Instytut Immunologii i Terapii Doświadczalnej im. Ludwika Hirszfelda,
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Zmienność genetyczna podjednostki katalitycznej telomerazy *TERT* w kontekście jej ekspresji i długości telomerów w modelu komórkowym *in vitro* oraz u pacjentów z chorobami nowotworowymi

Rozprawa doktorska

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Genetic variability of the catalytic subunit of telomerase (*TERT*) in the context of its expression and telomere length in an *in vitro* cell model and in cancer patients

Doctoral Thesis

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Składam serdeczne podziękowania

Prof. dr hab. Katarzynie Boguni-Kubik,
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okazane mi w trakcie realizacji pracy doktorskiej.

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którzy towarzyszyli mi na tym etapie mojej drogi naukowej.

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STRESZCZENIE

Telomeraza to polimeraza DNA zaangażowana w utrzymanie długości telomerów, proliferację komórek macierzystych i nowotworowych, apoptozę czy odnowę uszkodzonych tkanek. Komórki linii płciowej, krypty jelitowe, hepatocyty, komórki macierzyste, aktywowane limfocyty T i B oraz komórki zarodkowe charakteryzują się stałym wysokim poziomem aktywności telomerazy, natomiast nie identyfikuje się ekspresji tego enzymu w komórkach somatycznych. Cechą większości komórek nowotworowych jest aktywacja telomerazy, która umożliwia im osiągnięcie stanu nieograniczonej proliferacji. Telomeraza jest holoenzymem o właściwościach odwrotnej transkryptazy, której integralnym składnikiem jest matryca RNA (ang. *telomerase RNA component*; *TERC*), podjednostka katalityczna telomerazy (ang. *telomerase reverse transcriptase*; *TERT*) oraz kompleks sześciu białek nazywanych szelteryną (ang. *shelterin*). Podstawowym zadaniem telomerazy jest wydłużanie końców 3' chromosomów poprzez dodawanie powtarzającej się sekwencji sześciu nukleotydów $(5'\text{-TTAGGG-}3')_n$, które tworzą odcinki nazywane telomerami. Chromosomy pozbawione telomerów mogą łączyć się ze sobą w nieprawidłowy i niekontrolowany sposób, co w konsekwencji przekłada się na niestabilność genomową i pojawienie się zmian w kariotypie. Enzym ten pełni też szereg funkcji niekanonicznych, wśród których należy wymienić m.in.: kontrolę procesów metabolicznych, epigenetyczną regulację struktury chromatyny, udział w szlakach odpowiedzi na stres i szlakach sygnałowych. Chociaż wydaje się, że wzrost aktywności telomerazy nie jest niezbędny w inicjacji rozwoju nowotworu, pewnym jest jednak, że stymuluje on progresję i ekspansję komórek onkogennych poprzez utrzymanie długości telomerów powyżej krytycznej liczby powtórzeń, zapobiegając w ten sposób inicjacji procesów starzenia się lub śmierci.

Gen *TERT* zlokalizowany jest na krótszym ramieniu chromosomu 5 (5p15.33) i składa się z 15 intronów, 16 eksonów oraz rdzenia promotorowego o długości 260 pz. Do najczęściej występujących zmian genetycznych możemy zaliczyć mutacje somatyczne i zmiany związane z występowaniem polimorfizmów pojedynczych nukleotydów (ang. *single nucleotide polymorphisms*; SNPs). Wśród mutacji obszaru promotorowego *TERT* (*TERT*p) wyróżniamy dwie zlokalizowane w odległości -124 pz (*C228T*) i -146 pz (*C250T*) od miejsca startu transkrypcji. Występowanie tych wariantów skutkuje utworzeniem fragmentu nukleotydowego o długości 11 pz, który stanowi nowy motyw wiązania dla czynników transkrypcyjnych z rodziny ETS (ang. *E-twenty-six (ETS) transcription factors*).

Celem niniejszej rozprawy doktorskiej było: 1) oznaczenie i porównanie występowania mutacji somatycznych *TERT*p, aktywności telomerazy, długości telomerów i ekspresji *TERT* w liniach komórkowych reprezentujących nowotwory hematologiczne, guzy lite oraz linie prawidłowe hodowane *in vitro;* 2) określenie zależności pomiędzy wariantami genetycznymi *TERT* a długością telomerów w kontekście parametrów klinicznych u pacjentów z ostrą białaczką szpikową (ang. *acute myeloid leukaemia;* AML), przewlekłą białaczką limfocytową (ang. *chronic lymphocytic leukemia*; CLL) oraz rakiem gruczołu sutkowego; 3) zbadanie zależności pomiędzy zmiennością genetyczną *TERT* a ekspresją genów *MYC*, *SP1*, *TP53* w materiale z krwi kobiet z rakiem gruczołu piersiowego oraz w komórkach wyprowadzonych z tkanki nowotworowej – organoidach; 4) wykazanie roli zmienności genetycznej i ekspresji genu *TERT* oraz długości telomerów jako potencjalnych biomarkerów wybranych nowotworów hematologicznych i guzów litych.

Wyniki badań zostały przedstawione w czterech kolejnych publikacjach naukowych, w których poruszone zostały zagadnienia związane z regulacją poziomu ekspresji genu *TERT*, długości telomerów i aktywności telomerazy w modelu komórkowym *in vitro* i u pacjentów z chorobami hematologicznymi linii mieloidalnej i limfoidalnej, jak również u pacjentek z rakiem piersi oraz komórkach wyizolowanych z guzów raka gruczołu piersiowego - organoidach.

W pierwszej publikacji z wykorzystaniem panelu 27 linii komórkowych hodowanych *in vitro* przeanalizowano występowanie dwóch mutacji *TERT*p: *C228T* i *C250T*. Powyższe mutacje zidentyfikowano w 5 liniach komórkowych nowotworów litych (ang. *solid tumours*): glejaku, raku naskórka, czerniaku, raku pęcherza moczowego i raku gruczołu sutkowego. Linie komórkowe z obecną mutacją *TERT*p cechowały się krótszymi telomerami oraz liniowym wzrostem ekspresji *TERT* skorelowanym ze wzrostem aktywności telomerazy. Wykazano również, że hematologiczne linie komórkowe, w których nie stwierdzono występowania mutacji, charakteryzowały się najwyższą ekspresją *TERT* w porównaniu do linii guzów litych i linii prawidłowych, oraz występowaniem zależności pomiędzy ekspresją *TERT* a długością telomerów.

U pacjentów z chorobami hematologicznymi, ostrą białaczką szpikową (AML) i przewlekłą białaczką limfocytową (CLL) obserwowano krótsze telomery w porównaniu do grupy osób zdrowych. Chorzy z AML w wieku powyżej 61 lat charakteryzowali się dłuższymi telomerami w porównaniu do młodszych pacjentów.

Analizy całkowitego przeżycia (ang. *overall survival*) wykazały, że pacjenci z genotypem *CC TERT*p rs2853669 cechowali się krótszym przeżyciem niż pacjenci z allelem *T.* Chorzy z AML poniżej 61 r.ż., u których stwierdzono występowanie mutacji w genie kinazy tyrozynowej (*FLT3*) wyróżniali się krótszymi telomerami oraz gorszym przeżyciem całkowitym w porównaniu do pacjentów bez tej mutacji. Pacjenci z AML, u których wykazano obecność mutacji w genie kodującym nukleofosminę 1 (*NPM1*) i brak mutacji w *FLT3*-ITD mieli dłuższe telomery niż pacjenci z mutacją w *FLT3* i bez mutacji w *NPM1*.

W grupie pacjentów z CLL z mniej zaawansowanym stadium choroby (0–I wg. kryterium Rai) udokumentowano występowanie dłuższych telomerów niż w grupie chorych z zaawansowaną postacią choroby (II–IV). Ponadto, wśród pacjentów z CLL posiadających allel *C TERT* rs2736100 (intron 2) telomery były dłuższe w mniej zaawansowanym stadium CLL wg. kryterium Binet A oraz wg. Rai 0–I w porównaniu do chorych w stadium, odpowiednio Binet B-C oraz Rai II–IV.

W kolejnej pracy przeprowadzono badania dotyczące zmienności genetycznej *TERT*, długości telomerów i ekspresji panelu genów: *TERT*, *MYC*, *SP1*, *TP53* w grupie kobiet z nowotworem gruczołu piersiowego oraz w organoidach - materiale pochodzącym z hodowli *in vitro* komórek wyizolowanych z fragmentu guza gruczołu piersiowego.

Analiza wykazała występowanie korelacji pomiędzy ekspresją *TERT* i *TP53* oraz *SP1* i *MYC* w organoidach. Badana grupa pacjentek charakteryzowała się zależnością pomiędzy ekspresją genów *TERT* i *MYC* oraz *TP53* i *MYC.* Dalsza cześć badań dotyczyła zmienności genetycznej *TERT* w kontekście długości telomerów i parametrów klinicznych. Pacjentki z allelem *A TERT* rs10069690 (intron 4) oraz genotypem *GG TERT* rs2736100 miały dłuższe telomery niż kobiety z innymi wariatami genetycznymi. U chorych z allelem *T TERT* rs2736100 i *C TERTp* identyfikowano bardziej inwazyjne guzy (ustalone na podstawie badań histopatologicznych) niż u kobiet z genotypem *GG TERT* rs2736100 i *TT TERTp* rs2735940. Ponadto pacjentki z allelem *A TERT* rs10069690 miały rzadziej amplifikację genu receptora ludzkiego naskórkowego czynnika wzrostu - *HER2* (ang. *human epidermal growth factor receptor 2*). Analiza polimorfizmu zmiennej liczby powtórzeń tandemowych VNTR-MNS16A (ang. *variable number tandem repeats*) wykazała, że u kobiet z VNTR-234 występowały mniej inwazyjne nowotwory niż u chorych z pozostałymi genotypami MNS16A.

Cykl publikacji zamyka praca poglądowa na temat regulacji oraz roli genu *TERT* w rozwoju nowotworów.

Wyniki badań opisanych w powyższych publikacjach potwierdziły znaczenie zmienności polimorficznej genu *TERT* oraz długości telomerów w patogenezie przewlekłej białaczki limfocytowej, ostrej białaczki szpikowej i raka gruczołu sutkowego. Badania sugerują, że różnice w długości telomerów, obecność określonych mutacji i polimorfizmów w genie *TERT* oraz ekspresja *TERT* mogą być potencjalnymi biomarkerami w nowotworach układu krwiotwórczego i raku gruczołu sutkowego.

ABSTRACT

Telomerase is a DNA polymerase involved in the maintenance of telomere length, proliferation of stem and cancer cells, apoptosis and regeneration of damaged tissues. Germline cells, intestinal crypts, hepatocytes, stem cells, activated T and B lymphocytes, and germ cells have high levels of telomerase activity, but expression of this enzyme in somatic cells has not been identified. The activation of telomerase is a feature of most cancer cells, which enables them to reach a state of unrestricted proliferation. Telomerase is a holoenzyme with reverse transcriptase properties, an integral component of which is the RNA template (TERC), the telomerase reverse transcriptase catalytic subunit (TERT), and a complex of six proteins called shelterin. The primary task of telomerase is to extend the 3' ends of chromosomes by adding a repeated sequence of six nucleotides (5'-TTAGGG-3')n, which form segments called telomeres. Chromosomes lacking telomeres may assemble abnormally and uncontrollably, causing genomic instability and changes in the karyotype. Telomerase also performs a number of non-canonical functions, including: control of metabolic processes, epigenetic regulation of chromatin structure, participation in stress response pathways and signalling pathways. While an increase in telomerase activity does not appear to be essential for the initiation of cancer development, it is certain that it stimulates the progression and expansion of oncogenic cells by maintaining telomere lengths above a critical number of repeats, thus preventing the initiation of aging or death.

The *TERT* gene is located on the shorter arm of chromosome 5 (5p15.33) and consists of 15 introns, 16 exons and a 260 bp promoter core. The most common genetic variations include somatic mutations and changes associated with the occurrence of single nucleotide polymorphisms (SNPs). Among the mutations in the area of the *TERT* promoter (*TERT*p), we distinguish mutations located at -124 bp (*C228T*) and -146 bp (*C250T*) from the transcription start site. The occurrence of these variants results in the formation of an 11 bp nucleotide fragment, which is a new binding motif for E-twenty-six (ETS) transcription factors.

The aim of the doctoral dissertation was: 1) to determine and compare the presence of *TERT*p somatic mutations, telomerase activity, telomere length and *TERT* expression in cell lines representing haematological malignancies, solid tumours and normal lines cultured *in vitro*; 2) to determine the relationship between *TERT* genetic variants and telomere length in the context of clinical parameters in patients with acute myeloid leukaemia (AML), chronic lymphocytic leukaemia (CLL) and breast cancer; 3) to study the relationship between the genetic variation of *TERT* and the expression of *MYC*, *SP1*, *TP53* genes in the blood of women with breast cancer and in cells derived from cancer tissue - organoids; 4) to demonstrate the role of genetic variability and *TERT* gene expression as well as telomere length as potential biomarkers of selected haematological and solid tumours.

The results of the study were presented in four subsequent scientific publications, which addressed the issues related to the regulation of the *TERT* expression level, telomere length and telomerase activity in an *in vitro* cell model and in patients with haematological diseases of the myeloid and lymphoid linkage, women with breast cancer, and cells isolated from breast cancer tumours - organoids.

The first publication analysed the presence of two *TERT*p mutations, *C228T* and *C250T* using a panel of 27 *in vitro* cultured cell lines. These mutations have been identified in 5 solid tumour cell lines: glioblastoma, epidermal carcinoma, melanoma, bladder cancer and breast cancer. Cell lines with *TERT*p mutation were characterized by shorter telomeres, and *TERT* expression associated with an increase in telomerase activity. It was also shown that haematological cell lines without mutations were characterized by the highest expression of *TERT* compared to solid tumour lines and normal lines, and by an association between *TERT* expression and telomere length.

Patients with haematological diseases, acute myeloid leukaemia (AML) and chronic lymphocytic leukaemia (CLL), had shorter telomeres compared to healthy controls. AML patients over 61 years of age had longer telomeres compared to younger patients. Overall survival (OS) analyses showed that patients with the *CC TERT*p rs2853669 genotype had a shorter OS than patients with the T allele. AML patients under 61 years of age who had mutation in the tyrosine kinase gene (FLT3) had shorter telomeres and poorer OS compared to patients without this mutation. AML patients with mutation in the nucleophosmin gene 1 (*NPM1*) and no *FLT3*-ITD mutation had longer telomeres than patients with *FLT3*-ITD mutation and no *NPM1* mutation.

In the group of patients with CLL in a less advanced stage of the disease (0-I according to the Rai criterion), telomeres were noted to be longer than in the group of patients with the advanced form of the disease (II-IV). Additionally, among CLL patients with the *C* allele of *TER*T rs2736100 (intron 2), telomeres were longer in the less advanced stage of disease (according to Binet A and Rai 0–I), compared to patients in more advanced stages of Binet B-C and Rai II–IV.

In the last paper, the genetic variability of *TERT*, telomere length and the expression of a panel of genes: *TERT*, *MYC*, *SP1*, *TP53* in a group of women with breast cancer and in organoids (cells isolated from a fragment of a breast tumour) were investigated.

The analysis showed a correlation between the expression of *TERT* and *TP53,* as well as between *SP1* and *MYC* in organoids. The studied group of patients was characterized by a relationship between the expression of *TERT* and *MYC* genes, as well as *TP53* and *MYC* genes. The next part of the study concerned the genetic variability of *TERT* in the context of telomere length and clinical parameters of women with breast cancer. Patients with the *TERT* rs10069690 allele *A* (intron 4) and the *TERT* rs2736100 genotype *GG* had longer telomeres than women with other genetic variants. Patients with the *TERT* rs2736100 *T* and *TERT*p rs2735940 *C* allele, had more invasive tumours (determined by histopathology) than women with the *TERT* rs2736100 *GG* and *TERT*p rs2735940 *TT* genotypes. Additionally, patients with the *A TERT* rs10069690 allele had less frequent amplification of the human epidermal growth factor receptor 2 (HER2) gene. Analysis of the variable number of tandem repeats polymorphism VNTR-MNS16A showed that women with VNTR-234 had less invasive tumours than those with other MNS16A genotypes.

The series of publications ends with a review paper on the regulation and role of the *TERT* gene in the development of cancers.

The results of the studies described in the above publications confirmed the importance of polymorphic variability of the *TERT* gene and telomere length in the pathogenesis of chronic lymphocytic leukaemia, acute myeloid leukaemia and breast cancer. Studies suggest that differences in telomere length, the presence of specific mutations and SNPs in the *TERT* gene, as well as *TERT* expression may be potential biomarkers in haematological malignancies and breast cancer.

List of Publications

1. **Dratwa M**, Wysoczańska B, Turlej E, Anisiewicz A, Maciejewska M, Wietrzyk J, Bogunia-Kubik K. Heterogeneity of telomerase reverse transcriptase mutation and expression, telomerase activity and telomere length across human cancer cell lines cultured *in vitro*. Experimental Cell Research. 2020; 396(1):112298.
2. **Dratwa M**, Wysoczańska B, Butrym A, Łacina P, Mazur G, Bogunia-Kubik K. *TERT* genetic variability and telomere length as factors affecting survival and risk in acute myeloid leukaemia. Scientific Reports. 2021; 11(1):23301.
3. Wysoczańska B, **Dratwa M**, Gębura K, Mizgała J, Mazur G, Wróbel T, Bogunia-Kubik K. Variability within the human *TERT* gene, telomere length and predisposition to chronic lymphocytic leukemia. OncoTargets and Therapy. 2019; 12:4309–4320.
4. **Dratwa M**, Wysoczańska B, Brankiewicz W, Stachowicz-Suhs M, Wietrzyk J, Matkowski R, Ekiert M, Szelachowska J, Maciejczyk A, Szajewski M, Bagiński M, Bogunia-Kubik K. Relationship between Telomere Length, *TERT* Genetic Variability and *TERT*, *TP53*, *SP1*, *MYC* Gene Co-Expression in the Clinicopathological Profile of Breast Cancer. International Journal of Molecular Sciences. 2022; 23(9):5164.
5. **Dratwa M**, Wysoczańska B, Łacina, P, Kubik T, Bogunia-Kubik K. TERT-Regulation and Roles in Cancer Formation. Frontiers in Immunology. 2020; 11:589929.

Co-authors' Declarations

16.01.2023 r.

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Declaration

I hereby declare that my contribution to the following manuscript:

Marta Dratwa, Barbara Wysoczańska, Eliza Turlej, Artur Anisiewicz, Magdalena Maciejewska, Joanna Wietrzyk, Katarzyna Bogunia-Kubik. Heterogeneity of telomerase reverse transcriptase mutation and expression, telomerase activity and telomere length across human cancer cell lines cultured *in vitro*. *Experimental Cell Research*, 2020, 396(1):112298

is correctly characterized in the table below.

Contributor	Contribution [%]	Description of main tasks
Marta Dratwa	45%	conceptualization, methodology, performed experiments, formal analysis, writing - original draft, writing - review and editing
Barbara Wysoczańska	15%	conceptualization, methodology, supervision, formal analysis, writing - original draft, writing - review and editing
Eliza Turlej	10%	methodology, performed experiments, writing - review and editing
Artur Anisiewicz	5%	methodology, performed experiments, writing - review and editing
Magdalena Maciejewska	5%	methodology, performed experiments, writing - review and editing
Joanna Wietrzyk	5%	supervision, writing - review and editing
Katarzyna Bogunia-Kubik	15%	conceptualization, methodology, supervision, formal analysis, writing - original draft, writing - review and editing, project administration

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Declaration

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Contributor	Contribution [%]	Description of main tasks
Marta Dratwa	45%	conceptualization, methodology, performed experiments, formal analysis, writing - original draft, writing - review and editing
Barbara Wysoczańska	15%	conceptualization, methodology, supervision, formal analysis, writing - original draft, writing - review and editing
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Magdalena Maciejewska	5%	methodology, performed experiments, writing - review and editing
Joanna Wietrzyk	5%	supervision, writing - review and editing
Katarzyna Bogunia-Kubik	15%	conceptualization, methodology, supervision, formal analysis, writing - original draft, writing - review and editing, project administration

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Katarzyna Bogunia-Kubik	10%	conceptualization, methodology, formal analysis, resources, data curation, writing - original draft, writing - review and editing, supervision, project administration

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Oslo, 19.01.2023

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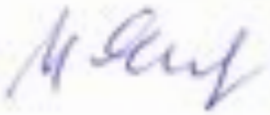

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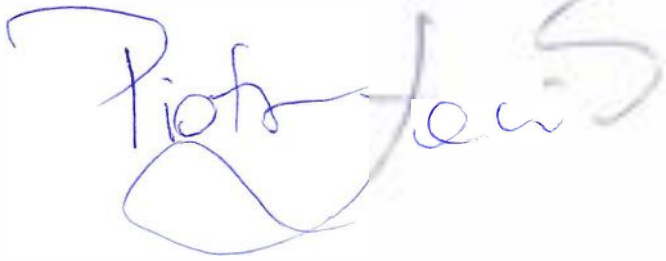

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Publications

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Research article

Heterogeneity of telomerase reverse transcriptase mutation and expression, telomerase activity and telomere length across human cancer cell lines cultured *in vitro*

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Telomere length
Neoplastic haematological and solid tumour human cell lines

ABSTRACT

Promoter region of the telomerase reverse transcriptase gene (*TERTp*) constitutes a regulatory element capable to affect *TERT* expression (TE), telomerase activity (TA) and telomere length (TL). *TERTp* mutation status, TL, TA and TE were assessed in 27 *in vitro* cultured human cell lines, including 11 solid tumour, 13 haematological and 3 normal cell lines. C228T and C250T *TERTp* mutations were detected in 5 solid tumour and none of haematological cell lines ($p = 0.0100$). As compared to other solid tumour cell lines, those with the presence of somatic mutations were characterized by: shorter TL, lower TA and TE. Furthermore, cell lines carrying *TERTp* mutations showed a linear correlation between TE and TA ($R = 0.9708$, $p = 0.0021$). Moreover, haematological cell lines exhibited higher TE compared to solid tumour cell lines ($p = 0.0007$). TL and TA were correlated in both solid tumour ($R = 0.4875$, $p = 0.0169$) and haematological ($R = 0.4719$, $p = 0.0095$) cell lines. Our results based on the *in vitro* model suggest that oncogenic processes may differ between solid tumours and haematological malignancies with regard to their *TERT* gene regulation mechanisms.

1. Introduction

Telomerase reverse transcriptase gene (*TERT*) encodes the telomerase catalytic subunit that is essential for maintenance of telomere length. *TERT*, which plays a crucial role in regulation of telomerase activity, is regulated by many different genes in response to a wide range of oncogenic and suppressive signalling pathways [1]. In various cancers, regulation of the complex *TERT* transcription process can be activated through a variety of mechanisms associated with changes at the genetic level, such as the presence of mutations, polymorphic variants, or variable number of tandem repeats of the *TERT* gene [2–4]. Wang et al. showed that MNS16A polymorphism, which is located downstream of *TERT*, has an effect on *TERT* expression and telomerase activity [5]. It was also reported that genetic variability within the *TERT* gene may affect telomere length and telomerase activity, thus affecting the risk, response to therapy, disease progression and survival in patients with various haematological and solid cancers [6–9].

Recent studies have demonstrated particular importance of transcriptional, post-transcriptional and post-translational *TERT* regulation, which may play a role in cancer development [1,10–12]. Two major somatic mutations located 124 and 146 bp upstream of the translation start site and referred to as C228T and C250T, respectively, were described in the promoter region of the *TERT* gene (*TERTp*) [13,14]. These hotspot mutations have been identified in over 50 distinct cancers and both of them may increase *TERTp* activity and *TERT* gene transcription [13,14].

TERTp mutations occur in the core promoter region (−124 C > T, C228T and −146 C > T, C250T) and functionally may create a new motif for transcription factors, e.g. E-twenty-six (ETS) family of transcription factors and GA binding protein (GABP) transcription factor, which have been linked to reactivation of epigenetically silenced *TERT* transcription [15]. These transcription factors may alter the ability to regulate *TERT* transcription in specific cell types and under various physiological conditions [13,15,16]. Also, these transcription factors could increase recruitment to the mutated site and build new chromatin interactions at the *TERTp* [17]. *TERTp* mutation rate varies significantly from

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undetectable to more than 90% among various human malignancies, and it remains poorly understood what causes such differential mutation distribution among different types of cancer [18–20]. *TERTp* mutations were found to be the most common mutations in tumours, and were detected in 83% of glioblastoma [21], 71% of melanoma [13,14], 66% of bladder cancer [22], and 47% of hepatocellular carcinoma patients [23]. Therefore, *TERTp* mutation status with its prognostic potential may be used as a clinical biomarker. It was documented that tumours bearing *TERTp* mutations may express higher levels of *TERT* mRNA and have increased telomerase activity, implying a stimulatory effect on *TERT* expression [18,24]. Experimental studies showed that *TERT* promoters with mutations are about twice as effective at driving *TERT* expression as the wild-type ones [25–27].

It is well established that dysfunctional telomeres trigger genomic instability [28,29] and contribute to pathogenesis of various human cancers, especially diseases originating from the haematopoietic system [30–32]. In haematological malignancies, shortening of telomere length is accelerated under increased proliferation pressure and telomeres are maintained at an extremely short length [33,34]. On the other hand, long telomeres suggest an early activation of *TERT* that may contribute to a delay in replicative senescence and prolonged time to acquire genetic alterations, critical for induction of a fully transformed phenotype [35]. Telomere length can play a different role in pathogenesis of melanoma, as it has been observed that short telomere length decreased risk of this malignancy [36,37]. On the other hand, Menin et al. noted that constitutive telomere length was significantly longer in familial melanoma cases, as compared to sporadic melanoma patients [38]. Results in breast cancer patients were inconclusive, as one study found increased risk of the disease to be associated with short telomeres [39], whereas another documented it to be associated with longer telomere length [40]. Bladder cancer and glioblastoma, tumours positive for the somatic *TERTp* mutation, have significantly shorter telomeres than tumours without those mutations [41,42]. Moreover, the presence of *TERTp* mutations have been associated with worse prognosis in melanoma patients [43].

