **KG1a** (DSMZ)[c] | Caucasian bone marrow acute myelogenous leukemia | 1.22 | |
| **HL-60** (ATCC)[b] | Caucasian promyelocytic leukemia | 0.47 | |
| **K562** (DSMZ)[c] | Caucasian chronic myelogenous leukemia | 0.97 | |
| **MDA MB-231** (ATCC)[b] | Caucasian breast cancer | 0.56 | RPMI + HEPES medium (PChO IITD PAN) with the addition of 10% FBS (Sigma-Aldrich, Steinheim, Germany) L-glutamine 2 mM (Sigma-Aldrich) |
| **Hs294T** (ATCC)[b] | Caucasian melanoma cell line | 1.22 | DMEM (Gibco, Paisley, UK) with 10% FBS (HyClone; GE Healthcare, Amersham, UK) and 2.0 mM L-glutamine (Sigma-Aldrich, Steinheim, Germany) |

**Notes:** [a]Sigma-Aldrich (Steinheim, Germany); [b]American Type Culture Collection (Rockville, MD, USA); [c]Leibniz-Institut DSMZ – German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). All culture media were supplemented with 100 units/mL penicillin (Polfa Tarchomin S.A., Warsaw, Poland) and 100 µg/mL streptomycin (Sigma-Aldrich, Steinheim, Germany).