In the present work, an *in vitro* human cell line model was applied to study the role of telomeres and telomerase in various cancers. Cell lines of both solid tumour and haematopoietic origin, representing the most common human cancers, were investigated. This approach allowed us to study the differences in two experimental groups indicating the relationship between *TERT* gene expression, telomerase activity, and telomere length in the presence of somatic mutations in the *TERTp* region.

2. Materials and methods

2.1. Culture of human cell lines

A panel of 27 established human cell lines cultured *in vitro*, including 11 solid tumours, 13 haematological and 3 normal cell lines was employed for evaluation of *TERTp* gene mutation status, *TERT* gene expression (TE), telomerase activity (TA) and telomere length (TL). The results are presented in Table 1.

All tested cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, Maryland, USA) and DSMZ (Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures) and maintained at the Cell Culture Collection of the Hirszfeld Institute of Immunology and Experimental Therapy (Wroclaw, Poland).

All cell lines used in the study were unstimulated and underwent 5 passages of culture. Human cancer cell lines were represented by: A-172, A-431, NCl–H358, A-549, HT-29, Hs294T, UM-UC-3, KU-19-19, MDA-MB-231, MCF-7, LoVo cell lines. Haematological cell lines included: MV-4-11, KG1a, HL-60, K562, THP-1, KOPN-8, SEM, RCH-ACV, Z-138, MOLM-13, REC-1, MAVER-1, and RS4; 11. In addition, 3 normal human cell lines: HaCaT, SC, MCF-10A, served as controls. Molecular

Table 1
Characteristics of human derived solid tumours, haematological, and normal cell lines investigated in the present study. The results of *TERT* expression telomerase activity and telomere length are given as mean values of 2, 3, and 3 measurements ± SD, respectively.

Cell lines symbols	Cell lines origin	*TERTp* Mutational status	*TERT* expression ($2^{-\Delta CT}$)	Telomerase Activity	Telomere length (kb)
Solid tumour cell lines (n = 11)					
A-172	Glioblastoma multiforme	C228T	0.0008 ± 0.0283	16.02 ± 0.04	0.69 ± 0.12
A-431	Epidermoid carcinoma	C250T	0.0003 ± 0.0566	10.91 ± 0.04	0.13 ± 0.02
NCl–H358	Bronchioalveolar carcinoma; non-small cell lung cancer	wild type	0.0006 ± 0.0283	0.05 ± 0.12	1.08 ± 0.52
A-549	Lung adenocarcinoma	wild type	0.0009 ± 0.0424	62.13 ± 0.01	3.38 ± 0.17
HT-29	Colon adenocarcinoma grade II	wild type	0.0004 ± 0.0011	2.49 ± 0.05	2.76 ± 0.15
Hs294T	Melanoma	C228T	0.0024 ± 0.0566	92.13 ± 0.11	1.22 ± 0.43
UM-UC-3	Bladder carcinoma	C228T	0.0006 ± 0.0424	20.83 ± 0.04	1.00 ± 0.32
KU-19-19	Urinary bladder transitional cell carcinoma	wild type	0.0015 ± 0.1202	107.23 ± 0.03	10.38 ± 0.25
MDA-MB −231	Breast adenocarcinoma	C228T	0.0004 ± 0.1202	11.25 ± 0.06	0.56 ± 0.17
MCF-7	Breast adenocarcinoma	wild type	0.0001 ± 0.0636	12.74 ± 0.04	0.27 ± 0.11
LoVo	Colorectal adenocarcinoma	wild type	0.0028 ± 0.0566	36.43 ± 0.01	0.82 ± 0.15
Haematological cell lines (n = 13)					
MV-4-11	Biphenotypic B myelomonocytic leukaemia (AML M5b)	wild type	0.0023 ± 0.0283	18.15 ± 0.02	0.78 ± 0.09
KG1a	Bone marrow acute myelogenous leukaemia (AML M0)	wild type	0.0061 ± 0.0212	0.65 ± 0.01	1.22 ± 0.43
HL-60	Promyelocytic leukaemia (AML M2/M3)	wild type	0.0020 ± 0.0011	2.42 ± 0.01	0.47 ± 0.06
K562	Chronic myelogenous leukaemia	wild type	0.0013 ± 0.0141	0.39 ± 0.03	0.97 ± 0.50
THP-1	Acute myeloid leukaemia (AML M4)	wild type	0.0056 ± 0.0071	0.99 ± 0.01	2.78 ± 0.30
KOPN-8	B cell precursor leukaemia	wild type	0.0428 ± 0.2758	153.49 ± 0.01	7.24 ± 1.20
SEM	B cell precursor leukaemia	wild type	0.0151 ± 0.9334	2.89 ± 0.01	0.55 ± 0.13
RCH-ACV	B cell precursor leukaemia	wild type	0.0516 ± 0.4031	75.43 ± 0.06	5.80 ± 0.31
Z-138	Mantle cell lymphoma	wild type	0.0090 ± 0.7495	85.75 ± 0.01	1.41 ± 0.57
MOLM-13	Acute myeloid leukaemia (AML M5a)	wild type	0.0019 ± 0.2121	31.93 ± 0.05	3.57 ± 0.62
REC-1	Mantle cell lymphoma; B cell non-Hodgkin's Lymphoma	wild type	0.0029 ± 0.0566	12.83 ± 0.08	0.37 ± 0.03
Maver-1	Mantle cell lymphoma	wild type	0.0032 ± 0.0919	73.62 ±0.02	0.55 ± 0.10
RS4; 11	B cell precursor leukaemia	wild type	0.0004 ± 0.0012	77.28 ± 0.04	2.01 ± 0.12
Normal cell lines (n = 3)					
HaCaT	Aneuploid immortal keratinocyte cell line	wild type	0.0006 ± 0.02828	2.29 ± 0.04	0.42 ± 0.21
SC	Human monocyte cell line	wild type	0.0004 ± 0.0071	1.08 ± 0.01	0.95 ± 0.17
MCF-10A	Normal human mammary epithelial cell line	wild type	0.00005 ± 0.25456	4.91 ± 0.05	0.83 ± 0.09

characteristics of the investigated cell lines are presented in supplementary Table S1. See also references [44–47] for additional information about biology of cell lines used in this study. All cell lines were maintained in media with specific supplements and incubated with 5% CO_2 at 37 °C (see cell line culture conditions in supplementary Table S2).

2.2. DNA extraction

Genomic DNA (gDNA) was isolated from 10^6 cells of cultured cell lines using the Qiagen DNA Isolation Kit (Qiagen, Hilden, Germany) following the recommendations of the manufacturer. DNA concentration and purity were quantified on DeNovix (DeNovix Inc., USA). Isolated DNA was used to assess and compare *TERTp* mutation status and telomere length in various human cell lines.

2.3. Mutation analysis

The presence of two somatic mutations (C228T and C250T) within the *TERTp* was analysed in gDNA samples isolated from 27 human cell lines cultured *in vitro*. *TERTp* mutations were detected using ddPCR™ Expert Design Assay: Unique Assay TERT C228T_113 (ID dHsaEXD72405942) and TERT C250T_113 (ID dHsaEXD46675715) according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, USA). Amplifications were carried out in 20 μl volumes containing 10 μl 2x ddPCR SuperMix for Probes (No dUTP) (Bio-Rad Laboratories, Hercules, USA), 0.25 μl (0.5 M, pH 8.0) EDTA (Thermo Fisher Scientific), 2 μl Betaine solution, 5 M PCR Reagent (Sigma Aldrich), 1 μl gDNA (concentration of 100 ng/μl), 1 μl (20x) primer/probe assay reagent and 6.75 μl deionized distilled water. The samples were transferred to an eight-channel DG8 cartridge and then placed in an Automated Droplet Generator (Bio-Rad Laboratories, Hercules, USA) for droplet generation. Afterwards, the droplets were transferred into a 96-well PCR plate and the plate was sealed with aluminium foil at 180 °C for 5 s using a Bio-Rad PX1 PCR Plate Sealer (Bio-Rad Laboratories, Hercules, USA). The amplification was performed in a T100™ Thermal Cycler (Bio-Rad Laboratories, Hercules, USA) with a ramp rate of 2 °C/s. The thermal cycling conditions were 95 °C for 10 min, followed by 50 cycles of: 96 °C for 30 s, 62 °C for 60 s and 98 °C for 10 min, and holding at 4 °C. After amplification, the sealed 96-well plate was read with QX200 Droplet Reader (Bio-Rad Laboratories, Hercules, USA) and data was analysed using QuantaSoft software version 1.7.4. Manual thresholds were applied for both *TERTp* mutations according to the manufacturer's instructions. Results are presented as a percentage of droplets positive for mutant and wild type *TERTp* DNA sequences (fractional abundance).

2.4. Quantification of telomere length

The average telomere length (TL) was measured in gDNA samples of 11 solid tumours, 13 haematological and 3 normal cell lines cultured *in vitro*. DNA samples were diluted with nuclease-free water to reach a concentration of 5 ng/μL. TL measurements were performed by employing real-time quantitative polymerase chain reaction (qPCR) in a LightCycler480 II Real-Time PCR system (Roche Diagnostics International, Rotkreuz, Switzerland) using qPCR 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 one 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. The qPCR conditions were as follows: 95 °C for 10 min followed by 32 cycles of: 95 °C for 20 s, 52 °C for 20 s and 72 °C for 45 s. Data analysis was conducted according to manufacturer's instructions. All reactions were run in three replicates. Quantitative results were expressed as mean ± 1.96 standard error of measurement (SEM). Results in the figures are presented as mean + 1.96SEM.

2.5. Quantification of telomerase activity

Relative telomerase activity of 27 human derived solid tumour, haematological and normal cell lines was assessed by qPCR in a LightCycler480 II Real-Time PCR system (Roche Diagnostics International, Rotkreuz, Switzerland) using SYBR® Green assay kits (ScienCell's Telomerase Activity Quantification qPCR Assay Kit [TAQ], Carlsbad, CA, USA) following the recommendations of the manufacturers.

Three million cells were harvested for each sample. For each experiment, two controls were used: a negative telomerase and positive cell lysates (Cat #8928e). PCR was performed in a final volume of 20 μL using 1 μL of post-telomerase reaction sample, 2 μL of TPS, 10 μL of 2 × qPCR FastStart Essential Green Master Mix (Roche Diagnostics International) and 7 μL of nuclease-free water. The PCR conditions were as follows: 95 °C for 10 min followed by 36 cycles of: 95 °C for 20 s, 52 °C for 20 s and 72 °C for 45 s. All reactions were run in three replicates. Data analysis was conducted according to manufacturer's instructions. Results in the figures are presented as mean + 1.96SEM.

2.6. Extraction of RNA, reverse transcription and TERT gene expression study

RNA of all cell lines was extracted from 1 × 10^6 cells resuspended in TRIzol Reagent® (Invitrogen™, Carlsbad, CA, USA) or RNA Extracol (EUR_X, Gdansk, Poland) according to the manufacturer's instructions.

1 μg/μl of isolated RNA was used for reverse transcription reaction. cDNA was synthesized using High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems™, Foster City, CA, USA) and 0.5 μl of RNase Inhibitor was added per sample (Applied Biosystems™, Foster City, CA, USA) to convert the extracted and purified RNA to cDNA. The conversion step was performed on a SimpliAmp™ Thermal Cycler (Applied Biosystems®, Foster City, CA, USA). After this step, the samples were stored in a freezer at −20 °C until further use.

Two genes were selected for evaluation of telomerase expression: *TERT* (Hs_00972,650_m1), and *GAPDH* (Hs02786624_g1), which was used as a housekeeping gene to normalize RNA expression data. The detection method was the TaqMan® Gene expression assay (Applied Biosystems Foster City, CA, USA) and qPCR was performed using LightCycler 480 II Real-Time PCR system (Roche Diagnostics International, Rotkreuz, Switzerland). For each PCR sample, the following protocol was used: 5 μl of cDNA, 1 μl (20x) each primer/probe, 10 μl (2x) TaqMan® Gene Expression Master Mix (Applied Biosystems™, Foster City, CA, USA), 4 μl of ultra-pure water. Amplification was performed under the following conditions: Initial denaturation for 10 min at 95 °C was followed by 40 cycles of denaturation for 15 s at 95 °C, and annealing for 1 min at 60 °C. Relative TERT gene expression levels were calculated using the $2^{-\Delta CT}$ method. Moreover, the fold change in gene expression was calculated by comparing values obtained in one group of cells to values from another group of cells $2^{-\Delta\Delta CT}$ [48]. Each sample was analysed in duplicate for the validation of the technique and the values of CT, according to the international standards for evaluation of gene expression by real-time PCR. Data in the figures are presented as mean + 1.96 SEM or median + confidence interval.

2.7. Statistical analysis

For each experiment, the data normality was verified with the Shapiro-Wilk test. Considering that distribution of some data deviated from the normal distribution, the non-parametric Mann-Whitney test was performed for comparison of the relative *TERT* expression in solid tumour, haematological and normal cell lines cultured *in vitro*. Remaining statistical analyses of differences between groups were carried out using one-way analysis of variance (ANOVA), post hoc

comparisons, and Bonferroni's T test (to determine significance of differences between the groups). The correlations were statistically evaluated using Pearson correlation (PC) tests. All statistical calculations were performed by GraphPad Prism software (GraphPad software, La Jolla, CA, version 8.0.1). Probability (p) values < 0.05 were considered statistically significant, while those between 0.05 and 0.10 as indicative of a trend.

3. Results

3.1. TERT promoter mutation status and characteristics of mutated cell lines

The presence of two somatic mutations, C228T (−124 bp) or C250T (−146 bp), within the *TERT* promoter region was analysed in haematological and solid tumour human cell lines cultured *in vitro* (Table 1).

Four types of haematological malignancies were distinguished among haematological cell lines: acute lymphoblastic leukaemia (ALL: KOPN-8, SEM, RCH-ACV, RS4; 11), mantle cell lymphoma (MCL: Z-138, Rec-1, Maver-1), acute myeloid leukaemia (AML: KG1a-M0, THP-1-M4, MV-4-11-M5b, MOLM-13-M5a, HL-60-M2/M3) and chronic myeloid leukaemia (CML: K-562).

Among solid tumour cell lines, 7 types of tumours were represented: bladder (UM-UC-3, KU-19-19), breast (MDA-MB-231, MCF-7), lung (NCl–H358, A-549) and colon cancers (LoVo, HT-29), melanoma (Hs294T), epidermoid carcinoma, (A-431) and glioblastoma (A-172).

The C228T and/or C250T somatic mutations in *TERTp* were detected only in 5 solid tumour cell lines (A-172, A-431, Hs294T, UM-UC-3 and MDA-MB-231), but no mutations were found in either of the haematological cell lines ($p = 0.0100$). The C228T (−124 bp) mutation was detected in 4 cell lines: A-172, Hs294T, UM-UC-3, MDA-MB-231 (glioma, melanoma, bladder cancer and breast cancer) while one C250T (−146 bp) mutation was observed in the A-431 (epidermal skin cancer) cell line.

Solid tumour cell lines characterized by the presence of somatic mutations (MUT+) had shorter average telomere length (0.72 ± 0.42 kb) and lower average telomerase activity (30.23 ± 34.84) compared to other solid tumour cell lines lacking these mutations (with telomere length and telomerase activity equalling 3.12 ± 3.75 kb and 36.85 ± 41.73, respectively). Moreover, *TERT* expression was 3-fold higher in *TERTp* mutation-positive cell lines than in normal cell lines. However, these differences did not reach statistical significance.

A linear correlation between *TERT* expression and telomerase activity was observed only in the cell lines carrying *TERTp* mutation ($R = 0.9708$, $p = 0.0021$) but not in cell lines negative for the presence of either C228T or C250T ($n = 6$, $R = 0.1773$, $p = 0.4057$). In contrast, a linear correlation between telomerase activity and telomere length was observed only in solid tumour cell lines without mutations ($n = 6$, $R = 0.7416$, $p = 0.0276$), but not in cell lines with mutations ($R = 0.5462$, $p = 0.1536$). Irrespective of *TERTp* mutation status, no correlation was found between *TERT* expression and telomere length (MUT+: $R = 0.5862$, $p = 0.1313$; MUT-: $R = 0.0200$, $p = 0.7892$). Additional correlations observed between TE, TA and TL in solid tumour cell lines and their subgroups with different C228T or C250T somatic mutation status are presented in supplementary Table S3.

3.2. TERT expression in haematological, solid tumour and normal cell lines

All haematological, solid tumour and normal cell lines cultured *in vitro* were analysed according to relative *TERT* expression level, telomerase activity and telomere length (Table 1).

Analysis of relative *TERT* expression showed significant differences between the haematological and solid tumour cell lines as well as between haematological and control cell lines. Haematological cell lines exhibited 11-fold higher relative *TERT* expression compared to solid tumour cell lines ($p = 0.0007$) and their mean expression was also 31-fold higher compared to normal cell lines ($p = 0.0071$) (Fig. 1). The 4-fold difference in expression level between solid tumour and control cell lines did not reach statistical significance ($p = 0.2418$).

3.3. Relations between TERT expression, telomerase activity and telomere length in solid tumour and haematological cell lines cultured in vitro

In the next step, we analysed the relationships between *TERT* expression, telomerase activity, and telomere length in both solid tumour and haematological cell lines.

A linear correlation between *TERT* expression and telomerase activity (Fig. 2A) was observed in both solid tumour cell lines ($R = 0.4010$, $p = 0.0365$) and in haematological cell lines ($R = 0.3648$, $p = 0.0288$). *TERT* expression was also found to be correlated with telomere length (Fig. 2B) but only in haematological cell lines ($R = 0.6829$, $p = 0.0005$). This relationship was not observed for solid tumour cell lines ($R = 0.032$, $p = 0.5934$). However, both solid tumour ($R = 0.4875$, $p = 0.0169$) and haematological ($R = 0.4719$, $p = 0.0095$) cell lines showed a linear correlation between telomerase activity and telomere length (Fig. 2C).

3.4. TERT expression, telomerase activity and telomere length in different subtypes of haematological cancer cell lines

TERT expression, telomerase activity, and telomere length were compared between cell lines representing various haematological malignancies (ALL, MCL, AML, CML). These four types of haematological cell lines were found to differ in the level of relative *TERT* expression (Fig. 3, right panel). As compared to the normal cells lines, the mean *TERT* expression was 115-times higher for ALL (KOPN-8, SEM, RCH-ACV, RS4; 11), 20 times higher for MCL (Z-138, Rec-1, Maver-1), 5 times higher for CML, and 15 times higher for AML (KG1a-M0, THP-1-M4, MV-4-11-M5b, MOLM-13-M5a, HL-60-M2/M3) cell lines. These relationships, however, did not reach statistical significance, except for the AML cell lines ($p = 0.0357$), as shown below (Fig. 3A, right panel).

Please note that the AML cell lines were selected to represent each stage of the disease (M0-M5) according to the French-American-British (FAB) classification. In general, *TERT* expression was higher in AML cell lines compared to normal cell lines ($p = 0.0357$, Fig. 3A, right panel). However, analysis of the subtypes of AML cell lines according to FAB criteria did not show significant differences regarding either *TERT* expression, or telomerase activity and telomere length.

In MCL (Z-138, Rec-1, Maver-1) cell lines, we observed shortened telomeres (0.78 ± 0.56 kb), higher telomerase activity (57.40 ± 39.07), and higher *TERT* expression (0.0050 ± 0.0034) as compared to normal cell lines (0.73 ± 0.28 kb; 2.76 ± 1.96; 0.0004 ± 0.0003 respectively, ns) (Fig. 3 B, C, right panel). Among MCL cell lines, the longest telomeres (1.41 ± 0.57 kb), highest telomerase activity (85.75 ± 0.007), and the

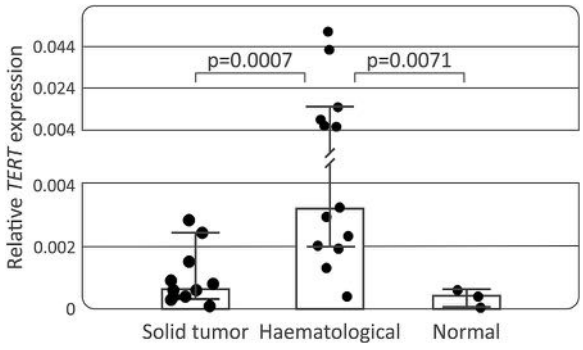

Fig. 1. Relative *TERT* expression in solid tumour, haematological and normal cell lines cultured *in vitro*. Bars in the figure reflect median expression levels. Individual points show expression of each analysed cell line. Mann-Whitney test was employed to assess significance of differences in *TERT* expression levels.

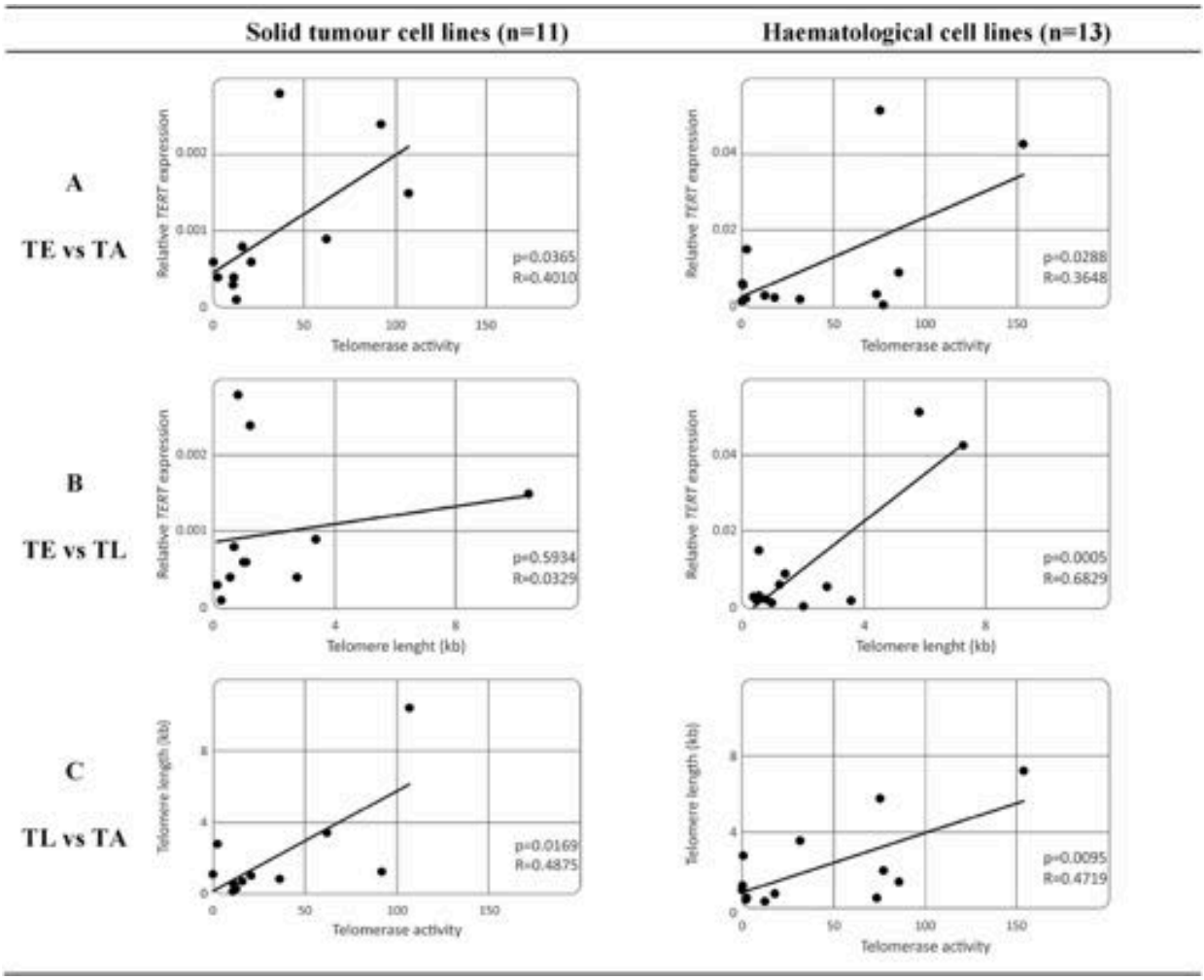

Fig. 2. Comparison of the relationships between relative TERT expression (TE), telomerase activity (TA) and telomere length (TL) in solid tumour (left panel, n = 11) and haematological (right panel, n = 13) cell lines cultured *in vitro*. Relations between TE and TA, TE and TL, and TL and TA are shown in rows marked as A, B, or C, respectively. Statistical analysis was performed using Pearson correlation (PC) tests.

highest *TERT* expression (0.009 ± 0.749) were all observed for the Z-138 cell line (Fig. 3 A, B, C, right panel).

Analysis of this group of haematological cancers did not show significant differences regarding either *TERT* expression, or telomerase activity and telomere length.

The following *TERT* expression pattern was observed when we compared *TERT* expression level in normal cell lines and AML cell lines: KG1a-M0 (25x higher), THP-1-M4 (23x higher), MV-4-11-M5b (9x higher), MOLM-13-M5a and HL-60-M2/M3 (8x higher). Interestingly, the MOLM-13-M5a cell line had the lowest level of *TERT* expression (0.002 ± 0.212), while it also had the longest telomere length (3.57 ± 0.620 kb) and the highest telomerase activity (31.93 ± 0.050). In contrast, the KG1a-M0 cell line had the highest relative *TERT* expression (0.006 ± 0.021), while its telomere length stayed at medium level (1.22 ± 0.433 kb) and telomerase activity was the lowest (0.65 ± 0.007).

3.5. *TERT expression, telomerase activity and telomere length in different subtypes of solid tumour cell lines*

Similar to the haematological cell lines, comparative analyses of *TERT* expression, telomerase activity, and telomere length were performed for the cell lines representing eight solid tumours (Fig. 3).

Solid tumour cell lines representing epithelial neoplasms, including bladder (UM-UC-3, KU-19-19), breast (MDA-MB-231, MCF-7), lung (NCl–H358, A-549), colon (LoVo, HT-29), skin epidermoid (A-431) as well as non-epithelial melanoma (Hs294T) and glioblastoma (A-172) cancers, presented with various levels of relative *TERT* expression (Fig. 3A, left panel). Among epithelial solid tumour cell lines, the highest *TERT* expression was detected in colon cell lines, especially in the LoVo cell line. The highest telomere activity and longest telomeres were observed for bladder cancer cell lines, with the highest values for the KU-19-19 cell line (Fig. 3B and C, left panel).

In this group of epithelial cancer cell lines, a linear correlation was observed between telomerase activity and telomere length (R = 0.9795, $p = 0.0013$). In the group of non-epithelial cancer cell lines, the highest expression of the *TERT* gene and the highest telomerase activity were detected in the melanoma cell line (Fig. 3A and B, left panel).

4. Discussion

In our present study, we compared the level of relative *TERT* gene expression, telomerase activity and telomere length in two independent groups of human cancer cell lines cultured *in vitro*, one derived from haematological cancers and the other from solid tumours. Furthermore, we analysed the presence of two somatic mutations, C228T and C250T, within the *TERT* promoter region in the aforementioned groups of cell lines.

TERT expression and telomerase activity can be upregulated *via* multiple molecular mechanisms including *TERTp* somatic mutations, *TERT* amplifications, *TERT* structural variants and epigenetic

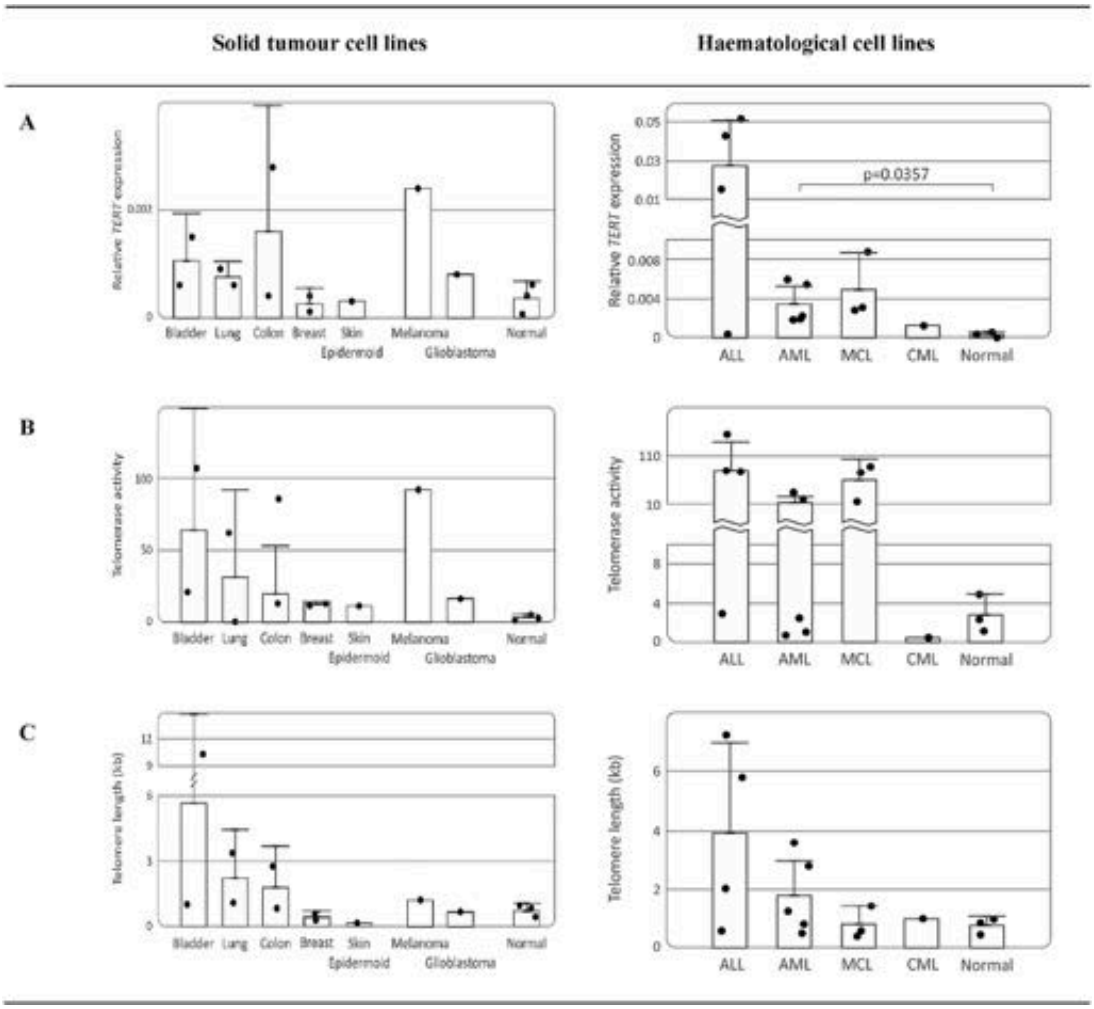

Fig. 3. Relative TERT expression (A), telomerase activity (B) and telomere length (C) in different subtypes of solid tumour (left panel) and haematological (right panel) cell lines cultured *in vitro*. Data are shown for 13 haematological cell lines: ALL - acute lymphocytic leukaemia (n = 4); MCL - mantle cell lymphoma (n = 3), AML - acute myeloid leukaemia (n = 5), CML - chronic myeloid leukaemia (n = 1). Among solid tumour cell lines 12 represent epithelial cell lines: bladder (n = 2), lung (n = 2), colon (n = 2), breast (n = 2), skin epidermoid (n = 1). In addition, melanoma (n = 1) and glioblastoma (n = 1) non-epithelial solid cancer lines, and normal cell lines (n = 3) are presented. Statistical analyses of differences between groups were carried out using one-way analysis of variance (ANOVA).

modifications [24,49]. *TERTp* mutation rate varies significantly among human malignancies, their histological types, and cancer cell lines origin. It has also already been shown that tumours carrying *TERTp* mutations frequently express higher levels of *TERT* mRNA and are characterized by increased telomerase activity as compared to wild-type ones [18–20]. *TERT* expression is regulated in tumours *via* multiple genetic and epigenetic changes known as Telomere Maintenance Mechanisms (TMMs). Two categories of TMMs can be distinguished: telomerase-mediated maintenance and alternative lengthening of telomeres (ALT). The majority of human cancers (80–90%) exhibit telomerase expression. Those which do not (~15% of cancers) may use ALT to maintain telomeres above a critical length. ALT is based on the homologous recombination (HR)-dependent replication exchange and the synthesis of telomeric templates [50].

In our present study, a linear correlation between *TERT* expression and telomerase activity was observed only in solid tumour cell lines with *TERTp* mutation but not in cell lines without mutation. However, it should be noted that we detected the presence of C228T/C250T mutations only in some of the solid tumour cell lines (glioma, melanoma, bladder cancer, breast and epidermal skin cancer), but not in others (lung, colon, breast), nor in any of the haematological cell lines (chronic or acute myeloid/lymphoid leukaemia, lymphoma). It is in line with clinical data documenting that some cancers (e.g. glioblastoma, malignant melanoma, urothelial bladder cancer) exhibit a high or intermediate *TERTp* mutation level (e.g. thyroid cancer, hepatocellular carcinoma, urinary carcinoma), whereas other tumours (e.g. lung, breast, gastrointestinal, prostate and kidney cancer) are characterized by a low mutation rate or lack of these mutations [20,21,24,26,51]. Our results regarding *TERTp* mutation in haematological cell lines are also consistent with data coming from patients with haematological diseases. Two research groups previously examined the presence of somatic mutations in patients with AML and non-Hodgkin's lymphoma, but they did not detect any of the *TERTp* hot-spot mutations [9,52]. Regarding haematological malignancies, only Panero et al. reported the presence of *TERTp* mutations in 33% patients with mantle cell lymphoma [53].

It is an interesting question why *TERTp* mutations consistently occur in some cancers, but not in others. One explanation comes from the study of Kiella et al. who documented that *TERTp* mutations usually occur in cancers with a low rate of self-renewal, e.g. brain tumours, liver, melanocytes and low-grade bladder cancers. *TERTp* mutations are also strongly implicated in triggering telomerase activation in these tumours [21]. This may explain why patients or haematological cell lines derived from myeloid and lymphoid origin (with a high proliferation index) do not show any presence of mutational changes within *TERTp,* but still are characterized with *TERTp* expression and telomerase activity. This may be due to the other mechanisms affecting telomerase activation in

haematological malignancies [26,49].

Analyses performed *in vivo* documented that in various tumours and cancer cell lines, the presence of telomerase with different levels of activity and *TERT* gene expression may play an important role in telomere lengthening [54,55]. Our results seem to confirm this, especially when solid tumour and haematological cell lines are separately compared. We observed that haematological cell lines exhibited significantly higher relative *TERT* mRNA expression levels as compared to solid tumour (11-fold) and normal cell lines (31-fold). Only in haematological, and not in solid tumour cell lines, was *TERT* expression correlated with telomere length. Results described by de Holanda et al. also showed higher *TERT* expression in haematological cell lines compared to solid tumour cell lines and normal cell lines [54]. Compared with the current work, their results are based on quite a narrow scope of analysis, limited only to the evaluation of *TERT* expression levels. We performed a deeper study and analysed the aforementioned relationships at the level of individual subtypes of solid tumour and haematological cell lines in terms of *TERT* expression, telomerase activity and telomere length. We paid close attention to the group of MCL cell lines (Z-138, Rec-1, Maver-1) due to their excessively shortened telomeres and at the same time high telomerase activity and *TERT* expression, which we observed in our study. Moreover, it should be noted that our MCL cell lines were not carrying any somatic mutations within their *TERT* promoter region. This is in contradiction to the results of Panero et al. who first demonstrated that acquired *TERTp* mutations are common in MCL patients [53]. Nevertheless, our present results are in line with observations of Panero et al. concerning the analysis of non-MCL types of haematological diseases. They did not find the presence of two hot spot mutations within the *TERT* promoter region in lymphoid neoplasms (including hairy cell leukaemia, follicular lymphoma, Waldenström macroglobulinemia, B-prolymphocytic leukaemia, plasma cell leukaemia, and T-cell lymphoma) other than MCL [53].

Our analysis of the subtypes of AML cell lines according to FAB criteria did not show significant differences regarding either *TERT* expression, or telomerase activity and telomere length. It has been well documented in clinical studies that shortened telomere length and increased telomerase activity may correlate with disease progression and relapse in patients with acute leukaemia [55]. Also, other genetic mutations, e.g. FLT3, and other mutations in signalling pathways have been associated with shorter telomere length in AML patients [56].

The selection of *TERTp* mutations at the transition from preneoplastic to malignancy suggests that telomere shortening acts as a critical barrier early in tumorigenesis of some cancers [49]. In our study, solid tumour cell lines harbouring *TERTp* mutations display lower telomerase activity compared to wild-type ones. It has also been proposed that the level of *TERT* is the limiting factor for telomerase activity [16]. Additionally, alternative splicing may play an important role in telomerase regulation. So far, ten different splice variants of *TERT* have been identified. Another important process of telomerase activation is posttranscriptional regulation. This regulation can occur *via* reversible phosphorylation of *TERT* at specific serine/threonine or tyrosine residues and may affect its structure, localization and enzyme activity [57]. In our analysis, solid tumour cell lines characterized by the presence of somatic mutations had shorter telomere length. Chiba et al. explain that the decreased telomere length in tumours with somatic *TERTp* mutations signifies delayed effect in stabilization of telomeres until after a generation of genomic instability [29].

In summary, by employing our simple cell line model, we wanted to highlight the significant role of *TERT* in different cancers. The principal aspect of our observations was the level of *TERT* expression, telomerase activity and telomere length in two groups of cell lines representing haematological cancers and solid tumours. We are aware that our research is based on a relatively small number of cell lines. Nevertheless, it allowed us to observe some differences between haematological and solid tumour cell lines, and to demonstrate the presence of telomerase activity and *TERT* expression in the absence of somatic mutations within the *TERTp* region.

5. Conclusions

It is important to understand how telomere length and telomerase activity are regulated in leukaemias, lymphomas, and in solid tumours *via TERT* expression. Our research based on the cell line model describes only a small fraction of possible interactions between *TERT* and other variables. Nevertheless, our findings contribute to a better understanding of telomere biology in the context of *TERT* mutations and expression. Focusing on selected parameters, such as *TERTp* mutation status, *TERT* expression, telomerase activity and length of telomeres, we were able to identify and describe some relationships present in haematological and solid neoplasms.

However, more extended studies are needed in order to more thoroughly understand the complex telomerase and *TERT* regulatory mechanisms, especially in haematological cancers lacking *TERTp* hot spot mutations. These mechanisms of the *TERT* gene remain a very attractive research subject in the context of anticancer therapy.

CRediT author contribution statement

Marta Dratwa: Conceptualization, Methodology, Performed experiments, have read and agreed to the published version of the manuscript, Formal analysis, Writing - original draft, Writing - review & editing. **Barbara Wysoczanska:** Conceptualization, Methodology, Supervision, Formal analysis, Writing - original draft, Writing - review & editing, have read and agreed to the published version of the manuscript. **Eliza Turlej:** Methodology, Performed experiments, Writing - review & editing, have read and agreed to the published version of the manuscript. **Artur Anisiewicz:** Methodology, Performed experiments, have read and agreed to the published version of the manuscript, Writing - review & editing. **Magdalena Maciejewska:** Methodology, Performed experiments, have read and agreed to the published version of the manuscript, Writing - review & editing. **Joanna Wietrzyk:** Supervision, Writing - review & editing, have read and agreed to the published version of the manuscript. **Katarzyna Bogunia-Kubik:** Conceptualization, Methodology, Supervision, Formal analysis, Writing - original draft, Writing - review & editing, Project administration, have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare no conflict of interest in this study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yexcr.2020.112298.

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TERT genetic variability and telomere length as factors affecting survival and risk in acute myeloid leukaemia

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Acute myeloid leukaemia (AML) is a neoplasm of immature myeloid cells characterized by various cytogenetic alterations. The present study showed that in addition to the *FLT3*-ITD and *NPM1* mutation status, telomere length (TL) and telomerase reverse transcriptase (*TERT*) gene polymorphisms may affect risk and overall survival (OS) in AML. TL was longer in healthy controls than in AML patients and positively correlated with age in the patients, but not in healthy subjects. TL was found to be independently affected by the presence of the *FLT3*-ITD mutation. As for the *TERT* gene polymorphism, AML patients with the *TERT* rs2853669 *CC* genotype were characterized by significantly shorter OS than patients carrying the *T* allele. Another observation in our study is the difference in TL and OS in patients belonging to various risk stratification groups related to the *FLT3*-ITD and *NPM1* mutation status. Patients with adverse risk classification (mutation in *FLT3*-ITD and lack of mutation in *NPM1*) presented with the shortest telomeres and significantly worse OS. In conclusion, OS of AML patients appears to be affected by *TERT* gene variability and TL in addition to other well-established factors such as age, WBC count, or *FLT3*-ITD and *NPM1* mutation status.

Acute myeloid leukaemia (AML) is a heterogeneous haematological malignancy, characterized by clonal expansion of abnormal immature leukaemic blasts[1–3]. Molecular changes in driver genes, polymorphic abnormalities and coexisting common mutational spectra occurring in AML are important prognostic and predictive markers in younger as well as in older AML patients[4–6]. Basic risk stratification and prognostic scoring of AML is based on the presence of mutation within nucleophosmin member 1 gene (*NPM1*) and/or signal transduction fms-like tyrosine kinase 3 (*FLT3*) gene, and groups patients into favourable, intermediate, and adverse risk categories[7]. There are many other gene mutations described in AML that are stratified according to the different functional pathways in which these genes are involved (e.g., oncogenes, transcription factors, tumour suppressors, epigenetic and chromatin modifying genes), most of which are important in diagnostics and modern therapy of AML patients[8].

The *FLT3*-internal tandem duplication (*FLT3*-ITD) is the most common genetic alteration and is identified in approximately 25% of AML patients[9,10]. It leads to proliferative activation by continuous phosphorylation of the FLT3 receptor, and at the same time suppresses apoptosis. In clinical practice, *FLT3*-ITD mutations are independent markers of poor prognosis in cytogenetically normal AML[10]. Moreover, they are associated with an aggressive disease phenotype and shorter overall survival[11]. Another important gene abnormality identified in both young and older AML patients is the *NPM1* mutation[12]. This mutation is found in almost one-third of newly diagnosed cases and leads to mislocalized NPM1 protein, found in the cytoplasm instead of the nucleolus[13]. In the absence of the *FLT3*-ITD alteration, *NPM1* mutation is associated with a favourable prognosis and possibility for complete remission of the disease in AML patients[14].

AML it the most common haematological neoplasm associated with short telomeres[15]. The occurrence of telomere shortening in leukaemias depends particularly on telomerase activity, telomerase reverse transcriptase catalytic subunit (*TERT*) expression, *TERT* promoter gene mutation (*TERT*p), and variability within the *TERT*

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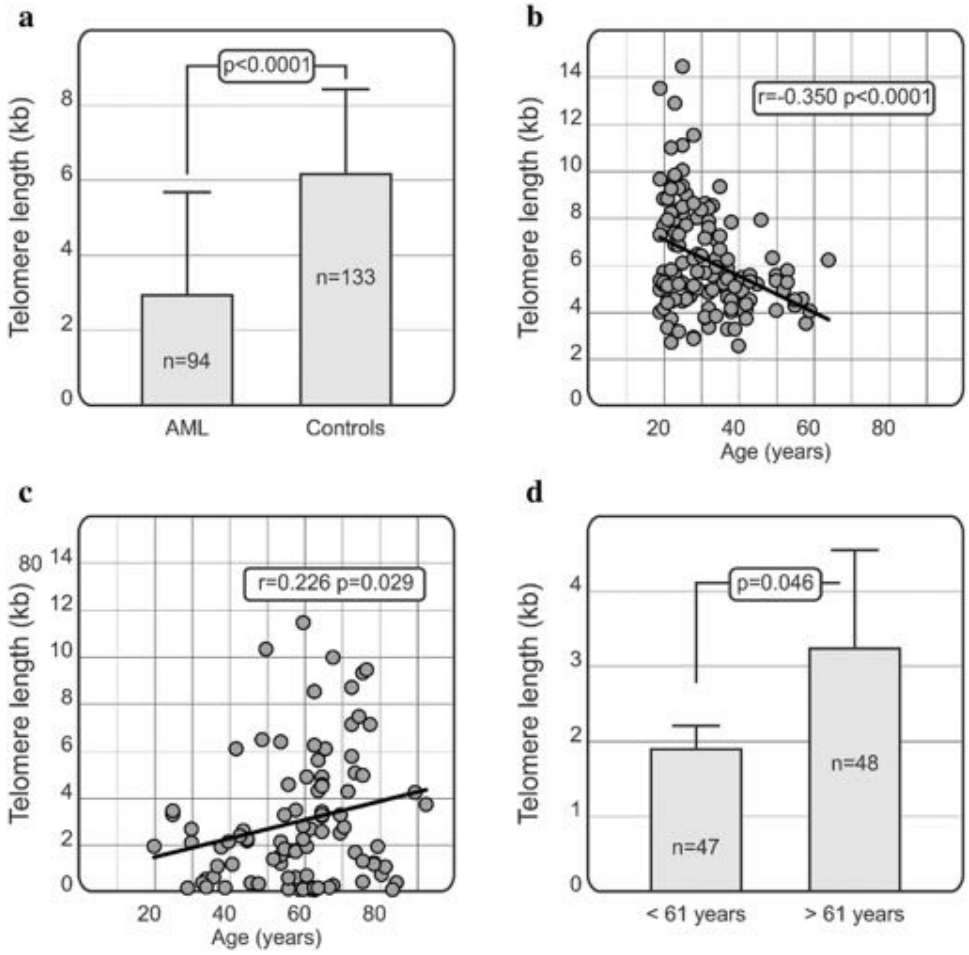

Figure 1. Comparison of telomere length in AML patients and controls (**a**) and relationships between age and telomere length in patients and controls. Telomere length correlates negatively with age in healthy controls (**b**) but not in AML patients (**c**). Statistical analysis was performed using Pearson correlation (PC) tests. Comparison of telomere length in AML patients below and above 61 years of age. Mann-Whitney U test was employed to assess the significance of differences in telomere length (**d**).

gene[16–19]. Reduction of telomerase activity and extremely short telomeres induce chromosomal instability, causing bone marrow failure. *TERT* overexpression is observed in 80–95% of malignant cells and this dysregulation in the cancer cells can be explained by factors that lead to modification e.g. in the *TERT* promotor structure[20].

A better understanding of the molecular mechanisms underlying AML led to development of drugs and new treatment strategies[21]. Standard clinical treatment of AML patients consists of high-intensity induction chemotherapy and/or haematopoietic stem cell transplantation[22]. However, many newly diagnosed AML patients do not qualify for intensive chemotherapy because of their age (> 75 years) or comorbidities[23,24]. Moreover, patients with *FLT3* mutations are characterized with a much worse response to chemotherapy. Nowadays, therapies using various FLT3 tyrosine kinase inhibitors are applied in AML patients with *FLT3* mutation[24]. Unfortunately, effective treatment of AML patients is challenging because of a very clonal heterogeneity of the disease and the occurrence of drug resistance.

In the present study we aimed to analyse AML patients in terms of the presence of *FLT3*-ITD and/or *NPM1* gene mutations, telomere length and genetic variability within catalytic subunit of telomerase (*TERT*) in younger and old AML patients with respect to the clinical data, including overall survival (OS).

Results

Telomere length in patients and controls. A significant difference ($p < 0.0001$) was observed between telomere length of healthy controls and AML patients (Fig. 1a), this was confirmed in an age-adjusted logistical regression analysis ($p < 0.0001$). In healthy controls, mean telomere length equalled 6.16 ± 2.27 kb (median: 5.56 kb, range from 2.58 kb to 14.43 kb) while in AML patients telomere length was shorter and equalled 2.94 ± 2.75 kb (median 2.09 kb, range from 0.02 kb to 11.48 kb). Moreover, telomere length declined with age in healthy subjects ($r = -0.350$, $p < 0.0001$), while it increased in AML cases ($r = 0.226$, $p = 0.029$) (Fig. 1b,c).

	AML patients (n = 91)	Healthy individuals (n = 133)
***TERT* rs2736100 (intron 2)**		
CC	21 (23.1%)	31 (23.3%)
AC	47 (51.6%)	58 (43.6%)
AA	23 (25.3%)	44 (33.1%)
***TERTp* rs2853669 (promoter)**		
TT	58 (63.7%)	81 (61%)
CT	24 (26.4%)	43 (32%)
CC	9 (9.9%)	9 (7%)

Table 1. Distribution of *TERT* genotypes in acute myeloid leukaemia (AML) patients and healthy individuals.

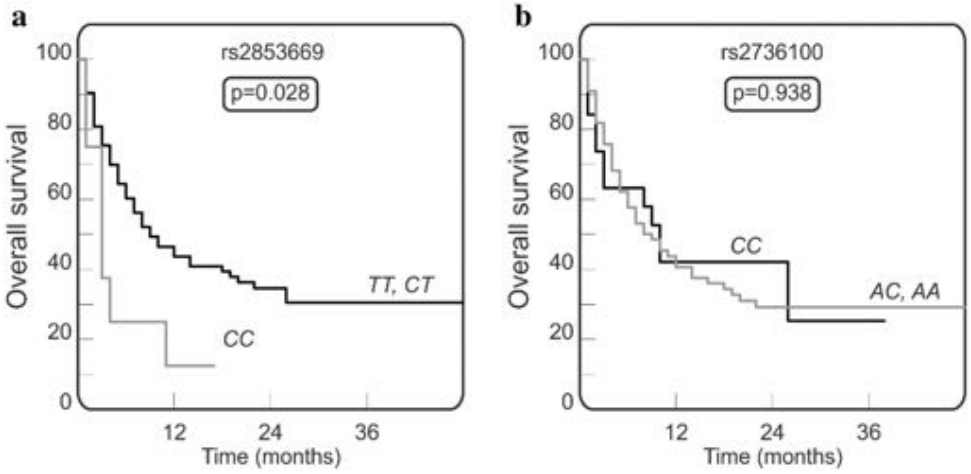

Figure 2. Overall survival in AML patients carrying various genotypes of *TERT* rs2853669 (**a**) and *TERT* rs2736100 (**b**). The homozygous rs2853669 *CC* genotype is associated with shorter overall survival (**a**). The Gehan-Breslow-Wilcoxon test was used for statistical analysis.

When the patients were subdivided with respect to the median age at diagnosis (61 years), it was observed that the older patients presented with longer telomeres as compared to younger patients (3.33 ± 2.84 vs 1.89 ± 3.73 kb, p = 0.046; Fig. 1d). There were 47 patients younger than 61 years and 48 older than 61 years.

Effect of the TERT gene polymorphism on overall survival. We did not observe any statistically significant differences in allele and genotype distribution between AML patients and healthy individuals for any of the *TERT* SNPs (rs2736100, rs2853669) studied (Table 1). Thus, in our AML patients, none of *TERT* (rs2736100, rs2853669) variants was found to affect the susceptibility of the disease.

Employing Kaplan-Meier curves, we compared the overall survival of AML patients carrying various *TERT* genotypes. Patients with rs2853669 *CC* homozygous genotype presented with a shorter overall survival than patients having the rs2853669 *T* allele (carriers of *TT* or *CT* genotypes; p = 0.028; Fig. 2a). The most favourable effect on survival was observed for the rs2853669 *CT* heterozygosity (p = 0.089; not shown). As for the second *TERT* SNP (rs2736100) investigated, no significant association between the presence of any of its genetic variants and overall survival was observed (p = 0.961; Fig. 2b), although there was a trend towards better OS in elderly patients over 61 years old carrying the rs2736100 *CC* genotype (p = 0.051).

***FLT3*-ITD and/or *NPM1* mutation status in relation to telomere length, overall survival and other clinical parameters.** As expected, analysis of the survival curves showed that younger patients (below median age of 61 years) lived longer than the older patients with a median overall survival of 12 and 5 months, respectively (p = 0.007).

Interestingly, some additional associations related to the presence of the unfavourable *FLT3*-ITD mutation were noted in the group of patients below 61 years of age. We observed, that among AML patients below 61 years, those carrying the *FLT3*-ITD mutation had significantly lower median telomere length 0.72 ± 0.81 kb (range from 0.02 to 2.19 kb) when compared to *FLT3*-ITD wild type cases with median telomere length of 2.07 ± 2.30 kb (range from 0.1 to 10.35 kb) (p = 0.003; Fig. 3a). Moreover, the presence of *FLT3*-ITD mutation in this group of AML patients was found to be associated with significantly worse overall survival (p = 0.038; Fig. 3b). On the other hand, no significant relationships were observed for *NPM1* mutation.

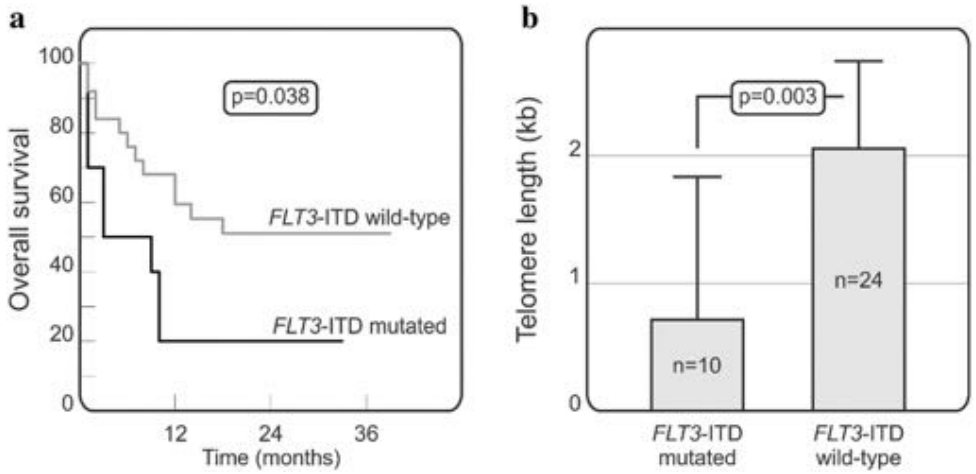

Figure 3. The effect of the presence of *FLT3*-ITD mutation on telomere length and survival in patients below 61 years of age. Kaplan-Meier curves for overall survival in patients stratified with respect to the presence of *FLT3*-ITD mutation. Gehan-Breslow-Wilcoxon test was used for statistical analysis (**a**). Differences in median telomere length in AML patients having or lacking *FLT3*-ITD mutation assessed by Mann-Whitney U test (**b**).

	HR (95% CI)	*p*-value
Age	1.054 (1.001–1.109)	0.0448
WBC	1.010 (1.004–1.014)	0.0002
FLT3-ITD, mutated vs wild-type	2.854 (0.826–9.866)	0.0975
NPM1, mutated vs wild-type	0.256 (0.055–1.193)	0.0827
Telomere length	0.709 (0.455–1.105)	0.1291
TERT rs2853669 (*CT* vs *CC* + *TT*)	0.324 (0.102–1.024)	0.0557

Table 2. Multivariate analysis of factors potentially affecting overall survival in patients with AML. *HR* Hazard ratio, *CI* confidence interval, *WBC* white blood cells count, *FLT3-ITD* internal tandem duplication of the *FLT3* gene, *NPM1* nucleophosmin member 1 gene.

Multivariate Cox regression analysis was employed to confirm independent associations of selected parameters with overall survival of AML patients. The following factors were considered: age, white blood cell (WBC) count, *FLT3*-ITD and *NPM1* mutation status (mutated vs wild-type), telomere length as well as *TERT* rs2853669 (*CT* vs *CC* + *TT*) polymorphism. This analysis demonstrated that variability within the *TERT* gene and mutation status may influence overall survival. The analysis documented that rs2853669 heterozygosity (p = 0.0557) and the presence of *NPM1* mutation (p = 0.0827) showed a positive association with overall survival, while higher WBC count (p = 0.0002) and more advanced age (p = 0.0448) showed an adverse effect (Table 2).

Additional multivariate Cox regression analysis including age, WBC count, *FLT3*-ITD and *NPM1* mutation status (mutated vs wild-type), as well as *TERT* rs2853669 (*CC* vs *CT* + *TT*) homozygosity showed that *CC* genotype was also associated with overall survival in AML patients (HR 8.066, p = 0.0230). As expected, the higher WBC count (HR 1.009, p < 0.0001) and advanced age (HR 1.053, p = 0.0352) showed a negative impact on overall survival. This analysis also confirmed that the presence of the *FLT3*-ITD (HR 3.518, p = 0.0410) and *NPM1* (HR 0.272, p = 0.0680) mutations influence overall survival in an opposite way.

To assess whether the *FLT3*-ITD and/or *NPM1* mutation status could act as an independent risk factor affecting telomere length in AML patients, a multiple linear regression model considering the presence of the *FLT3*-ITD and *NPM1* mutation and both SNPs was employed. It was found that the presence of *FLT3*-ITD significantly affected telomere length that it was shorter in the *FLT3*-ITD positive cases (p = 0.002), while patients positive for the *NPM1* mutations tended to have longer telomeres; p = 0.074). In this analysis, none of the two SNPs appeared to be an independent factor for telomere length.

As for the associations with other clinical parameters, we observed that patients positive for *FLT3*-ITD mutation showed increased lactate dehydrogenase (LDH) levels (with an average of 763.5 U/l; $p = 0.048$) and a tendency towards a higher WBC count (112.5×10^9/l; $p = 0.072$).

As the *FLT3*-ITD and *NPM1* mutations are included, among other parameters, in the 2017 European LeukemiaNet (ELN) criteria[7] of AML patients, we decided to check if telomere length and overall survival differ between patients in ELN risk groups (favourable, intermediate, adverse). As shown in Fig. 4a, AML patients with favourable risk classification characterized with longer telomeres as compared to AML patients with adverse risk. The mean telomere length equalled 4.08 ± 4.39 vs. 0.66 ± 0.94 kb, for patients with favourable and adverse risk respectively, p = 0.019; Fig. 4a). We also observed that patients with intermediate risk classification had longer telomeres as compared to the adverse group (2.15 ± 1.66 vs. 0.66 ± 0.94 kb, p = 0.013; Fig. 4a). Moreover, AML

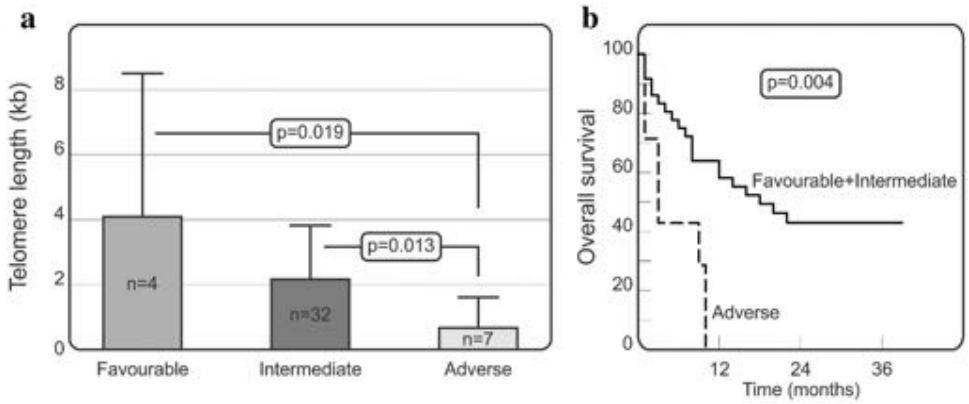

Figure 4. Telomere length and overall survival of patients in various ELN risk groups. Kruskal–Wallis test with the Original FDR method of Benjamini and Hochberg were employed to assess the significance of differences in mean telomere length in AML patients with favourable, intermediate, and adverse risk stratification (**a**). Gehan–Breslow–Wilcoxon test for statistical analysis of overall survival curves in AML patients was performed (**b**).

patients with adverse risk were characterized by worse overall survival as compared to patients of the other two risk groups ($p = 0.002$; Fig. 4b).

Discussion

Acute myeloid leukaemia arises from the expansion of haematopoietic stem and progenitor cells which acquire numerous somatic mutations[25]. Approximately half of all AML patients are characterized by normal karyotype and have losses or duplications in terminal regions of chromosomes which may affect telomere stability[26]. Moreover, about 40–50 genes were found to harbour recurrent somatic mutations in various AML subtypes[25]. We hypothesised, that telomere length or polymorphisms within the *TERT* gene may also be related to disease risk, and survival.

In the present study we assessed telomere length in healthy blood donors and AML patients. We observed that AML patients were characterized by significantly shorter telomeres than healthy subjects. Similar relationships were shown by Aalbers et al. in the group of children with AML. They observed that telomere length in leukaemic cells was very short as compared to healthy control peripheral blood mononuclear cells[16]. Ventura Ferreira et al. also demonstrated that AML patients were characterized by significantly shorter telomeres than healthy controls[27]. Moreover, in our present study we have also noticed that telomere length decreases with age in healthy controls, but not in AML cases. Previously published data show inconsistent results. For example, a study by Menshawy et al. demonstrated a lack of correlation between age and telomere length in patients with AML[28]. On the contrary, Williams et al. reported a correlation between telomere length and age of diagnosis in AML patients[29]. Interestingly, both studies, our present one and that of Williams et al., report differences in telomere length between patients at different ages. We divided our AML patients into two groups, below and above 61 years of age (reflecting the median age in our group of patients). Our analysis showed that younger patients did have shorter telomere length as compared to older patients. Similar results were reported by Williams et al., who observed that younger AML patients (< 60 years old) had significantly shorter telomeres than patients at a more advanced age[29].

In several studies, genetic variability within the *TERT* gene was analyzed in order to look for association with predisposition to the disease, its progression/outcome, or survival. We analyzed two *TERT* SNPs, one in the *TERT* promoter region (rs2853669, C/T) and the other located in intron 2 (rs2736100, C/A). We did observe some associations between *TERT* polymorphism and overall survival, although no significant relationship with the risk for the development of AML was found. In our study, patients carrying homozygous rs2853669 *CC* genotype were characterized with shorter overall survival than patients with *T* allele while the *CT* heterozygosity seemed to play more favourable role (see Fig. 3). Our results are consistent with those of Mosrati et al. in Swedish patients with AML. Additionally, Mosrati et al. found an interesting association between *TERT* rs2853669 *CC* homozygosity and increased expression of IL-6 and TNFα, cytokines known as markers for inflammation and cancer progression[19]. Furthermore, they reported that the rs2736100 SNP generated a modest risk for AML, although it had no effect on survival in their AML cohort. The latter observation confirms our results on the lack of association between rs2736100 and survival. On the other hand, it has been reported that in Chinese population, rs2736100 is associated with increased susceptibility to non-small cell lung cancer and myeloproliferative neoplasm[30–32] as well as AML risk[33]. Tong et al. observed a higher frequency of the *CC* genotype and *C* allele of rs2736100 polymorphism in the Chinese AML patients. However, no significant differences were detected in either genotype or allele distributions between patients and control groups regarding the second SNP (rs2853669)[33]. The above observations may suggest that the effect of *TERT* SNPs may vary between patients from different populations and may be dependent on the broader genetic background of examined populations.

AML is the most common haematologic neoplasm that is associated with short telomeres. Watts et al. suggested that shorter telomere length leads to disconnection of proteins [e.g., telomeric repeat-binding factor 2 (TRF2)] from telomere complex. This biological process may be correlated with loss of major non-telomeric

functions such as DNA damage and activation of important repair pathways. Therefore, it seems that telomere length could confer resistance to cytotoxic chemotherapy by affecting DNA repair mechanisms[34].

Mutations in *FLT3*-ITD and *NPM1* genes are frequently identified in AML, especially in patients with de novo AML and normal karyotype. Both of these genes are involved in important cellular processes, such as differentiation and apoptosis of haematopoietic progenitor cells. The occurrence of mutation in the *NPM1* gene may be beneficial for the health and survival of patients[2]. On the other hand, excessive proliferation and survival of *FLT3*-ITD mutant cell clones have adverse effects for AML patients and is associated with poor prognosis[35]. In our present study, we found some differences between patients having and lacking the *FLT3*-ITD mutation in terms of telomere length and overall survival. Similar relationships were observed also in some previous studies[16,29,34]. Moreover, Molina Garay et al. demonstrated that patients with the *FLT3*-ITD mutation characterized with significantly shorter lifespan as compared to patients lacking the *FLT3*-ITD mutation or with a mutation in the tyrosine kinase domain (*FLT3*-TKD)[36].

As for the *NPM1* mutation, no association between *NPM1* mutation status and telomere length was found either in our present work or in previous studies[16,29]. However, in our logistic regression model, *NPM1* mutation showed a slight trend towards association with survival.

The novelty of our study is the observation regarding the differences between telomere length in patients belonging to various risk stratification groups (according to the 2017 European LeukemiaNet criteria by Döhner et al.[7]). AML patients carrying *NPM1*, but not *FLT3*-ITD mutation (favourable risk group) were characterized by longer telomeres as compared to AML patients of the adverse risk group (with *FLT3*-ITD mutation and *NPM1* wild-type). Moreover, we observed that patients of the intermediate risk group (*FLT3*-ITD mutation and *NPM1* mutation or *FLT3*-ITD wild-type and *NPM1* wild-type) similarly had significantly longer telomeres in comparison to the adverse risk group of patients. Our AML patients from the adverse risk group were characterized by worse overall survival as compared to patients of the other two risk groups.

Logistic regression analysis showed that WBC count is a strong independent risk factor for survival and the presence of *FLT3*-ITD mutation was associated with higher WBC counts in our AML patients. The latter relationship between WBC count at diagnosis and *FLT3*-ITD mutation status was also reported in a meta-analysis published by Picharski et al.[37].

In summary, outcomes of patients with the *FLT3*-ITD mutation were significantly worse than of those without this mutation, and higher WBC count was associated with poor prognosis, probably because of the presence of *FLT3* mutation that is likely associated with higher WBC count. We also observed a correlation between LDH levels and *FLT3*-ITD mutation status. Patients positive for *FLT3*-ITD mutation showed higher levels of LDH, suggesting an unfavourable role of LDH in AML patients. In line with this observation, the study of Djunic et al. showed that serum LDH level was the most significant predictor of poor complete remission ratio in AML patients[38].

In conclusion, overall survival of AML patients depends on various factors such as age, telomere length, mutation status, and *TERT* variability. The presence of *FLT3*-ITD and *NPM1* mutations is used for estimating survival and response to treatment, although new prognostic genetic factors could be used to construct a more detailed risk stratification system. Adverse risk patients (positive for *FLT3*-ITD but negative for *NPM1* mutation) need novel approaches to improve their overall survival and to get a better response to treatment. To clearly demonstrate the role and significance of telomere length in AML patients with normal karyotype, a clinical study involving a larger number of patients may be needed. It seems necessary to explore the new genetic and environmental factors that could be involved in leukaemogenesis.

Methods

Characteristics of the study groups. The study involved 95 Polish patients diagnosed with de novo AML with normal karyotype (57 males and 38 females, age range 20–93, median 61 years). Blood samples were collected at diagnosis after obtaining informed consent from patients. All methods were according to the Declaration of Helsinki. Approval of the Bioethical Committee of Wroclaw Medical University was obtained for the study (No. KB-368/2019). WBC count range was $0.7–510.5 \times 10^9$/l (median = 21.5×10^9/l). Risk stratification groups included 11% patients with favourable risk, 49% patients with intermediate risk and 40% patients with adverse risk according to 2017 European LeukemiaNet criteria[7]. There were 11 AML patients with *FLT3*-ITD mutation and 34 without it (n = 45). Additionally, there were 8 patients with *NPM1* mutation and remaining 35 without it (n = 43). The median overall survival was 9 months (range 1–122 months). Additionally, 133 blood donors (84 males and 49 females, age range 19–64, median 30 years) served as a control group for the *TERT* polymorphisms and telomere length studies. Due to difference between patients and controls, an age adjusted logistic regression analysis was additionally performed.

DNA extraction. Genomic DNA was isolated from 10 mL of peripheral blood taken on EDTA using the Qiagen DNA Isolation Kit (Qiagen, Hilden, Germany) following the recommendation of the manufacturer. DNA concentration and purity were quantified on DeNovix (DeNovix Inc., USA). Isolated DNA was used to for *TERT* genotyping and assessment of the telomere length in AML patients.

Genotyping of *TERT* gene polymorphisms. The selection of investigated single nucleotide polymorphisms (SNPs) within the *TERT* gene was based on results from the SNP Function Prediction tool of the National Institute of Environmental Health Sciences (NCBI Database) 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 Caucasians above 10%, change in RNA and/or amino acid chain, potential splicing site and/or miRNA binding site.

Based on the above criteria, 2 SNPs were selected for the study. *TERT* rs2736100 (G > T), located in intron 2, is a susceptibility factor for a variety of cancers and myeloproliferative neoplasms. The *TERT* rs2853669 (T > C) SNP located at − 245 bp (Ets2 binding site) in the promoter region, suppresses *TERT* expression and is associated with the enzymatic activity of telomerase. The *TERT* rs2736100 SNP was determined with the use of LightSNiP typing assays (TIB MOLBIOL, Berlin, Germany) while a TaqMan assay was employed for rs2853669 SNP genotyping (ThermoFisher Scientific, USA). Both assays are based on real-time polymerase chain reactions (PCR). Amplifications were performed on a LightCycler480 II Real-Time PCR system (Roche Diagnostics International AG, Rotkreuz, Switzerland) according to the recommendations of the manufacturer. The PCR conditions were as follows: 95 °C for 10 min followed by 45 cycles of: 95 °C for 10 s, 60 °C for 10 s and 72 °C for 15 s. PCR was followed by one cycle of: 95 °C for 30 s, 40 °C for 2 min, and gradual melting from 75 °C to 40 °C.

Quantification of telomere length. The average telomere length was measured in genomic DNA samples of 91 AML patients and 133 controls. DNA samples were diluted with nuclease-free water to reach a concentration of 5 ng/mL. Telomere length measurements were performed on a LightCycler480 II Real-Time PCR system (Roche Diagnostics International, Rotkreuz, Switzerland) using quantitative polymerase chain reaction (qPCR) assay kits (ScienCell's Absolute Human Telomere Length Quantification qPCR Assay Kit [AHTLQ], Carlsbad, CA, USA), as previously described[39]. The PCR conditions were as follows: 95 °C for 10 min followed by 32 cycles of: 95 °C for 20 s, 52 °C for 20 s and 72 °C for 45 s. Data analysis was conducted according to manufacturer's instruction. All reactions were run in three replicates.

Statistical analysis. The null hypothesis that there is no difference between allele and genotype frequencies between patients and controls was tested with the Fisher's exact test, calculated using the web-based tool http://vassarstats.net/tab2x2.htm. Survival was assessed using the Gehan-Breslow-Wilcoxon test and Kaplan-Meier survival curves. The remaining statistical analyses of differences between groups were performed using one-way analysis of variance (ANOVA; to determine the significance of differences between the groups), and the resulting p-values were FDR-adjusted using the Benjamini and Hochberg method. For each experiment, data normality was verified with the Shapiro–Wilk test. Considering that distribution of some data deviated from normal distribution, the non-parametric *U* Mann-Whitney test was performed for comparison of telomere length. Statistical calculations were performed by GraphPad Prism software (GraphPad Software, La Jolla, CA, version 8.0.1) and Real Statistics Resource Pack for Microsoft Excel 2019 (version 16.0.10369.20032, Microsoft Corporation, Redmont, Washington, USA). RStudio (RStudio, PBC, Boston, Massachusetts, USA) was used for multiple linear regression model analyses and logistic regression model (Cox regression model) analyses. The r value for the correlation was determined using Pearson correlation (PC) tests. Probability (p) values < 0.05 were considered statistically significant, while those between 0.05 and 0.10 as indicative of a trend. Data in the figures are presented as mean + Standard Deviation (SD) or median + 95% Confidence Interval (CI).

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Competing interests

The authors declare no competing interests.

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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]

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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

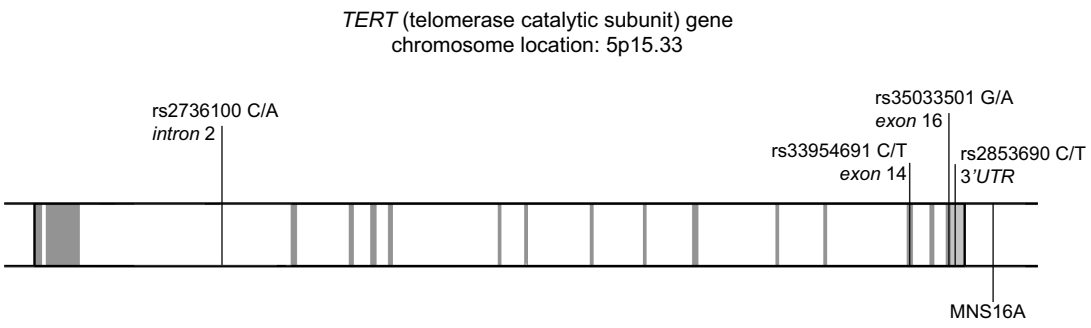

Figure 1 Genomic structure of the human telomerase gene. Exons are marked in gray while intronic regions are in white.

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://vassarstats.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

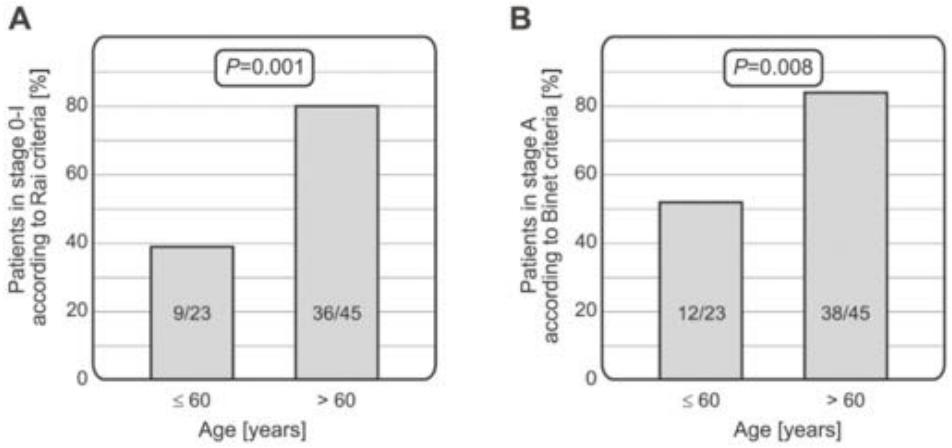

Figure 2 Relationship between age of patients and CLL stage at diagnosis. Patients older than 60 years had lower stage of the disease at diagnosis than those aged 60 or younger, using both the Rai (**A**) and Binet (**B**) staging systems.
Abbreviation: CLL, chronic lymphocytic leukemia.

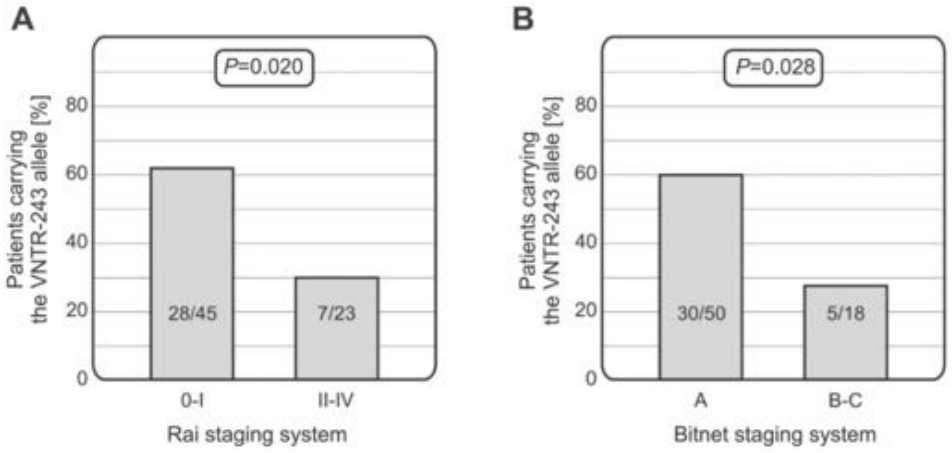

Figure 3 Relationships between the *TERT* VNTR polymorphism and CLL stage at diagnosis. Patients carrying the MNS16A-243 allele were characterized with lower stage of the disease according to both the Rai (**A**) and Binet (**B**) criteria.
Abbreviations: VNTR, variable number of tandem repeats; CLL,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

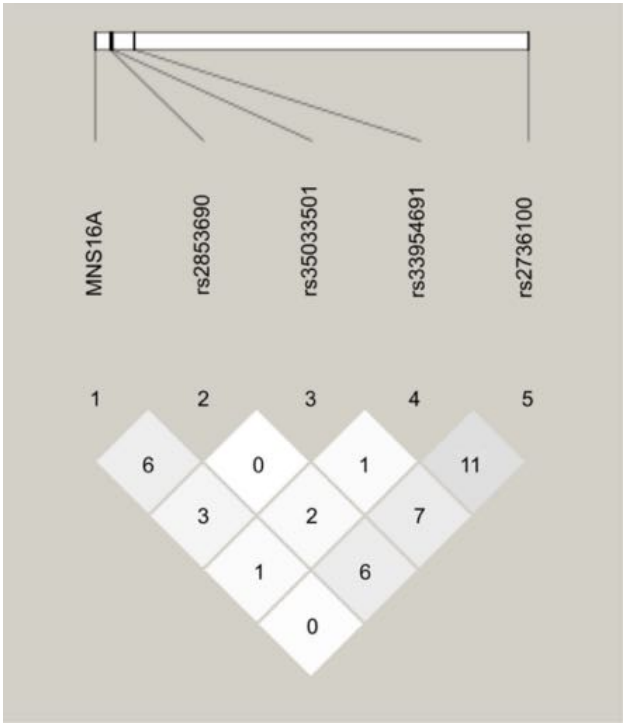

Figure 4 Lack of LD between polymorphic variants under study. MNS16A VNTR is shown to the left, and *TERT* SNPs to the right. Darker color shows higher r^2 values, while the value shown inside the squares is $r^2 \times 10^2$. LD was considered medium for r^2>20 and strong for r^2>80. The graph was created 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 threefold 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

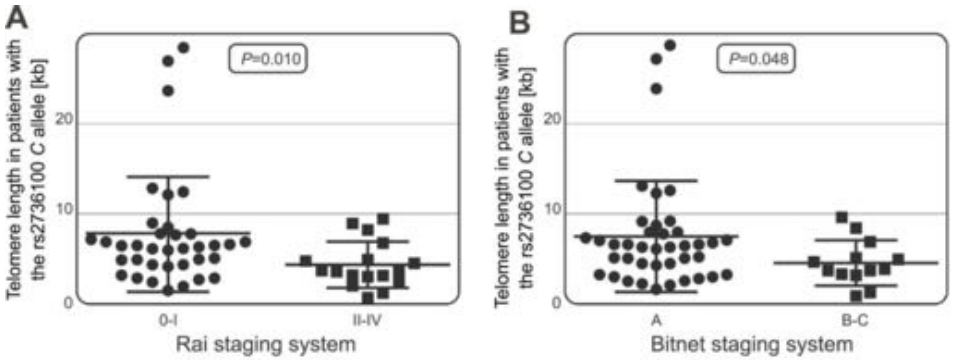

Figure 5 Telomere length in patients with varying severity of the disease. In patients carrying the *TERT* rs2736100 *C* allele, longer telomeres were associated with less advanced disease according to the Rai staging system (**A**) and the Binet criteria (**B**).

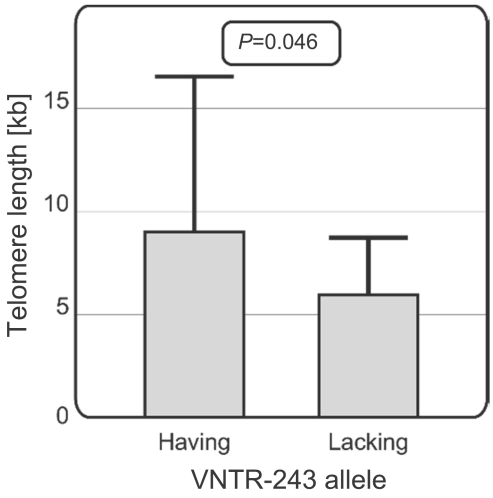

Figure 6 Relationship between telomere length and the presence of VNTR alleles in older patients. Among CLL patients over 60 years of age, those carrying the VNTR-234 allele had longer telomeres than patients lacking this allele (with an average telomere length of 9.01 vs 6.03 kb, p=0.046).
Abbreviations: 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, immunoglobulinheavy chain variable; L, long VNTR allele; qPCR, quantitative polymerase chain reaction; S, short VNTR allele; SCR, single-copyreference; SNP, single-nucleotide polymorphism; VNTR, variable number of tandem repeats.

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Author contributions

All authors contributed toward data analysis, drafting and revising the paper, gave final approval of the version to be published and agree to be accountable for all aspects of the work.

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).

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Relationship between Telomere Length, *TERT* Genetic Variability and *TERT*, *TP53*, *SP1*, *MYC* Gene Co-Expression in the Clinicopathological Profile of Breast Cancer

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Abstract: The molecular mechanisms of telomerase reverse transcriptase (*TERT)* upregulation in breast cancer (BC) are complex. We compared genetic variability within *TERT* and telomere length with the clinical data of patients with BC. Additionally, we assessed the expression of the *TERT*, *MYC*, *TP53* and *SP1* genes in BC patients and in BC organoids (3D cell cultures obtained from breast cancer tissues). We observed the same correlation in the blood of BC patients and in BC organoids between the expression of *TERT* and *TP53*. Only in BC patients was a correlation found between the expression of the *TERT* and *MYC* genes and between *TP53* and *MYC*. We found associations between *TERT* genotypes (rs2735940 and rs10069690) and *TP53* expression and telomere length. BC patients with the *TT* genotype rs2735940 have a shorter telomere length, but patients with *A* allele rs10069690 have a longer telomere length. BC patients with a short allele VNTR-MNS16A showed higher expression of the *SP1* and had a longer telomere. Our results bring new insight into the regulation of *TERT*, *MYC*, *TP53* and *SP1* gene expression related to *TERT* genetic variability and telomere length. Our study also showed for the first time a similar relationship in the expression of the above genes in BC patients and in BC organoids. These findings suggest that *TERT* genetic variability, expression and telomere length might be useful biomarkers for BC, but their prognostic value may vary depending on the clinical parameters of BC patients and tumor aggressiveness.

Keywords: breast cancer telomerase reverse transcriptase (*TERT*); telomere length; expression of transcription factors genes; single nucleotide polymorphism (SNP)

1. Introduction

Breast cancer (BC) is the most common malignant tumor neoplasm in women worldwide [1]. About ten percent of BC cases are associated with a genetic predisposition or family history, with variations by country and ethnicity [2]. BC is a heterogeneous and

polygenic disease, and treatment strategies vary depending on the molecular subtype as well as the most common differentially expressed genes that exist in different disease subtypes [3].

The relationship between telomerase reverse transcriptase (*TERT*) and the risk of BC has been investigated in several publications in the contexts of gene polymorphism, telomere length and the mechanism of gene expression regulation [4]. Various mechanisms, including genetic mutations and epigenetic changes, have been proposed to explain the pleiotropic association of the 5p15.33 region in which the *TERT* gene resides with telomerase activity and cancer predisposition [5,6].

The *TERT* gene encodes the catalytic subunit of telomerase, which is a key enzyme for the maintenance of telomere length; therefore, genetic variations in this region likely influence BC risk through multiple distinct biological pathways, with telomere length being only one of the implied mechanisms [7,8]. The upregulation of the *TERT* gene in BC leads to the activation of telomerase, which contributes to the growth advantage and survival of tumor cells. The molecular mechanisms of *TERT* upregulation are complex, tumor subtype specific and may be clinically relevant [9,10]. The transcriptional regulation of the *TERT* gene is a complex process, and several mechanisms that may play a role have been described, including mutations in the *TERT* promoter that can alter the binding sites of transcription factors, e.g., MYC, SP1 and ETS family proteins [11,12].

In BC, mutation of the *TERT* promoter is rare; therefore, other genetic changes have been described such as gene amplification and the presence of gene copy number gains or single nucleotide polymorphisms (SNPs), which may play a regulatory function in *TERT* expression and be associated with different telomere lengths [13–15].

The present study investigated the relationship between *TERT* gene polymorphisms, both SNPs and a variable number of tandem repeats (VNTR), in the context of mRNA *TERT* gene expression and telomere length and clinical parameters in female patients with BC. Additionally, we assessed the expression of the *TERT*, *MYC*, *TP53* and *SP1* genes in patients with BC and in BC organoids.

In our study, the same correlation was found between the relative expression of *TERT* and *TP53* in the whole blood of BC patients and in BC organoids. Moreover, we observed that the two *TERT* polymorphisms (rs2735940 and rs10069690) correlated with *TP53* expression and telomere length. Additionally, BC patients with a short allele (S) within VNTR-MNS16A showed higher expression of the *SP1* and had longer telomeres. Our results provide more information on the regulation of *TERT* in terms of mRNA expression as well as the genetic variability of *TERT* and telomere length in patients with BC. We have also shown that the *TERT* related genes *MYC*, *TP53* and *SP1* play an important role in BC carcinogenesis.

2. Results

2.1. Disparities of Single Nucleotide and VNTR-MNS16A TERT Gene Polymorphisms in BC

BC patients and healthy individuals were genotyped for TERT single nucleotide polymorphism (SNP; rs10069690, rs2735940, rs2736100 and rs2853669) and variable number tandem repeats MNS16A (VNTR-MNS16A). Their location in the TERT gene is shown in Figure 1. The genotype frequencies for all the SNPs were consistent with the Hardy–Weinberg equilibrium in both study groups. Table 1 shows the distribution of the TERT genotypes in our study group (BC women) and the control group (healthy women) and the frequency of these polymorphisms in the European population (using data from the Ensembl database, accessed on 2 February 2022). There was no difference in the distribution of alleles and genotypes between BC patients and healthy controls in any of the SNPs tested.

Four different VNTR-MNS16A alleles were detected in our BC patients and in the healthy controls (VNTR-333, VNTR-302, VNTR-274 and VNTR-234; Table 2). Patients with BC carried eight different genotypes (long (LL): 302/302, 302/333; short/long (SL): 243/302, 243/333, 274/302; short (SS): 243/243, 274/274, 243/274), but seven genotypes were noted in the control group (no 274/274 genotype as compared to BC patients). The

tandem repeats rates were consistent with the Hardy–Weinberg equilibrium in the patients group, but an imbalance was observed in healthy subjects (Table 2). BC patients and healthy individuals showed no significant differences in the VNTR-MNS16A genotypes and allele frequencies.

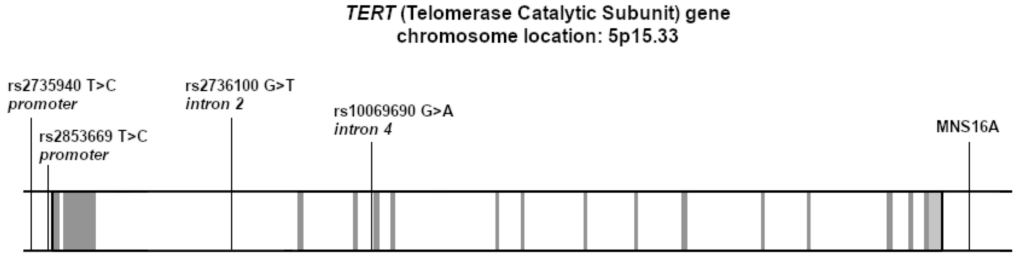

Figure 1. Genomic structure of the human telomerase TERT gene and the location of the studied SNPs and VNTR polymorphism. The exons are shown in grey, while the intronic regions are in white.

Table 1. Distribution of *TERT* genotypes in our group of patients with BC, the control group and the European population.

TERT Genetic Polymorphism	Genotype	BC Patients Frequency	Control Group Frequency	EUR Population Frequency
rs10069690 (intron 4)	*GG*	59 (53.2%)	46 (48.4%)	265 (52.7%)
	AG	48 (43.2%)	42 (44.2%)	198 (39.4%)
	AA	4 (3.6%)	7 (7.4%)	40 (8.0%)
rs2735940 (promoter region)	*CC*	35 (30.9%)	22 (23.2%)	127 (25.2%)
	TC	54 (47.8%)	54 (56.8%)	238 (47.3%)
	TT	24 (21.2%)	19 (20.0%)	138 (27.4%)
rs2736100 (intron 2)	*GG*	28 (23.7%)	24 (22.6%)	134 (26.6%)
	TG	52 (44.1%)	52 (49.1%)	234 (46.5%)
	TT	38 (32.2%)	30 (28,3%)	135 (26.8%)
rs2853669 (promoter region)	*CC*	11 (9.8%)	8 (7.5%)	49 (9.7%)
	CT	40 (35.7%)	39 (36.8%)	192 (38.2%)
	TT	61 (54.5%)	59 (55.7%)	262 (52.1%)

Table 2. *TERT* VNTR-MNS16A genotype distribution and telomere length in BC patients and healthy controls.

TERT VNTR-MNS16A Genotypes	BC Patients (*n*)	Telomere Length (Mean ± Std. Deviation) [kb]	Health Controls (*n*)	Telomere Length (Mean ± Std. Deviation) [kb]
Long VNTR-MNS16A (LL)				
302/302	41	4.21 ± 2.85	36	3.79 ± 1.59
302/333	2		3	
Short/Long VNTR-MNS16A (SL)				
243/302	40	4.95 ± 3.05	46	4.66 ± 1.48
243/333	1		2	
274/302	5		6	
Short VNTR-MNS16A (SS)				
243/243	11	6.72 ± 5.48	6	7.80 ± 5.33
274/274	3		not detected	
243/274	2		1	

2.2. Relationships between the Expression of TERT, SP1, MYC and TP53 Genes in BC Patients and BC Organoids

In this part of the study, we analyzed the relationships between *TERT*, *SP1*, *MYC* and *TP53* expression, *TERT* polymorphisms and telomere length in both patients with BC (n = 50) and BC organoids (n = 9). We observed a correlation between the relative expression of *TERT* and *TP53* in BC organoids (r = 0.8404, p = 0.0046; Figure 2a) and a trend towards this association in BC patients (r = 0.3097, p = 0.0646; Figure 2b). Moreover, we found a relationship between the expression of the *SP1* and *MYC* genes only in BC organoids (r = 0.6214, p = 0.0116; Figure 2c) and not in BC patients (r =−0.2328, p = 0.1026; Figure 2d).

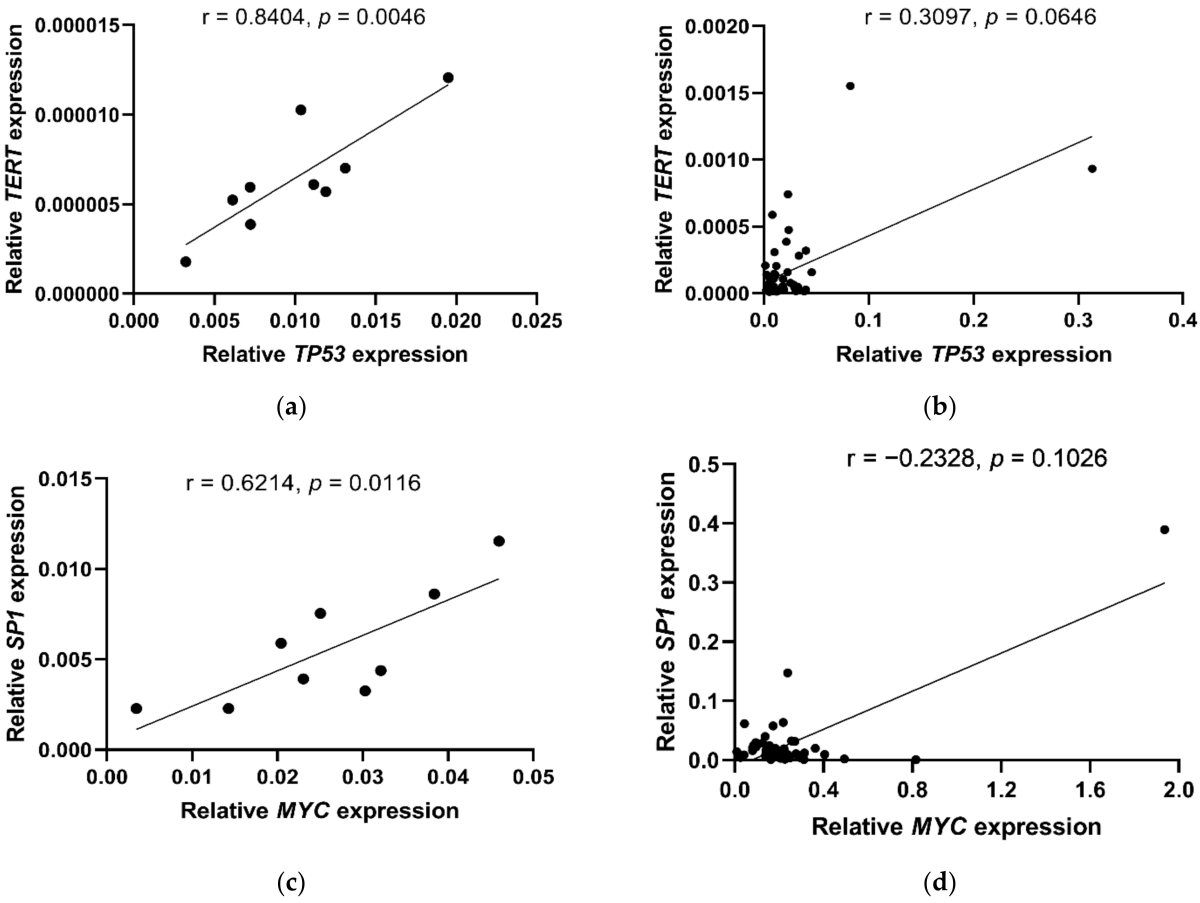

Figure 2. Relationships between expression of *TERT*, *TP53*, *SP1, MYC* genes observed in BC organoids (**a**,**c**) and BC patients (**b**,**d**). Statistical analysis was performed using the Pearson correlation (PC) test (**a**,**c**) and the Spearman r correlation test (**b**,**d**).

A correlation between the expression of the *TERT* and *MYC* genes (r = 0.3097, p = 0.0296; Figure 3a) and between the expression of the *TP53* and *MYC* genes (r = 0.7892, p < 0.0001; Figure 3c) was also found, but only in BC patients and not in BC organoids (*TERT*/*MYC*: r = 0.0008, p = 0.9416; Figure 3b and *TP53*/*MYC*: r = 0.0469, p = 0.5759; Figure 3d).

Additionally, we only observed a trend toward associations between the relative gene expression of *TERT* (p = 0.0817) and *SP1* (p = 0.0774) in the context of BC subtypes (Luminal with *HER2* gene amplification, Luminal without *HER2* gene amplification and Triple Negative BC). We observed no such associations between *MYC* and *TP53* expressions. In addition, we observed a trend towards high estrogen receptor expression in patients with increased *TP53* expression (above average) (p = 0.0894). Moreover, BC patients with low *SP1* and *MYC* (below average) expression were characterized by high progesterone receptor expression (p = 0.0504 and p = 0.0897, respectively).

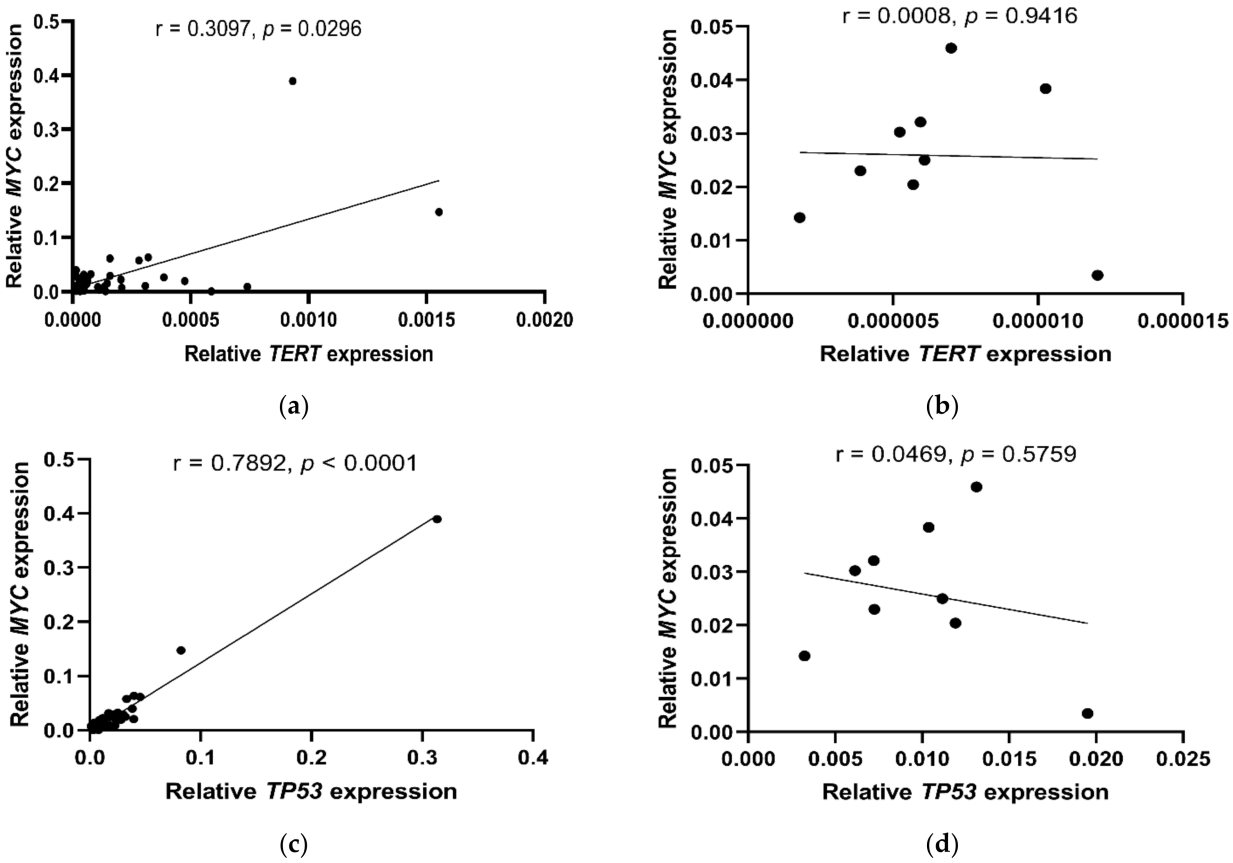

Figure 3. Relationships between the expression of *TERT*, *MYC* and *TP53* genes observed in the blood of BC patients (**a**,**c**) and BC organoids (**b**,**d**). Statistical analysis was performed using the Spearman r correlation test (**a**,**c**) and the Pearson correlation (PC) test (**b**,**d**).

2.3. Genetic Variation in TERT, Telomere Length and Expression Level of TP53 and SP1 in BC Patients

We found a link between the expression level of *TP53* and *SP1*, the genetic variability in *TERT* and telomere length. BC patients with the *TERT* (rs10069690) *A* allele ($p = 0.0266$; Figure 4a) and patients with the *TERT* (rs2735940) *TT* genotype had the highest relative expression of the *TP53* gene ($p = 0.0340$; Figure 4b). Additionally, patients with the *TERT* (rs10069690) *A* allele had the longest telomeres ($p = 0.0056$) as compared to patients with the *GG* genotype (Figure 4c). However, patients with the *TT* genotype in *TERT* (rs2735940) did not have the longest telomeres compared to the other rs2735940 genotypes (*CC* vs. *CT*, $p < 0.0001$; *CC* vs. *TT*, $p = 0.0562$; *CT* vs. *TT*, $p = 0.0074$, Figure 4d). No significant associations were observed between either *TERT* rs2736100 (*GG* vs. *TG*, $p = 0.5334$; *GG* vs. *TT*, $p = 0.3780$; *TG* vs. *TT*, $p = 0.7571$) or *TERT* rs2853669 (*CC* vs. *CT*, $p = 0.6034$; *CC* vs. *TT*, $p = 0.9039$; *CT* vs. *TT*, $p = 0.4233$) and the relative expression levels of *TP53*. However, we observed that BC patients with the *GG* genotype rs2736100 had longer telomeres than women with the *TG* and *TT* genotypes (*GG* vs. *TG*, $p < 0.0001$; *GG* vs. *TT*, $p = 0.0360$; *TG* vs. *TT*, $p = 0.0125$).

We noticed a trend for a relationship between *SP1* gene expression and the *TERT* VNTR-MNS16A gene polymorphism in BC patients. BC patients with SL (243/302, 243/333, 274/302) and SS (243/243, 274/274, 243/274) VNTR-MNS16A genotypes had a higher relative expression of *SP1* ($p = 0.0670$, Figure 5a) and the longest telomeres compared to the patients with LL genotypes (302/302, 302/333; $p = 0.0551$; Figure 5b).

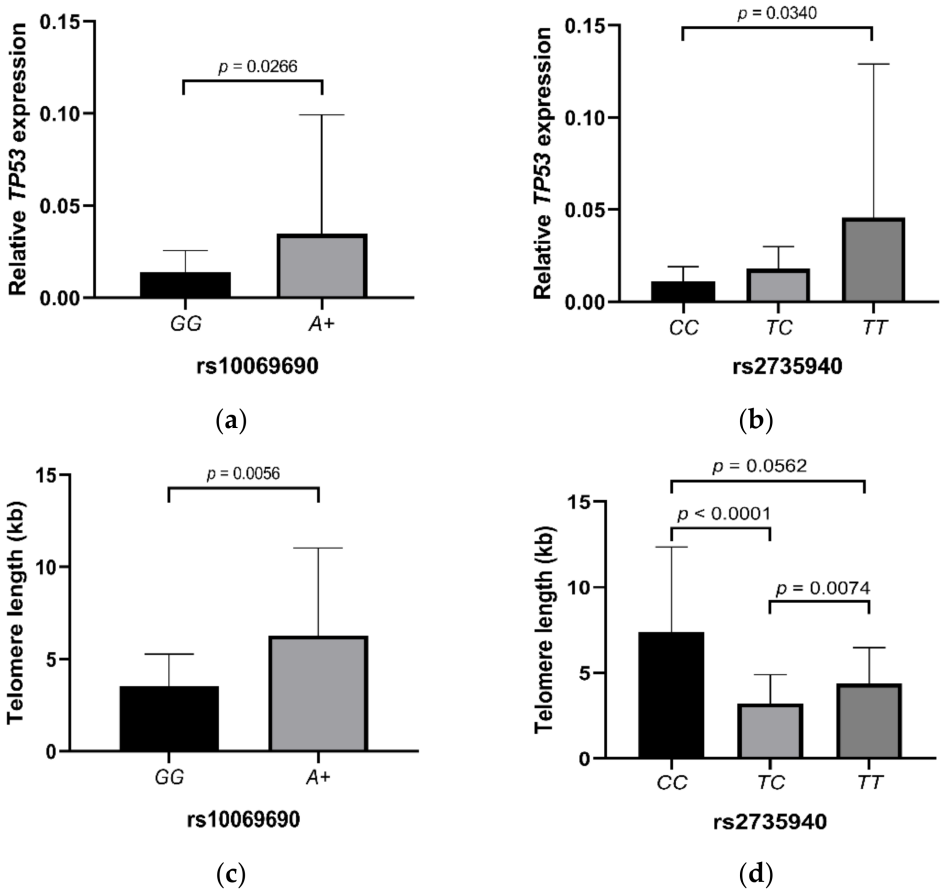

Figure 4. Associations between the *TERT* gene polymorphisms (10069690 and rs2735940), relative expression of the *TP53* gene (**a**,**b**), and telomere length (**c**,**d**) in patients with BC. The Mann–Whitney U test was employed to assess the significance of differences in the expression levels of *TP53* and rs10069690 (**a**) and in telomere length (**c**). The Kruskal–Wallis test with the Original FDR method of Benjamini and Hochberg was used to assess the significance of the relative expression of *TP53* and the genotypes in rs2735940 (**b**), as well as differences in telomere length (**d**).

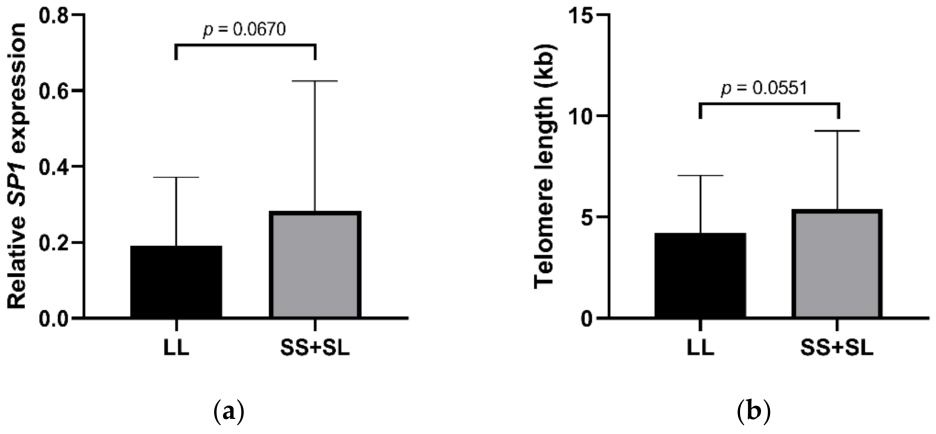

Figure 5. Relationship between the *TERT* VNTR-MNS16A polymorphism, relative *SP1* expression and telomere length. High relative expression of the *SP1* gene is associated with short allele (S) *TERT* VNTR-MNS16A (**a**), which was associated with long telomeres (**b**). The Mann-Whitney U test was employed to assess the significance of differences in the expression level of SP1 (**a**) and the differences of telomere length (**b**).

2.4. Relationship between Gene Expression, TERT Genetic Variability, Telomere Length and Clinicopathological Hallmarks of Breast Cancer

In the present study, telomere length was measured in three independent groups: BC patients (n = 108), BC organoids (n = 9) and a group of healthy women (n = 100). We did not observe any significant differences between the telomere length in BC patients (4.95 ± 3.61 kb), healthy females (4.43 ± 2.26 kb) (Table 3) and BC organoids (3.75 ± 1.42 kb). We also did not notice any significant differences between the *TERT* genotypes (rs10069690, rs2735940, rs2736100, rs2853669, VNTR-MNS16A) and telomere length; the details are presented in Table S1 in the Supplementary Materials. In addition, no relationship was observed between telomere length and main clinical features (shown in Table 3).

Table 3. Relationships between telomere length and various clinical parameters in patients with BC.

	BC Patients	*n*	Telomere Length Median (IQR) [kb]	*p*-Value
Age (range)	18–59 years	108	5.53 (2.68–5.94)	0.4903
Estrogen receptor	Positive	93	3.44 (2.64–5.76)	0.2502
	Negative	7	5.02 (3.15–5.87)	
HER2 amplification	Positive	15	4.24 (2.78–6.99)	0.3299
	Negative	83	3.36 (2.64–5.57)	
Progesterone receptor	Positive	88	3.53 (2.65–5.92)	0.3261
	Negative	14	5.08 (2.93–6.46)	
Molecular subtypes	Luminal with HER2 gene amplification	15	4.70 (2.75–7.09)	0.4797
	Luminal without HER2 gene amplification	76	3.37 (2.62–5.86)	
	Triple Negative BC	7	5.02 (2.93–5.86)	
UICC TNM stage	I	48	3.83 (2.41–5.67)	0.9433
	II	40	3.27 (2.69–6.28)	
	III	5	3.26 (3.13–4.08)	
Pathologic lymph nodes status	pN0	77	3.39 (2.64–5.15)	0.4666
	pN+	24	4.12 (2.71–5.94)	
Germline mutation (*BRCA1*, *BRCA2*, *CHEK2*, *PALB2*)	Positive	8	6.66 (2.74–6.05)	0.6727
	Negative	74	4.02 (2.66–6.28)	

It was observed that BC patients with an intermediate Ki67 proliferation index (25–50%) had the lowest relative expression of *TP53*—lower than patients with low (2–20%) and high (60–85%; p = 0.0221) levels of Ki67. Similarly, intermediate levels of Ki67 were characterized by the lowest expression of *TERT*, although this was not statistically significant. In addition, BC patients lacking the expression of the estrogen receptor tended to have lower relative *TP53* expression (p = 0.0894).

Analysis of the *TERT* polymorphisms showed that BC patients with *T* allele rs2736100 and *C* rs2735940 had more invasive tumors (assessed according to histologic grade (G), describing the aggressiveness and dynamics of tumor development) than patients with the *GG* genotype (rs2736100, p = 0.0008) and *TT* genotype (rs2735940, p = 0.0055). Moreover, *TERT* rs10069690 polymorphism showed that patients with the *A* allele had *HER2* gene amplification less frequently (p = 0.0268).

Additionally, BC patients with the *GG* genotype (rs2736100) had higher parathyroid hormone (PTH) levels (40.64 ± 16.78 pg/mL) than heterozygotes (28.11 ± 10.67 pg/mL; p = 0.0400) and *TT* homozygotes (35.36 ± 10.82 pg/mL; p = 0.0469). However, in the case of rs2735940 *TERT* polymorphism, it was observed that the heterozygous group of patients

(28.73 ± 10.52 pg/mL) had the lowest concentration of PTH in the blood (p = 0.0408). For the *TERT* rs2853669 polymorphism, we only observed that BC patients with the *TT* genotype had higher blood estradiol levels (62.41 ± 61.90 pg/mL) compared to patients with C allele (25.80 ± 53.43 pg/mL; p = 0.0051).

The VNTR-MNS16A analysis showed that women with SS genotypes showed fewer invasive tumors classified by G feature than women with the LL or SL genotypes (p = 0.0181). Moreover, BC patients with heterozygous genotypes (SL) had less *HER2* amplification/overexpression than patients with homozygous genotypes (SS + LL) (p = 0.0097).

Additionally, we performed a linkage disequilibrium (LD) analysis and found that the two *TERT* SNPs (rs2736100 and rs2735940) were in a medium LD (r^2 = 0.54 in BC patients; Figure 6). Moreover, three *TERT* SNPs (rs2736100, rs2853669 and rs2735940) were in a low LD (r^2 = 0.10 in BC patients; Figure 6).

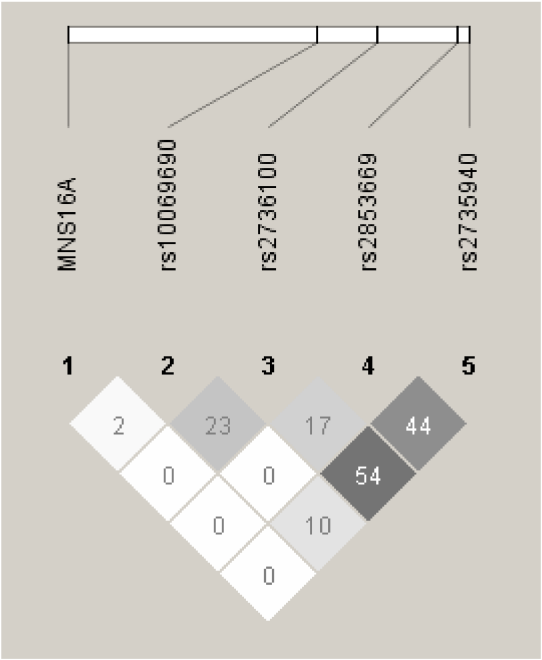

Figure 6. Analysis of linkage disequilibrium in patients with BC. Darker color shows higher r^2 values, while the value shown in the squares is r2 × 102. LD was considered to be medium for r^2 > 20 and strong for r^2 > 80. The chart was created using the Haploview 4.2 software.

We observed an association between the two SNPs rs2853669 (allele *C*) and rs2735940 (genotype *TT*) by which BC patients with this combination of *C* allele and *TT* genotype presented higher levels of estradiol (54.58 ± 63.87 vs. 11.33 ± 11.04 pg/mL; p = 0.0484).

Additionally, further analysis showed that patients with the *TCC* (rs2736100, rs2853669 and rs2735940, respectively) were characterized by G feature (p = 0.0317). In addition, we observed a relationship between the combination of VNTR-MNS16A (L alleles) and *TCC* (rs2736100, rs2853669, rs2735940), showing that the BC patients with L*TCC* had more invasive tumors classified by G feature (p = 0.0029). Another combination showed that BC patients with the alleles *T* (rs2736100) and *A* (rs10069690) and with the SL genotype VNTR-MNS16A had a lower frequency of *HER2* amplification/overexpression (p = 0.0008).

3. Discussion

Breast cancer (BC) is characterized by a high level of gene heterogeneity. The determination of the molecular/biologic subtypes of BC is an important issue for the classification of this disease according to the status of hormone receptors (estrogen and progesterone), the human epidermal growth factor receptor 2 (HER2) and the Ki67 proliferation index.

All these variables, together with the presence of somatic and/or germline mutations, are important for the prognosis and individual treatment of BC patients.

TERT appears to play a significant role in the description of BC [16,17]. Therefore, our research covered *TERT* gene expression and telomere length, as well as the expression of the transcription factors *MYC*, *SP1* and *TP53* detected at the mRNA level. Moreover, the genetic variability of the *TERT* gene was detected at the level of SNPs and VNTR in the context of telomere length and the clinical parameters of patients with BC.

The *TERT* gene is a major functional subunit of telomerase, and telomere length is critical to genome stability. Although the molecular mechanisms of *TERT* regulation have been described in detail in many cancers, it is not well understood in BC. It is known that many cellular processes are related to the presence of telomerase and are associated with apoptosis, uncontrolled cell division, the breakdown of the division cycle and the repair of damaged DNA. In this context, the choice to examine *TERT* and *TP53* gene expression seems justified. Molecular disruptions, e.g., mutation in both *TERT* and *TP53* genes, can alter expression and often lead to aberrant telomerase activation that can induce uncontrolled cell proliferation and oncogenesis in BC.

In the present study, we showed that BC patients with a high Ki67 proliferation index (60–80%) had an increased relative expression of the *TP53* gene compared to patients with a low Ki67 index (25–50%), who had a lower *TP53* expression. Similar data, although not statistically significant, were observed in the expression of the *TERT* gene, where high levels of Ki67 were characterized by high *TERT* expression (see the Results section).

The *TP53* gene is a well-known tumor suppressor gene—also known as the "guardian of the genome"—and its mutations may be considered a major biomarker of cancer. Its role has been associated with the regulation of apoptosis, cell cycle control and DNA damage repair processes [18].

We used cells from two sources, the blood of BC patients and BC organoids, to compare the expression of the *TERT, TP53, MYC* and *SP1* genes. We found correlations within the genes *TERT* and *TP53* in both of these two independent cell models.

It is important to know that under physiological conditions, the exposure of cells to various stress signals activates the p53 signaling pathway, allowing cells to activate several transcriptional programs, including cell cycle arrest, DNA repair, senescence and apoptosis, leading to the suppression of tumor growth [19,20]. It should be noted that all these processes are related to telomerase activity and the expression of *TERT*. Inactivation of the *TP53* gene caused by mutation drives cell invasion, proliferation and survival, thereby facilitating cancer progression and metastasis [21]. Marei et al. highlights recent advances in the understanding of the regulatory network by which mutant p53 proteins can modulate the molecular signaling pathways involved in cancer progression and/or protection [22]. A mutation in the *TP53* gene is detectable in approximately 50% of human breast, colon, lung, liver, prostate, bladder and skin cancers [23]. Many of these mutant p53 proteins are oncogenic and therefore modulate the ability of cancer cells to proliferate, escape apoptosis, invade and metastasize [24]. *TP53* has also been documented to be involved in the cellular responses to dysfunctional telomeres. Guièze et al. showed that patients with chronic lymphocytic leukemia (CLL) with impaired *TP53* have severe telomere dysfunction and high genomic instability. This group found that each type of *TP53* alteration was associated with very short telomeres and high *TERT* expression. Additionally, the disruption of *TP53* was characterized by the downregulation of the shelterin complex genes within the telomerase complex [25].

In our study, we observed a dual role of telomere length in the context of *TP53* expression and *TERT* variability. BC patients with the *TT* genotype in the *TERT* promoter (rs2735940) have a shorter telomere length and higher *TP53* expression. The opposite effect was observed in BC patients with *A* allele in intron 4 (rs10069690), who had a longer telomere length and higher *TP53* gene expression (see Figure 4).

The relationship between telomere length and BC risk is contradictory. First, no significant association was found between telomere length and the risk of BC [26–28].

Secondly, some recent reports have suggested that longer telomere lengths have been associated with an increased risk of BC [29,30]. Pellat et al.'s study strongly suggests that both telomere length and telomere related genes influence BC risk and that the tumor estrogen and progesterone receptors appear to be important modifiers of the associations with telomere related genes and BC risk [8]. However, other studies found that a shorter telomere length was associated with an increased risk of BC [31]. Shen et al. observed that, overall, telomere length was not significantly associated with the risk of BC. However, they noted that a shorter telomere length may be associated with an increased risk of BC in premenopausal women [31]. Additionally, Pooley et al. found a strong association between a shorter telomere length and BC risk [32]. One study found that both shorter and longer telomeres were associated with an increased risk of BC [33]. Oztas et al. reported that the rs2736100 *TERT C* allele is not associated with BC risk, but Aydin et al. observed the opposite [34,35]. De Souza Rodrigues et al. showed that the *TERT* variants rs2736098, rs10069690 and rs2853676 were associated with an increased risk of BC [17]. Additionally, it was observed that the VNTR-MNS16A influences the risk of BC in the Iranian population but not in the Greeks and Americans [36]. A meta-analysis by Aziz et al. did not show any significant associations of rs2853669 (located in the promoter region of *TERT*) genotypes in Caucasian BC patients [37]. Moreover, Varadi et al. found no clear association between a reduction in hereditary or occasional BC risk with rs2853669 in a cohort of Swedish patients [38].

In our study, we did not observe any significant differences in telomere length in BC patients with the *TERT* rs2736100 and rs2853669 alleles and genotypes. However, we noticed that patients with *TERT* VNTR-MNS16A with a short (S) allele had longer telomeres and higher expression of *SP1* mRNA (see Figure 5).

In an earlier study, Hofer et al. discussed the role of the VNTR-MNS16A polymorphism in the context of transcription factors and showed that transcription activity depends on various VNTR-MNS16A length variants presenting a different number of transcription factor binding sites for the GATA binding protein 1 [39].

In our study, we noticed a trend towards association between the expression of the *SP1* gene and the *TERT* VNTR-MNS16A gene polymorphism. Our BC patients with the S allele had a higher relative expression of *SP1* and longer telomeres than the patients with LL genotypes (see Figure 5a,b).

When we compared the genetic variability of *TERT* with the clinical data of the BC patients, we showed that BC patients with more invasive tumors were characterized by VNTR-MNS16A L allele and *TCC* (rs2736100, rs2853669 and rs2735940, respectively). Additionally, BC patients with the *T* allele (rs2736100), *A* allele (rs10069690) and SL genotype VNTR-MNS16A had a lower frequency of *HER2* amplification/overexpression. Moreover, patients with the *TT* genotype (rs2735940) and with the *C* allele (rs2853669) were characterized by lower levels of estradiol and higher levels of progesterone. Regarding the analysis of clinical data, Bojesen et al. showed that *TERT* rs10069690 is associated with a risk of estrogen receptor negative BC and BC in *BRCA1* mutation carriers, which is consistent with another observation that showed that most incidents of BC arising from *BRCA1* mutation carriers are estrogen receptor negative [40]. In our present study, we did not observe any significant association of genotype and risk of BC or *TERT* SNP with estrogen and progesterone receptor status and *BRCA1* mutation.

Interesting results documented by Gay-Bellile et al. presented the role of the *TERT T349C* (rs2853669) promoter polymorphism, which was not correlated with *TERT* expression, but carriers of the *TC* and *CC* genotypes had a significantly shorter disease-free survival [14]. Our present results confirm their observation of *TERT* expression in both BC patients and BC organoids, as *TERT* rs2853669 was not associated with *TERT* expression. Additionally, Gay-Bellile et al. showed that *TERT* gains found in 15–25% of cases were strongly correlated with increased *TERT* mRNA expression and worse patient prognosis in terms of disease-free and overall survival [14].

Our study provides definitive evidence of the genetic control of telomere length by some of the genetic variants in the *TERT* locus (e.g., VNTR-MNS16A, rs2735940, rs10069690). Additionally, we showed that *TERT* genetic variants could be potential prognostic biomarkers of BC associated with tumor invasiveness. Given the limitations of this study, future studies with a larger sample size to validate the current findings are needed, as well as functional studies to reveal the role of the *TERT* gene polymorphism and mRNA expression in BC carcinogenesis.

4. Materials and Methods

4.1. Patients and Controls

The study included 108 Polish women (age range at diagnosis: 32–86 years, median 61 years) treated for invasive breast cancer at the Lower Silesian Oncology, Pulmonology and Hematology Center (Wroclaw, Poland). The blood samples were collected at diagnosis after obtaining informed consent from the patients. All methods were according to the Declaration of Helsinki. The approval of the Bioethical Committee of Wroclaw Medical University was obtained for the study (No. KB—808/2019). Additionally, 100 healthy blood donors (age range: 18–59, median 21 years) served as a control group for the study of *TERT* polymorphisms and telomere length. Relationships between telomere length and the various clinical parameters of the studied group are presented in Table 3. Our study group included 8 women with different variants of germline mutations in the *BRCA1* (c.181T > G (p.Cys61Gly); c.5266dupC)), *BRCA2* (c.9227G > A; c.10202C > T (p.Thr3401Met)), *CHEK2* (c.444 + 1G > A (IVS3 + 1G > A)) and *PALB2* (c.172_175del) genes, 74 BC patients without these germline mutations and 26 BC patients who were not tested for germline mutations. All BC patients and control subjects were Polish Caucasians recruited from the population of Lower Silesia (south-western province of Poland, ≈ 2.9 M population in 2019).

4.2. Breast Cancer Organoids

The sample was the tissue from eight BC patients (age range at diagnosis: 37–76 years old, median 47 years) with infiltrating duct carcinoma [(NOS) 8500/3] G1, 2, 3 before radiotherapy, chemotherapy and other treatment. The tissues were delivered as a postoperational material from the Gdynia Oncology Center of the Polish Red Cross Maritime Hospital. The human material was sampled according to the local bioethical commission guidelines (but no particular permission was required since the material was obtained within regular surgery operations removing carcinoma). However, according to the bioethical commission guidelines, the informed consent of the patient was necessary and was obtained each time. The tissues were then washed using phosphate buffer saline (PBS 1 ×, Gibco, Waltham, MA, USA) and preserved in the transfer medium consisting of DMEM/F12, +10% Fetal Bovine Serum (FBS, Sigma-Aldrich, Saint Louis, MO, USA) + 100 µg/mL Penicillin/Streptomycin + 5 µg/mL Piramycin + 50 U/mL Polymyxin B before being isolated. After that, the tissues were washed with 1 × PBS in a Petri dish and then cut into small pieces using a surgical scalpel. The sample fragments were washed again with 1 × PBS, inserted into a 15 mL falcon tube (Sigma-Aldrich, Saint Louis, MO, USA) containing the mixed enzyme solution and then incubated for 16 h, 300 rpm, 37 °C. After incubation, the samples were filtered using 100 µm and 40 µm cell strainer (Corning, New York, NY, USA) and then centrifuged at 600× *g* for 5 min. The supernatant was discarded, and the pellet containing tissue fragments was washed with 1 × PBS and centrifuged at 600× *g* for 5 min. One part of the material was frozen using RNA later (Thermo Fisher Scientific, Waltham, MA, USA) or 50% DMEM/F12 + 44% FBS + 6% Dimethyl sulfoxide (DMSO, Sigma-Aldrich, Saint Louis, MO, USA) and Nunc type freezing ampoules (Thermo Fisher Scientific, Waltham, MA, USA). The remnant pellet was then resuspended with the culture initiation media and cultured in a 6-well plate (37 °C, 5% CO_2) for 48 h. Afterwards, the media mix was removed and the stimulation medium was added, which was replaced every 3 days. Next, the cells were transferred into T75 flasks and cultured in the stimulation medium until reaching a confluence of 80%. The

cultured cells were then detached using trypsin (Sigma-Aldrich, Saint Louis, MO, USA) and incubated for 1–3 min at 37 °C, and medium containing FBS was added to neutralize trypsin. The detached cells were centrifuged at 600× *g* for 5 min at room temperature. The cells were counted using a Z series Coulter Counter by Beckman Coulter, Indianapolis, IN, USA. Eventually, the cells were frozen using RNA later or 75% stimulation medium + 15% FBS + 10% DMSO and Nunc type freezing ampoules. The ampoules were stored at −80 °C until further analysis.

4.3. DNA Extraction

Genomic DNA was isolated from 200 µL of peripheral blood taken on EDTA using the NucleoSpin Blood kit (MACHEREY-NAGEL GmbH & Co. KG, Dueren, Germany) according to the manufacturer's instructions. Genomic DNA from the BC organoids was isolated using NucleoSpin Tissue XS kits (MACHEREY-NAGEL GmbH & Co. KG, Dueren, Germany). DNA concentration and purity were quantified on a DeNovix DS-11 spectrophotometer (DeNovix Inc., Wilmington, DE, USA). The isolated DNA was then stored at −20 °C until *TERT* genotyping and evaluation of the telomere length in patients with BC and BC organoids.

4.4. Genotyping of TERT Gene Polymorphisms

The selection of the studied single nucleotide polymorphisms (SNPs) within the *TERT* gene was based on results of the SNP Function Prediction tool available on the website of the National Institute of Environmental Health Sciences (NCBI Database), as well as other auxiliary databases (https://snpinfo.niehs.nih.gov/snpinfo/snpfunc.html (accessed on 2 February 2022); https://www.ncbi.nlm.nih.gov/snp/ (accessed on 2 February 2022); https://www.ensembl.org/index.html (accessed on 2 February 2022). The following criteria were used: minor allele frequency in Caucasians above 10%, change in RNA and/or amino acid chain, potential splicing site and/or miRNA binding site.

Based on the above criteria, four *TERT* SNPs were selected for the study: rs10069690 (G > A) located in intron 4; rs2736100 (G > T) located in intron 2; rs2853669 (T > C) and rs2735940 (T > C), both located in the promoter region at −245 bp (Ets2 binding site) and 1327 bp upstream of the transcription start site, respectively. The *TERT* polymorphisms were determined by LightSNiP typing assays (TIB MOLBIOL, Berlin, Germany) using quantitative polymerase chain reaction (qPCR). Amplifications were performed on a LightCycler480 II Real-Time PCR system (Roche Diagnostics International AG, Rotkreuz, Switzerland) according to the recommendations of the manufacturer. The PCR conditions were as follows: 95 °C for 10 min followed by 45 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 15 s. PCR was followed by one cycle of 95 °C for 30 s, 40 °C for 2 min and gradual melting from 75 °C to 40 °C.

4.5. VNTR-MNS16A Genotyping of the TERT Gene

The presence of the VNTR-MNS16A *TERT* gene polymorphism was assessed in BC patients and in healthy women by PCR amplification followed by electrophoresis in sequencing gel, as described by Wysoczanska et al. [41]. PCR was performed in a 2720 Thermal Cycler instrument (Applied Biosystems, Foster City, CA, USA) using the forward and reverse primer sequences (5′-AGGATTCTGATCTCTGAAGGGTG-3′ and 5′-TAMRA-TCTGCCTGAGGAAGGACGTATG-3′) prepared by Genomed (Warsaw, Poland). The amplification procedure included an initial denaturation step for 5 min at 95 °C, followed by 35 cycles: 30 s at 95 °C, 30 s at 65 °C, 30 s at 72 °C and a final extension step for 10 min at 72 °C. The PCR products were diluted with formamide and a GeneScan™500 ROX™ dye Size Standard (Applied Biosystems, Foster City, CA, USA). The samples were denatured at 95 °C for 5 min and analyzed on the 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) with an eight-capillary system filled with POP7 polymer (Applied Biosystems, Foster City, CA, USA). The alleles were identified using the GeneMapper software version 4.2 (Applied Biosystems, Foster City, CA, USA).

4.6. Quantification of Telomere Length

Mean telomere length was measured in the genomic DNA samples of 108 BC patients, 100 controls and 9 BC organoids. The DNA samples were diluted with nuclease-free water to a concentration of 5 ng/mL. Telomere length measurements were performed on a LightCycler480 II Real-Time PCR system (Roche Diagnostics International, Rotkreuz, Switzerland) using qPCR test kits (ScienCell's Absolute Human Telomere Length Quantification qPCR Assay Kit [AHTLQ], Carlsbad, CA, USA), as previously described by Dratwa et al. [42]. The PCR conditions were as follows: 95 °C for 10 min followed by 32 cycles of 95 °C for 20 s, 52 °C for 20 s and 72 °C for 45 s. Data analysis was conducted according to the manufacturer's instructions. All reactions were run in three replicates.

4.7. Extraction of RNA, Reverse Transcription and TERT, SP1, MYC and TP53 Genes Expression Study

The RNA of 50 patients with BC and 9 BC organoids was extracted from 10^6 cells suspended in RNA Extracol (EURx, Gdansk, Poland) or RNA later (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. RNA purity and integrity were verified on a DeNovix DS-11 spectrophotometer (DeNovix Inc., Wilmington, DE, USA) and gel electrophoresis. A total of 1 µg/µL of the isolated RNA was used for the reverse transcription reaction. cDNA was synthesized using the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems™, Foster City, CA, USA), and 0.5 µL of RNase Inhibitor (Applied Biosystems™, Foster City, CA, USA) was added per sample to convert the extracted and purified RNA into cDNA. The conversion step was performed on a SimpliAmp™ Thermal Cycler (Applied Biosystems®, Foster City, CA, USA). After this step, the samples were stored in a freezer at −20 °C until further use.

Four genes were included in the expression experiments: *TERT* (Hs_00972,650_m1), *SP1* (Hs_00916521_m1), *MYC* (Hs_00153408_m1) and *TP53* (Hs_01034249_m1). *GAPDH* (Hs02786624_g1) and *ACTB* (Hs_01060665_g1) were used as housekeeping genes to normalize RNA expression data. TaqMan® Gene expression assays were used for detection (Applied Biosystems Foster City, CA, USA), and qPCR was performed using the LightCycler 480 II Real-Time PCR system (Roche Diagnostics International, Rotkreuz, Switzerland). The following protocol was used for each PCR sample: 5 µL of cDNA, 1 µL (20×) each primer/probe, 10 µL (2×) of TaqMan® Gene Expression Master Mix (Applied Biosystems™, Foster City, CA, USA), 4 µL of ultra-pure water. Amplification was performed under the following conditions: initial denaturation for 10 min at 95 °C was followed by 40 cycles of denaturation for 15 s at 95 °C and annealing for 1 min at 60 °C. Relative genes' expression levels were calculated by the $2^{-\Delta CT}$ method. Each sample was analyzed in triplicate to validate the technique and CT values, according to the international standards for the evaluation of gene expression by real-time PCR.

4.8. Statistical Analysis

The null hypothesis that there is no difference between the frequency of alleles and genotypes between patients and controls was verified with the Fisher's exact test, calculated using the online tool http://vassarstats.net/tab2x2.htm (version as of 2 February 2022). In each experiment, the normality of the data was verified with the Shapiro-Wilk test. The remaining statistical analyses of the differences between the groups were performed using one-way analysis of variance (ANOVA) to determine the significance of intergroup differences, and the obtained *p*-values were corrected by the Benjamini and Hochberg method. Taking into account that the distribution of some data deviates from the normal distribution, the non-parametric U Mann–Whitney test was performed for the comparison of telomere lengths and gene expression. The correlations were statistically evaluated using the Pearson correlation (PC) test or the Spearman r test. The statistical calculations were performed by the GraphPad Prism software (GraphPad Software, La Jolla, CA, USA, version 8.0.1) and the Real Statistics Resource Pack for Microsoft Excel 2019 (version 16.0.10369.20032, Microsoft Corporation, Redmont, Washington, DC, USA). The probability

(p) values < 0.05 were considered statistically significant, while the trend index was between 0.05 and 0.10.

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Institutional Review Board Statement: This study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Ethics Committee of Wroclaw Medical University (protocol code: 808/2019).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available upon request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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REVIEW
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TERT—Regulation and Roles in Cancer Formation

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Telomerase reverse transcriptase (TERT) is a catalytic subunit of telomerase. Telomerase complex plays a key role in cancer formation by telomere dependent or independent mechanisms. Telomere maintenance mechanisms include complex *TERT* changes such as gene amplifications, *TERT* structural variants, *TERT* promoter germline and somatic mutations, *TERT* epigenetic changes, and alternative lengthening of telomere. All of them are cancer specific at tissue histotype and at single cell level. *TERT* expression is regulated in tumors *via* multiple genetic and epigenetic alterations which affect telomerase activity. Telomerase activity *via TERT* expression has an impact on telomere length and can be a useful marker in diagnosis and prognosis of various cancers and a new therapy approach. In this review we want to highlight the main roles of *TERT* in different mechanisms of cancer development and regulation.

Keywords: telomerase reverse transcriptase, cancer progression, *TERTp* mutations, telomere maintenance mechanisms, *TERT* structural variants, *TERT* epigenetic alterations, *TERT* transcriptional activators and repressors

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INTRODUCTION

In most human cancers, telomerase is reactivated during carcinogenesis by expression of the catalytic subunit telomerase reverse transcriptase (TERT). TERT plays a key role in cancer formation, ensuring chromosomal stability by maintaining telomere length, and allowing cells to avert senescence. It constitutes a limiting factor for formation of the telomerase complex in cancer cells (1). TERT is one of two major components of the larger telomerase complex, which extends telomeres by adding specific short repetitive DNA sequences. These tandem repeats are bound by the shelterin complex, which is composed of six proteins: telomere repeat factor 1 and 2 (TRF1, TRF2), protection of telomeres 1 (POT1), TRF1-interacting nuclear protein 2 (TIN2), tripeptidyl peptidase I (TPP1), and repressor/activator protein 1 (RAP1) (**Figure 1**) (2). The Shelterin complex plays a fundamental role in protecting chromosome ends and in telomere length regulation (3, 4).

The *TERT* gene is situated at chromosome 5p15.33 in humans, and is an integral and essential part of the telomerase holoenzyme. *TERT* gene is 42 kb long and consists of 15 introns and 16 exons with a 260 bp promoter core (5). The reverse transcriptase domain is encoded by 5–9 exons. The TERT transcript can be spliced into 22 isoforms (6). *TERT* promoter (*TERTp*) region contains GC

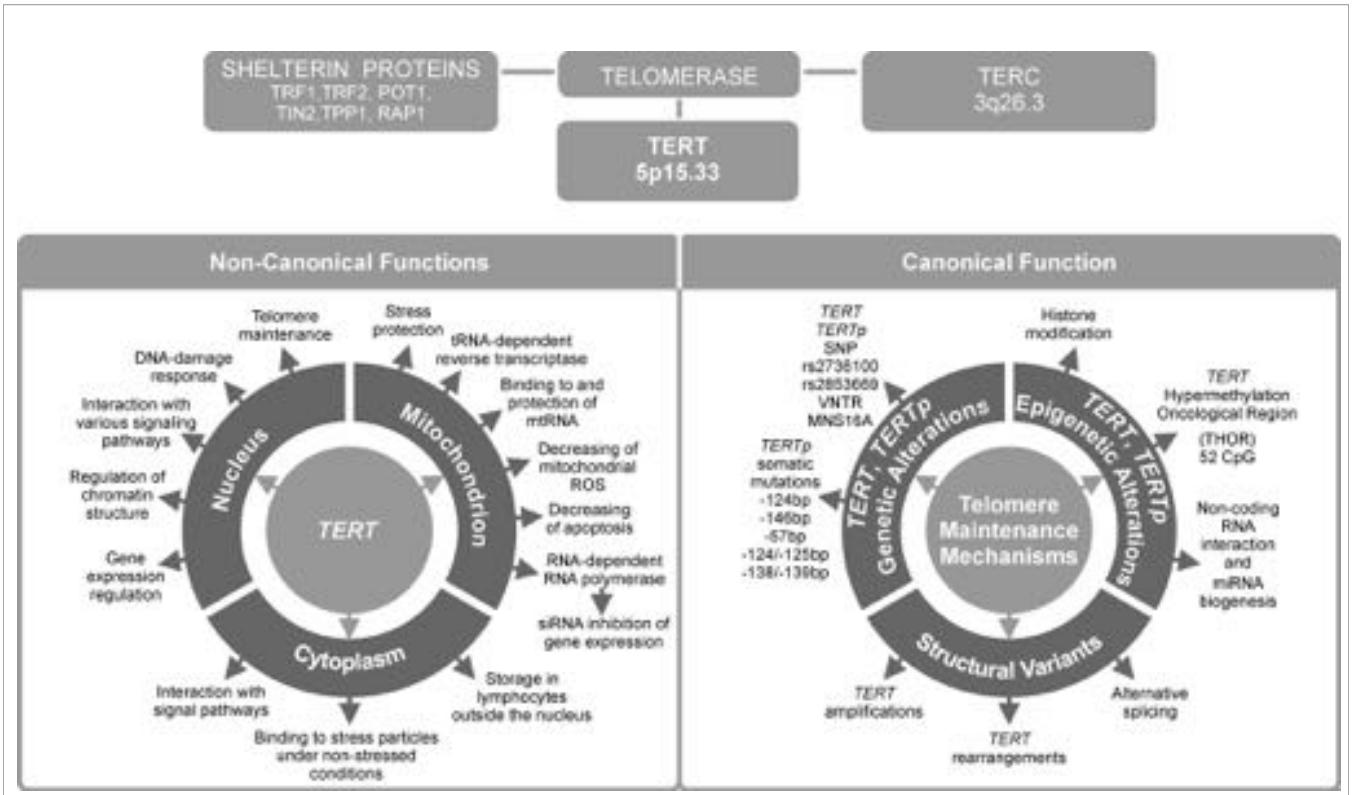

FIGURE 1 | Telomerase reverse transcriptase (TERT) is the most important telomerase subunit and plays a major role in telomerase activity and in other telomere-unrelated processes in cancer development. Telomerase is a complex reverse transcriptase that comprises, besides TERT, an RNA template for telomere repeats (TERC), and a group of proteins called shelterin complex (upper panel). While the primary function of TERT is telomere lengthening (canonical function, lower panel, on the right), there are also other, telomere-unrelated functions (non-canonical functions, lower panel, on the left).

boxes that bind the zinc finger transcription factor Sp1, which increases *TERT* transcription, and E-boxes that bind both transcriptional enhancers and repressors. *TERTp* lacks a TATA box but it contains binding sites for many different transcription factors (7).

Another major component of the telomerase complex is telomerase RNA component (TERC). It is an RNA sequence, which functions as a template for synthesis of telomeres by TERT. These two main components of telomerase are accompanied by a host of auxiliary proteins, including dyskerin (DKC1), telomerase Cajal body protein 1 (TCAB1), non-histone chromosome protein 2 (NHP2), nucleolar protein 10 (NOP10), glycine arginine rich 1 (GAR1), heat shock protein 90 (HSP90) and serine and arginine rich splicing factor 11 (SRSF11) (8). This complex is essential for maintaining telomere homeostasis, which is crucial in regulation of aging and cancer development (9).

Over 80% of tumors adopt various regulatory strategies, known as telomere maintenance mechanisms (TMMs). They maintain telomere length by reactivating telomerase, and therefore are known as TERT canonical functions (10). Individual TMMs are specific for cancer type, tissue histotype, and cell lines. The most important TMMs are (1) *TERT* gene rearrangements and *TERT* and *TERC* gene amplification, (2) *TERTp* somatic mutations, (3) epigenetic alterations, (4) transcription factor binding, (5) polymorphic variants within *TERT* gene body and *TERTp*, and (6) alternative splicing (**Figure 1**). Each of these mechanisms will be described in detail in subsequent sections of this manuscript.

Approximately 10–15% of tumor cells acquire immortality through a telomerase-independent mechanism, namely alternative lengthening of telomeres (ALT) (11). On the other hand, the so called non-defined telomere maintenance mechanism (NDTMM) are activated when both telomerase (or TERT) expression and ALT are absent (10, 12). While telomere lengthening is considered a major function of telomerase, it can also modulate expression of various genes, such as nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) and Wnt/β-catenin signaling pathway genes (13, 14). Such alternative, non-telomere-related roles are known as non-canonical functions of TERT. They will be presented in the last two chapters of this review (**Figure 1**), together with potential consequences of TERT telomere-unrelated functions for the development of anti-cancer strategies and applications of TERT as a potential therapeutic target.

CHROMOSOMAL REARRANGEMENTS

Chromosomal rearrangements are a type of mutation that results in a change in chromosome structure. They may involve duplications, amplifications, insertions, interchromosomal changes, inverted orientations, or deletions (15). A concept

associated with chromosomal rearrangements is copy number variation (CNV). CNV describes the fact that some sections of the genome may be repeated and the number of these repeats may be different between individuals. CNVs involve 50 bp to over 1,000,000 bp fragments of gene regulatory regions (16). They are associated with gene expression and phenotype by affecting gene copy number (17). Chromosomal rearrangements may affect *TERT* gene copy number and are a known TMM. They may involve insertion of active enhancers close to the *TERT* gene and increasing *TERT* expression. A common process is *TERT* amplification, which can arise from telomere dysfunction (18). It results from a dysfunctional telomere, promoting fusion of chromosome ends, and subsequently forming a dicentric chromosome (19). Several studies showed that chromosomal rearrangements at the *TERT* locus may be associated with cancer development and as was observed, e.g., in the case of neuroblastoma (20–22). Furthermore, a major study specifically focusing on *TERT* gene amplification found it to occur in many cancers, such as esophageal, ovarian cancer, and squamous cell carcinoma (12). In addition, other authors found telomerase activity to be the highest in tumors with *TERT* amplification (22, 23). Gay-Bellile et al. observed increased number of *TERT* gene copies in breast cancer cells, and upregulation of *TERT* gene was associated with worse prognosis in breast cancer, thyroid carcinoma, and lung adenocarcinoma (24). This suggests that *TERT* rearrangement could be a critical step in cancer development.

TERT PROMOTER HOT-SPOT MUTATIONS

TERT somatic mutations are the most common non-coding mutations in human cancer cells. While they are documented to occur in the coding region, they are far more common in the promoter region. Some *TERTp* mutations were shown to affect *TERT* expression, telomere length and telomerase activity by abrogating telomerase silencing (25). *TERTp* mutations occur in specific clinical and phenotypic subtypes of various cancers and cell lines, and recurrent mutations have been identified in 19% of cancers (26). In cancer cells, *TERTp* mutations are generally associated with higher *TERT* expression level.

The two most common *TERTp* mutations are C>T transitions, located at -124 bp, and -146 bp from the transcription start site (TSS). They are also referred to as C228T and C250T, respectively (27, 28). These mutations result in an 11 bp nucleotide fragment providing a new consensus binding site for E-twenty-six (ETS) transcription factors (29). Many other somatic mutations were detected that occur in the *TERTp* in cancer, although less frequently than C228T and C250T and they also may contribute to increased *TERT* transcription. A group of CC>TT substitutions, located at −124/−125 and −138/−139 bp relative to the TSS, result in an ETS binding site in skin cancers (30). In melanoma patients, the −138/−139 mutation correlated with more adverse survival (31). In basal cell carcinoma, Maturo et al. observed additional *TERTp* alterations other than the recurrent *TERTp* hotspot mutations (32).

TERTp mutations were found in several tumor types with different frequencies. Generally, two types of tumors can be distinguished: those with low and high proliferative potential (33). Tumors with high levels of *TERTp* mutation, such as, melanoma, glioblastoma, bladder cancer or hepatocellular carcinoma (somatic mutation levels of 64–80%, ~84%, ~65%, and 32–45%, respectively) are characterized by low proliferative potential (28, 33–36). Tumors with low or undetectable level of *TERTp* mutation have high proliferative potential, e.g., breast cancer 0.9% (37), testicular cancer 3% (38), intestinal cancer (34) and acute myeloid leukemia and non-Hodgkin's lymphoma (39, 40). It is important to note that *TERTp* mutation was not detected in hematological cell lines cultured *in vitro* (41), as well as in a group of patients with hematological malignances, with the exception of mantle cell lymphoma patients (42). In the case of cancers with low proliferative potential, *TERTp* mutation is considered a late tumorigenic event (33). In some other cancers, e.g., basal cell carcinoma, *TERTp* mutations may appear as a result of environmental factors, such as contact with carcinogens, in which case it is considered as an early tumorigenic event (10, 26). *TERTp* mutations are thought to contribute to tumorigenesis in two distinct phases. In the first phase, *TERTp* mutations heal the shortest telomeres, thus extending life span of cells containing them, but they fail to avert general telomere shortening. This leads to the second phase, where the critically short telomeres result in genomic instability, causing further increase in telomerase expression needed for continued cell proliferation (43).

Another interesting aspect of *TERTp* mutation is the possible cooperation with mutations, such as those in genes coding for BRAF, FGFR3, and IDH (44–48). BRAF is a serine/threonine kinase and its mutation results in activation of the mitogen-activated protein kinase (MAPK) and/or phosphatidylinositol 3-kinase–serine threonine protein kinase (PI3K-AKT) pathways. This leads to upregulation of the ETS system and induction of *TERT* expression. Out of a variety of *BRAF* mutations, *V600E* (a glutamic acid to valine substitution) is the most frequent. This mutation leads to increased GABPA-GABPB complex formation and activation of *TERT* expression (29, 49). Co-existence of *TERTp* mutation and *V600E* is associated with poor prognosis in patients with thyroid cancer, particularly papillary thyroid cancer (8, 50). Fibroblast growth factor receptor 3 (*FGFR3*) is another example of genetic alterations interacting with *TERTp*. Its mutation is well described in urothelial carcinoma (51). FGFR3 belongs to the tyrosine kinase receptor family and stimulates the RAS-mitogen-activated protein kinase and PI3K-AKT pathways. *TERTp* and *FGFR3* mutations are more often present together than alone (47). Co-occurrence of these mutations may support creation of tumors with poor prognosis (10). Additionally, tumors with *TERTp* and/or *FGFR3* mutations had shorter telomeres when compared to tumors without these mutations (47). Malignant gliomas, acute myeloid leukemia and cholangiocarcinoma, are often associated with mutations in isocitrate dehydrogenase 1 and/or 2 (*IDH1* and/or *IDH2*) (52). These somatic mutations occur at arginine residues of the IDH active site (namely, $IDH1^{R132H}$, $IDH2^{R140Q}$, and $IDH2^{R172K}$) (53). According to Diplas et al., *TERTp* and *IDH* mutation status can be used together to classify over 80% of all diffuse gliomas (54). A previous study suggested that presence of *TERTp* mutation and additional 1p/19q co-deletion and also mutation within the *IDH* gene led to a better response to chemotherapy and better outcome in glioma patients (55).

In conclusion, *TERTp* mutation status, alone or in combination with mutations in other genes, can be used to characterize distinguish various types of tumors, as well as predict prognosis and outcome. While *TERTp* mutation status appears to significantly impact cancer development, some cancers, such as prostate, lung, breast, colorectal, and hematological malignancies display telomerase activity, even though they contain few *TERTp* mutations (24, 39, 40, 56, 57). Consequently, other undefined or epigenetic mechanisms of *TERT*-upregulating are expected to exist.

EPIGENETIC MODIFICATIONS

DNA Methylation

Epigenetics describes stable, and possibly heritable changes in activity and expression, which are not associated with any underlying changes in DNA sequence (58). DNA methylation is a common epigenetic mechanism that is essential for regulation of gene expression. It occurs primarily at non-coding regions of DNA characterized by high frequency of CG repeats. Such regions, called CpG islands, are most commonly found in gene promoters. 60–70% of genes contain promoters with these CpG islands (59).

Tissue-specific DNA hypo- or hypermethylation is considered to be important in regulation of gene expression during development. Such tissue-specific DNA hypermethylation is present at promoters rich in CpG islands (60, 61). Promoter DNA methylation is ubiquitous in human cells and is one of the most commonly encountered mechanisms of gene expression regulation. Promoter methylation generally causes gene silencing by interfering with transcription factor bindings sites. Therefore, promoters of actively transcribed genes are normally unmethylated (62). However, DNA hypermethylation may occur at introns/exons (rather than promoters) of actively transcribed genes, as well as at intra- and intergenic enhancers (63). Having an important role in tissue-specific regulation of transcription, DNA hypermethylation may be considered as a marker for a broad variety of diseases and cancers (64, 65).

Promoter methylation is also a major regulatory element of *TERT* expression, correlating both with *TERT* mRNA levels and telomerase activity (66). An approximately 300 bp part of *TERTp* situated on either site of the TSS is unmethylated in actively transcribed *TERT*. However, Castelo-Branco et al. and, more recently, Lee et al. documented that hypermethylation of the *TERT* gene correlates with telomerase activity in different types of cancers (67–69). A study on patients with pediatric brain tumors brought to light a new group of 5 CpG islands located upstream of the TSS, which were hypermethylated and correlated with *TERT* expression. On the other hand, healthy tissues without *TERT* expression did not have this hypermethylation (59). This pattern is counter to the generally established functions of DNA methylation (63). Lee et al. discovered that it is due to presence of a new, larger region known as the *TERT* Hypermethylated Oncological Region (THOR). It is located distal to the TSS and is composed of 52 CpG islands (69, 70). This means that there are two regions of *TERTp* regarding methylation status in telomerase-positive cells: the unmethylated proximal *TERT* core promoter, which is where transcription factors are usually bound, and the hypermethylated THOR, located further away from the core promoter (67, 69, 71) (**Figure 2**). The unusual nature of THOR methylation is due to it acting as a transcription repressor in its unmethylated state. Recently, several authors documented an association between THOR hypermethylation and cancer progression coupled with *TERT* upregulation in pancreatic and gastric cancers (72, 73). Interestingly, both THOR and the *TERTp* region proximal to TSS were mostly unmethylated in normal thyroid tissue (49).

Regarding *TERTp* mutation status, it appears that it does interfere with effects of THOR hypermethylation in cancers where *TERTp* mutation is common. Furthermore, presence of both of these factors may have a synergistic effect on *TERT* expression. In a study on urothelial bladder cancer patients, co-occurrence of THOR hypermethylation and *TERTp* mutation was a marker of higher risk of disease recurrence and progression (74). Likewise, a study on melanoma patients showed a similar effect on reduced recurrence-free survival (75). These and other examples show that *TERTp* mutation coupled with THOR hypermethylation is a better marker of disease progression than *TERTp* mutation alone. Nevertheless, it should be noted that THOR hypermethylation does not associate with progression in a small group of cancers such as esophageal cancer, meningioma or pituitary adenoma (76).

Another interesting issue is the possible interplay between *TERTp* mutation, methylation, and histone modifications, which constitute yet another epigenetic mechanism affecting chromatin accessibility. A study by Stern et al. on monoallelic cancers showed that cancers without a specific *TERTp* mutation at −124 from the TSS had promoter hypermethylation, which was accompanied by repressive histone H3K27me3 methylation, leading to gene inactivation. They hypothesized that presence of this mutation coupled with low *TERTp* methylation discourages H3K27me3 histone methylation in transcriptionally active *TERT* (70). Interestingly, one study showed that *TERTp* hypermethylation was present in both melanoma and normal skin cells. However, only in melanoma cells with *TERTp* mutation did this hypermethylation correspond to increased *TERT* expression and chromatin accessibility (77). A further study by McKelvey et al. on thyroid cancer cell lines heterozygous for *TERTp* mutation demonstrated conclusively that *TERTp* methylation was allele-specific, whereby *TERTp* with mutation was significantly less methylated than wildtype promoter. Moreover, MYC, a transcription activator, bound only to the hypomethylated mutated *TERTp*, resulting in monoallelic expression (MAE) in heterozygous cells (29). MAE is one of two TERT expression categories as described by Huang et al., the other being biallelic expression (BAE, both alleles transcriptionally active). These two expression patterns appeared to be specific for many cancers, although some cancers exhibited variation between MAE and BAE in differed cell lines (34). However, a later study by Rowland et al. showed that this simple classification into MAE and BAE-specific cancer cell lines does not sufficiently describe the complex nature of *TERT* expression. In a study conducted on a single cell-level, they found great heterogeneity in TERT expression between various cells, within both the cell lines described as MAE, and those described as BAE by Huang et al. (78).

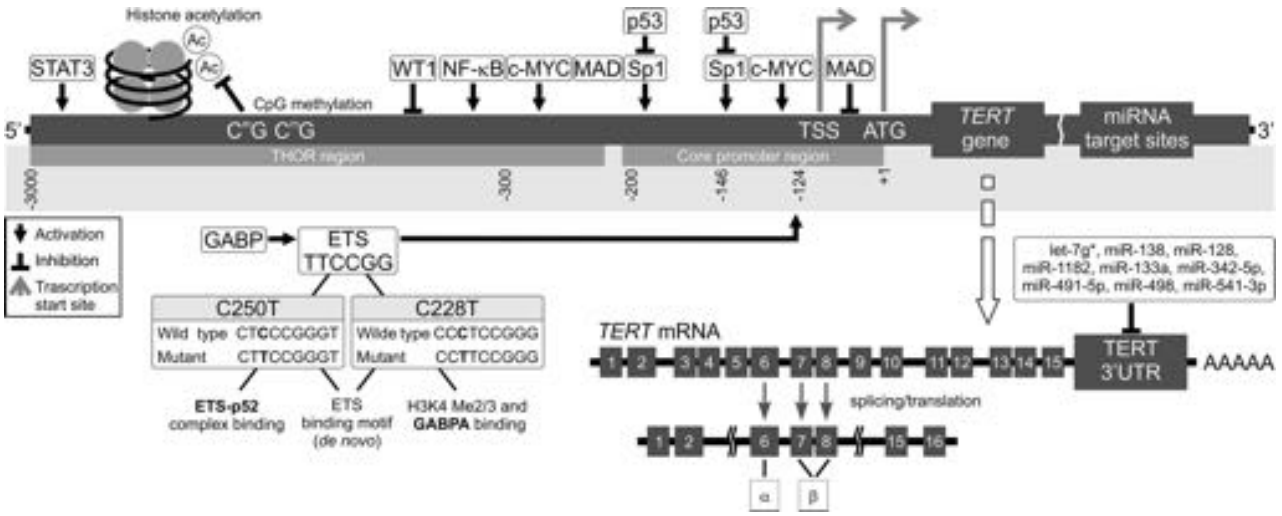

FIGURE 2 | Mechanisms of *TERT* transcription regulation. The figure shows various mechanisms regulating *TERT* expression at the transcriptional level. Transcription factors: activators (e.g., c-MYC, SP1, STAT3, NF-κB, and ETS), repressors (e.g., MAD, p53, and WT1), and their respective binding sites are shown. Binding of these transcriptional agents to *TERT* could be controlled by DNA methylation (CpG sites) in the TERT Hypermethylation Oncological Region (THOR). Two main hotspot mutations within *TERTp*, -146C > T (C250T) and -124C > T (C228T) upstream of the transcription start site (TSS) generate new E-twenty-six (ETS) binding sites, leading to GABP recruitment and, eventually, *TERT* transcription. Alternatively spliced variants of *TERT*, which do not have telomerase activity, could be also generated. Most tissues and organs express no or very low levels of *TERT* mRNA, dependent on histone markers that are correlated with passive or active transcription in many cells. The figure also shows different miRNAs at the 3'UTR that inhibit translation of *TERT*.

micro-RNA

Most recent studies focus on *TERT* regulation at the transcriptional level. Meanwhile, post-transcriptional regulation by microRNAs (miRNAs), has not been expensively studied. miRNAs are a class of small non-coding RNAs (~22–24 nucleotides) (79). miRNA recognition sites are typically located in 3′ untranslated regions (3′UTRs) of mRNA (**Figures 1** and **2**). miRNAs binding to 3'UTR generally silences the transcript, thus reducing gene expression. miRNAs are ubiquitous elements of gene regulation, and control many different biological processes. In cancer, miRNAs function as gene regulatory molecules, acting as tumor suppressors or oncogenic drivers (18, 80).

Various miRNAs are known as regulators of *TERT*. In particular let-7g-3p, miR-128, miR-133a, miR-138-5p, miR-498, miR-541-3p, and miR-1182, downregulate expression of *TERT* and telomerase activation (18, 81). Functional analyses indicated that overexpression of miR-138-5p and miR-422a significantly inhibit *TERT* expression through interaction with *TERT* 3'UTR in colorectal cancer cells (79, 82). Moreover, miR-138-5p represses *TERT* protein expression in human anaplastic thyroid carcinoma and cervical cancer cells (79, 83). Likewise, miR-1182, miR-1266, miR-532, miR-1207-5p, and miR-3064 suppress gastric, bladder, ovarian cancer growth and invasion by binding to the *TERT* 3'UTR (10, 79, 84, 85). Furthermore, miR-128 was found to control TERT expression in HeLa and teratoma cell lines (81, 86).

miRNAs can also regulate *TERT* indirectly by controlling expression of various transcription factors. Accordingly, c-MYC, a major regulator of TERT, was regulated by miR-494 and miR-1294 in esophageal squamous cell carcinoma and pancreatic cancer. Additionally, c-MYC and FoxM1 were targeted by a known tumor suppressor, miR-34a, causing senescence in cells (18). Interestingly, the study of Lassmann et al. suggested that *TERT* is able to regulate miRNA levels at the early phase of miRNA processing. They demonstrated that deletion of *TERT* resulted in a decrease of most mature miRNAs (87).

TRANSCRIPTION FACTORS

Transcriptional Activators

TERTp contains binding sites for a huge number of transcriptional activators and repressors that directly or indirectly regulate gene expression. Multiple pathways, such as RAS/RAF/MEK/MAPK, PI3K/Akt/mTOR, IKK/NF-κB, transforming growth factor β/Smads, PKC, and the JAK-STAT pathway regulate *TERT* expression and telomerase enzymatic activity (88). In fact, most transcription factors have been identified as possible *TERT* gene regulators, such as protein kinases, growth factors, and oncogenic proteins. Canonical positive regulators of *TERT* transcription include the oncogene c-MYC, Sp1, NF-κB, STAT family of proteins, AP-2, and GSC. These activatory transcription factors will be described in detail in the following section.

MYC encodes a basic helix-loop-helix leucine zipper (bHLH-LZ) transcriptional factor called c-MYC (89, 90). The *MYC* gene family regulates expression of genes implicated in many processes, such as proliferation, cell growth, differentiation, self-renewal, apoptosis (91, 92). It is essential for embryonic development and it is expressed in normal somatic cells. There are several ways for healthy cells to control MYC levels, such as targeted degradation by the ubiquitin-proteasome system (92).

Chromosome translocations, gene amplification, retroviral insertion or mutations of *MYC* gene are tumorigenic in mice and correlate with development of most human cancers (93, 94). c-MYC functions is dependent on heterodimerization with MAX (90, 95). While *MYC* gene contains a transcription activation domain, no such regulatory domain has been reported for MAX (96). The c-MYC/MAX heterodimers can bind to specific DNA sequences located within the core promoter region, known as E-box motifs (5′-CACGTG-3′), thus activating various genes (90, 92). c-MYC activates telomerase by inducing expression of *TERT* (90, 94). In addition, *TERT* is responsible for maintenance of c-MYC levels and regulates c-MYC proteasomal degradation (97).

The core promoter of *TERT* also contains specificity protein 1 (Sp1) binding sites that are necessary for *TERT* expression. Sp1 belongs to the family of nuclear proteins called Sp/KLF (specificity protein/Krüppel-like factor) that binds GC-(GGGGCGGGG) and GT-(GGTGTGGGG) rich elements (98, 99). It is one of the best characterized transcriptional activators of housekeeping genes and other TATA-less genes (89, 99). Sp1 regulates processes such as inflammation, carcinogenesis, senescence, hormonal activation, apoptosis and angiogenesis (98). Transcriptional activity of Sp1 is regulated by a few post-translational modifications (glycosylation, acetylation, phosphorylation) and by direct interaction with other proteins, including other transcription factors, nuclear factors, oncogenes, and tumor suppressors. Sp1-silencing completely inhibits telomerase activity by suppressing *TERT* expression, leading to apoptosis. Furthermore, mutations in Sp1 binding sites (GC-boxes) significantly decrease transcriptional activity of *TERTp*, suggesting that Sp1 protein is involved in *TERT* transcription (100). Some reports indicated that cooperation between Sp1 and c-MYC drives cell type-specific *TERT* expression. This is further substantiated by the fact that normal cells have lower levels of Sp1 and c-MYC than cancer cells. However, Sp1 would be a weak candidate for a biomarker of cancer-specific *TERT* expression because of its ubiquitous expression in normal cells (89, 100).

NF-κB is well known for playing a major role in inflammation, tumorigenesis, cytokine and chemokine expression, stress regulation, cell division and transformation (101, 102). NF-κB regulates expression of apoptosis inhibitors. The NF-κB signaling pathway is a master regulator of *TERT* activation in cancer cells. It initiates expression of *TERT* by binding to either of two potential motifs in *TERTp* (101). Additionally, *TERT* can directly regulate expression of NF-κB-dependent genes through binding to the p65 subunit. Studies have demonstrated that telomerase can directly regulate recruitment to promoters of NF-κB target genes, such as those encoding interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) that are critical for inflammation and cancer progression (103).

The signal transducer and activator of transcription (STAT) family of cytoplasmic proteins are direct mediators of signaling from the extracellular environment to the nucleus (104). Seven STAT proteins have been identified as STAT 1–4, 5A, 5B, and 6 (105–107). They are normally inactive, but can be activated by phosphorylation. Of the seven human STAT encoding genes, STAT3 has drawn the most interest for its association with a wide variety of human cancers (104, 108).

In addition, these proteins are able to regulate *TERT* expression in tumor and normal cells (104). *TERTp* contains binding sites STAT3 and is overexpressed in prostate, breast, head, neck, and hematologic cancers, which implicates STAT3 as an important anticancer target (105).

The adipocyte protein 2 (AP-2) family of transcription factors contains five isoforms: AP-2α, AP-2β, AP-2γ, AP-2δ, and AP-2ϵ (109, 110). They are encoded by the *FABP4* gene. These isoforms have a major role in gene regulation and have different biological functions. They are required for morphogenesis during embryonic development (109). AP-2β specifically binds to the *TERTp* and activates telomerase in human cancer cells, but not normal cells. Two E-box sites in a 320-bp region of *TERTp* (320 bp upstream of the translational ATG site) have been observed to regulate promoter activity in human rhabdomyosarcoma cells (110).

A recent showed that goosecoid homebox protein (GSC) may be a new potential activator of *TERT* expression (49). It is normally involved in embryonic development and interacts with TGF-β and Wnt/β-catenin signaling pathways, which are implicated in tumor invasion (111). It was found to be overexpressed and to correlate with metastasis in patients with breast carcinoma (112), and was also associated with poor prognosis and chemoresistance in ovarian carcinoma (111). An analysis of *TERTp* areas with locally decreased methylation in thyroid cancer cells revealed a GSC biding site. GSC is a TERT activator and was variously expressed in both thyroid cancer and normal thyroid cells. Additionally, GSC was overexpressed in thyroid cancer (49).

Transcriptional Repressors

Transcriptional repressors are proteins that attach to DNA at specific silencer sites and block transcription of nearby genes. In the following section, we are going to briefly discuss repressors that have been shown to downregulate *TERT* transcription, such as MAD1/2, p53, WT1, CTCF, and MZF-2.

The mitotic checkpoint is a crucial mechanism in maintaining chromosomal stability. It guarantees precise chromosome segregation by delaying separation of replicated sister chromatids (113, 114). Mitotic arrest deficient 1 (MAD1) is a major element of the mitotic checkpoint, and it recruits its binding partner MAD2 to nuclear pores (113, 115). During mitosis, MAD1 localizes to unattached kinetochores, where it serves as a docking site for MAD2. Kinetochore-bound MAD1–MAD2 act as a catalyst for conformational change of free MAD2 (114, 116). MAD1 upregulation serves as a marker of poor prognosis, as it tends to be overexpressed in cancers (116). Upregulation of MAD1 leads to chromosomal instability and resistance to microtubule poisons that are currently used as chemotherapeutic agents (116). MAD1 is recognized as an important cellular antagonist of c-MYC (117, 118). In addition, c-MYC and MAD1 are involved in regulation of *TERT* expression because they bind to the same promoter sites (E-box) to activate *TERT* expression (119). There two E-boxes in *TERTp*, and both of them constitute binding sites for c-MYC/MAX or MAD1/MAX heterodimers (117, 120). A switch from c-MYC/MAX to MAD1/MAX, triggers decrease in H3 and H4 histone acetylation at *TERTp* (119, 120).

p53 is the best known human tumor suppressor which is a member of a larger p53 family of tumor suppressors (121, 122). Other than p53, this family also includes p63 and p73 (123, 124). p53 acts primarily as an inducer of cell cycle arrest, cell differentiation, senescence, and apoptosis in response to numerous intrinsic and extrinsic stress signals (122, 125). It has a major role in the control of genomic stability, DNA replication, and DNA repair. The p53 encoding *TP53* gene is mutated in approximately 50% of human cancers. *TERTp* contains two p53 binding motifs (123). Several findings showed that p53 suppresses telomerase activity by inhibiting *TERT* expression (125). This inhibition may be caused indirectly, by an interaction between Sp1 and overexpressed p53 (125, 126). Furthermore, this inhibition of *TERT* could be possibly independent of other p53 functions, such as those associated with apoptosis (125).

Another protein implicated in inhibition of *TERT* is the Wilms' tumor 1 (WT1) tumor suppressor (125). It contains four zinc fingers and an RNA-binding protein that directs the development of several organs (heart, diaphragm) and genitourinary tissues (127, 128). It is normally expressed in kidney, testes, ovaries, and spleen (129). Most neoplasms, including lung carcinomas, renal cell carcinoma, pediatric sarcomas, and breast, ovarian, colon, melanoma, and pancreas cancers, and exhibit a possible oncogenic activity of WT1 (130, 131). In addition, it is overexpressed in most acute myeloid leukemia patients, and is considered to be an independent marker of minimal residual disease (132). A WT1 binding site is located in *TERTp* (−352 upstream of the TSS), and its mutation significantly reduced telomerase activity and *TERT* mRNA expression in 293 embryonic kidney cells but not in HeLa cells (1, 89, 125). Additionally, WT1 inhibited *TERT* transcription during differentiation. This inactivation may influence activation of telomerase in the tumorigenesis phase. Furthermore, WT1 binding to *TERTp* suppresses c-MYC level at both protein, and mRNA level (1, 2).

CCCTC-binding factor (CTCF) is a zinc finger transcription factor which is ubiquitously expressed in human (133). Its binding sites are located in the first two exons of the *TERT* gene, and are located in a CpG island. Earlier studies showed that CTCF does not bind to TERT in telomerase-positive cells, which is correlated with methylation of exon 1 in these cells (134). Hypermethylation in this exonic region is common in most cancers, and CTCF is considered a major TERT repressor in normal cells. Methylation at specific CpG dinucleotides in exon 1 results in a change in secondary structure of DNA and creation of a four-strand structure known as G-quadruplex, which disrupts CTCF binding (135). Interestingly, CTCF was observed not to bind to TERT in normal thyroid tissue despite the presence of methylation, while thyroid cancer cell lines exhibited both partial methylation and CTCF binding (49).

The myeloid zinc finger protein (MZF)-2 is a Krüppel-like C_2H_2 zinc finger protein expressed predominantly in myeloid progenitor cells and involved in growth, differentiation, and tumorigenesis (136). The mechanisms involved in MZF-2-induced suppression of *TERTp* activity are still unclear (137). There are multiple binding sites for MZF-2 within the *TERTp* region, and upregulation of MZF-2 inhibits *TERTp* activity. This suggests a role for MZF-2 in transcriptional downregulation of *TERT* (125, 137).

TERT GENE POLYMORPHISMS

Single Nucleotide Polymorphism

Single nucleotide polymorphisms (SNPs) have been described as being associated with increased risk for developing various cancers. They may be located both in intronic and exonic sequences of *TERT*, as well as in *TERTp*. Some common *TERT* SNPs found may modify survival and prognosis of certain cancers. A number of studies have recently been conducted to identify new SNP *loci* related to telomere length, which have shown a relationship between the risk of disease, its severity and the survival time in various cancers (138–140). In this section, we will discuss four common *TERT* polymorphisms that may be associated with gene expression.

Located at intron 2 of the *TERT* gene, rs2736100 A>C is a important non-coding SNP (141, 142). It has been associated with multiple cancers, especially with lung adenocarcinoma, which is characterized by significantly increased *TERT* gene expression, telomerase activity and gene copy number (143). Other solid cancers that are associated with this SNP include gliomas, bladder cancer, melanoma. rs2736100 has been identified as a major predisposing factor to sporadic and familial myeloproliferative neoplasms (MPNs), independently of the major diagnostic and molecular MPN subtypes. The *C* allele of rs2736100 and JAK2 46/1(GGCC) haplotype are major factors predisposing to MPN (141–143). Interestingly, the two alleles of rs2736100 seem to be associated with different types of diseases. While the *C* allele is primarily associated with cancers, the *A* allele, which is linked to shorter telomeres, is generally associated with predisposition to degenerative diseases (144). Furthermore, rs2736100 *C* is linked to increased blood cell count in the Japanese population (145).

Another *TERT* SNP, rs2853669 A>G is located in the *TERT*p region. It obstructs an ETS2 binding site, located close to an E-box. Previous studies showed that *TERTp* mutations creating a putative binding site for ETS, resulted in *TERT* upregulation and increased telomerase activity, while mutations at the ETS2 binding site suppressed c-MYC binding to the E-box (146, 147). Studies on rs2853669 showed that it is significantly associated with poor survival and increased cancer risk rate in hepatocellular carcinoma patients (146). In contrast, it was also observed to correlate with improved survival in patients with clear cell renal cell carcinoma, melanoma and glioblastoma (148). The *C* variant of this functional polymorphism results in decreased telomerase activity. Several studies suggest that rs2853669, in the presence of certain *TERTp* mutations, may also affect development of cancers (149). It was reported that it could influence telomere length and telomerase activity (150, 151). Furthermore, a study by Rachakonda et al. demonstrated that, in patients with urothelial bladder carcinoma, *TERT* rs2853669 may correlate with survival, prognosis, and tumor recurrence (152).

The two SNPs described above were located in non-coding regions of *TERT*, there are however also SNPs situated in exonic regions, of which rs2736098 G>A is a notable example. It is a synonymous A305A substitution located in exon 2, and was found to correlate with telomere length (153). Genotype *GG* was found to be associated with longer telomeres and decreased cancer susceptibility in patients with renal cell carcinoma (154). In another study, Xiao et al. showed that Chinese males harboring allele rs2736098 *A* had a greater risk of developing lung cancer than those with allele *G* (155). Allele *A* was also found to be significantly associated with risk of bladder cancer in the North Indian population (156). Further studies showed that it may impact risk for many other cancers, such as breast, esophageal, prostate, and basal cell carcinoma (153, 157).

Variable Number of Tandem Repeats Polymorphism

It was demonstrated that *TERT* may be regulated *via* a variable number of tandem repeats (VNTR) polymorphism named MNS16A (**Figure 1**). It is located upstream of promoter region of an antisense *TERT* transcript. Depending on the number tandem repeats, promoter activity is affected differently. There are two MNS16A variant alleles: short (*S*) and long (*L*). The *L* allele correlates with higher promoter activity in the antisense strand and increased expression of the antisense *TERT* transcript. This increased expression of antisense *TERT* leads to silencing of functional *TERT* (158). As a result, the *S* allele is associated with higher telomerase activity, while *LL* homozygotes have lower telomerase activity (158). Our previous work showed that the *S* variant was more frequent in non-Hodgkin's B-cell lymphoma patients how did not respond to treatment, as well as those with intermediate/high International Prognostic Index (159). In contrast, the *S* variant was less frequent in chronic lymphocytic leukemia patients with high disease stage (160).

ALTERNATIVE SPLICING

TERT regulation is a multifarious process, which involves not only the transcriptional mechanisms described in the previous sections, but also posttranscriptional ones. This includes pre-mRNA alternative splicing of the *TERT* gene (161–163). There as many as 22 potential alternative splicing sites in the *TERT* gene, but the function of many of them is unclear (164–168). One of the most commonly studied splicing sites are deletions at two sites, α and β (**Figure 2**). The β splice site results in a major deletion (182 bp) and creates a non-functional, truncated protein. The α splice site generates a smaller (36 bp) deletion, which produces an impaired protein. Both of these splice sites result in TERT proteins that are incapable of telomere elongation (169–172). In many cancers, the full length TERT transcript (α+β+) correlated with tumor development and shorter survival in patients (173). However, the α variant alone is known to cause decreased telomerase activity and shorter telomeres, while the β splice variant was reported to not only inhibit telomerase activity but also the ability of cancer cells to induce apoptosis (174, 175). Another splice *TERT* variant may be generated by a deletion of exons 4–13, resulting in an inactive protein lacking its catalytic domain. This deletion was observed in both telomerase-negative and -positive cells, and was associated with increased cell proliferation (6).

INVOLVEMENT OF TERT IN NON-TELOMERE-RELATED MECHANISMS

In the previous sections, we described *TERT* regulation and telomerase reactivation mechanisms that are involved in telomere maintenance. Telomere-related functions of TERT, also known as canonical, may likewise entail prevention of chromosome fusions (176, 177). However, telomerase also has non-canonical (telomere-independent) roles (**Figure 1**). These roles can be grouped into two broad categories: a) involving telomerase activity but not telomere elongation and b) involving neither telomere elongation nor telomerase activity (177). The telomere-independent roles contribute to the regulation of metabolic mechanisms, epigenetic regulation of chromatin, stress response, RNA silencing, signal transduction pathways (Wnt and c-MYC signaling pathways), enhanced mitochondrial function, cell adhesion, and migration (176, 178, 179).

TERT is found in cytoplasm and mitochondria, alongside its usual nuclear localization (176, 180) (**Figure 1**). In humans, mice and rats, TERT contains two specific targeting sequences that regulate its transport in and out of organelles: a nuclear targeting signal sequence, and a mitochondrial targeting sequence (181). In inactive $CD4^+$ lymphocytes, TERT is mainly cytoplasmic but after activation it is transported to the nucleus in a process controlled by the kinase Akt (182). Additionally, shuttling TERT out of the nucleus may be promoted by oxidative stress, and this mechanism is dependent on phosphorylation of tyrosine 707 by Src kinase. Translocation of TERT into mitochondria improves mitochondrial potential which eventually leading to cancer cell survival (183). The extra-nuclear TERT functionalities are generally thought of as non-telomere related, i.e. non-canonical, and will be described below (179).

Cytoplasmic TERT exhibits many functions, including interacting with signaling pathways such as Wnt/β-catenin signaling. In addition, TERT binds to stress particles under non-stress conditions, and in lymphocytes, it is stored outside the nucleus without stimulation. TERT may also form a part of a TERT–NF-κB subunit p65 complex, which can move from the cytoplasm to the nucleus in multiple myeloma cells, upon TNF-α induction (184). NF-κB, in turn, controls expression of a variety of genes involved in inflammation, immune responses, and cell differentiation (179). Zhou et al. demonstrated that the endoplasmic reticulum transiently activates the expression of *TERT* in cancer cell lines (185).

As much as 10–20% of total TERT is localized in mitochondria (176, 179). Therein, TERT binds to mitochondrial DNA (mtDNA) and improves respiratory chain activity, protecting mitochondrion from environmental damage and decreasing reactive oxygen species (mtROS) production (180, 186). mtROS production leads to mitochondrial damage and telomere shortening. Neutralization

of mtROS does not recover the mitochondrial function but reduces telomere shortening (187). Additionally, telomere and mitochondrial disfunction is mediated by p53, which induces growth arrest, senescence and apoptosis in cells (188). TERT import depends on membrane potential and it is located close to the inner membrane (181). *TERT* binds to mtDNA in the region coding for NADH ubiquinone oxidoreductase subunits 1 (ND1) and 2 (ND2) and protects mtDNA from environmental damage (181). Mitochondrial TERT plays a role in decreasing apoptosis and improving mitochondrial membrane potential. Furthermore, it has unusual DNA- and RNA-dependent RNA polymerase activities, upon interaction with tRNAs (189). TERT can also interact with mitochondrial RNA processing endoribonuclease (RMRP) and use the RNA-dependent RNA polymerase to synthesize dsRNA. Mutations in RMRP can interfere with RMRP-TERT binding, contributing to pleiotropic syndrome cartilage–hair hypoplasia (190).

TERT AS A POTENTIAL THERAPEUTIC TARGET

The unique feature of telomerase is its low or nonexistent expression in somatic cells, but overexpression in most cancer cells (191). Thus, telomerase and other telomere components offer a highly attractive diagnostic and prognostic biomarker of cancer and a target for development of therapeutics. Several strategies have been devised to target telomerase functions: telomerase inhibition, telomerase peptide vaccines, and suicide gene therapy. Epigenetic processes were suggested as another promising target for therapeutic purposes (192). Some of these are already used in treatment of patients as part of clinical trials (193).

TERT inhibition has been regarded as a promising therapeutic strategy, as earlier *in vitro* studies showed that TERT silencing cell proliferation (194, 195). An early approach was to design compounds that would interact with DNA at the 3' overhang, stabilizing telomeric G-quadruplex secondary structures, and thus blocking telomerase access to DNA. Telomestatin, BRACO-19, RHPS4, TMPyP4 are some of the most commonly studied G-quadruplex binding proteins (191, 196, 197). Telomestatin (OBP-301) is a natural product isolated from *Streptomyces anulatus* (198). The primary mechanism of telomestatin action involves a highly specific interaction with the G-quadruplex to stabilize its structure (199). These DNA-binding compounds are now less popular due to discovery of better molecular strategies, such as targeting the TERT active site directly. Studies on such inhibitors led to discovery of 2-[[(E)-3-naphthalen-2-ylbut-2-enoyl]amino]benzoic acid (BIBR1532), which inhibits telomerase by binding non-competitively to the TERT active site (197, 200). This binding leads to increased oxidative stress and decreased nitrogen monoxide bioavailability in favor of H_2O_2. However, BIBR1532 has not yet progressed to clinical tests (201). Aside from synthetic compound, various naturally occurring compounds, such as allicin (from garlic), curcumin (from turmeric), silibinin (from thistle), and epigallocathechin gallate (EGCG, from tea) were found to have telomerase inhibitory properties (202). A synthetic, more stable derivative of EGCG, MST-312, was shown to inhibit telomerase in various cancer, although its mechanism of action remains unknown (203–205).

Some peptide vaccines can possibly target the telomerase active site (199). GV1001 (KAEL-GemVax Co. Ltd., Gangnam-gu Seoul, Republic of Korea) is the only such vaccine to enter clinical trials (206). Its structure is based on a peptide sequence from TERT active site and it capable of binding multiple HLA class II molecules. It functions by stimulating tumor-reactive $CD8^+$ and $CD4^+$ T-cell immunity specific for TERT (199, 207, 208). GV1001 is used in treatment of patients with advanced stage melanoma, lung, hepatocellular carcinoma and pancreatic cancer (196). Two other TERT-based peptide vaccines, p540 and p675 were also observed to elicit TERT-specific cytotoxic T cell HLA-A*02:01- restricted immunity (208, 209). Other TERT-based vaccines are composed of more than one separate peptide sequence. An example of such a vaccine is GX301, composed of four peptides. This multi-peptide character means that it recognizes more HLA haplotypes, binding to both class I and II HLA molecules (210). GX301 is currently (October 2020) in phase II of a clinical trial on patients with prostate cancer (211). GRNVAC1 is a dendritic cell vaccine, which was created by transfecting dendritic cells with mRNA encoding TERT-chimeric protein, and then returning the transfected cells to the patient (196). These cells would then target telomerase-expressing tumor cells. The clinical trial is in phase I/II, and the vaccine is currently used in treatment of patients with metastatic prostate cancer (196, 207).

Another strategy are the suicide gene therapies. They include oncolytic virotherapy, the predominantly used strategy to treat cancer, which has potential to specifically lyse the tumor, and not healthy cells. This approach involves adenoviruses replicating selectively in cancer cells, and subsequently killing them (212). This viral system relies on the highly active *TERC*/*TERT* promoter controlling expression of a bacterial protein nitroreductase. Neither this nor any other suicide gene therapy has entered into clinical trials (193).

Recent studies increasingly suggest that epigenetic mechanisms may be targeted in new therapeutic strategies. Chidamide, an inhibitor of the enzyme histone deacetylase, was shown to decrease telomerase expression through miR-129-3p up-regulation in non-small cell lung cancer cells. This leads to subsequent ROS accumulation and subsequent cell cycle arrest (213). Epigenetic mechanisms may also be exploited in potential therapies using personalized approach. A study on effects of all-trans retinoic acid (ATRA) in treatment of ovarian carcinoma patients showed that the efficacy of therapy correlated inversely with methylation level of *TERTp*. This was of particular interest in a large subgroup of serous ovarian carcinoma patients, who had hypomethylated *TERTp*, and could therefore be treated effectively with ATRA (214).

As shown by the examples described above, telomerase is an attractive target for cancer immunotherapy. The main advantage of TERT is its high cancer-specific expression. Results from clinical trials have been encouraging, because of the safety and good tolerability of telomerase inhibitors (215). As a final point, it

should be noted that using just one type immunotherapy may not suffice to eliminate cancer cells. Therefore, new studies should focus on strategies integrating various types of therapies (216).

SUMMARY

TERT is normally actively transcribed only in early embryonic development and in cells with high proliferative potential, while it is inactive in most somatic cells in adults. However, in most cancers, *TERT* undergoes reactivation, and by extending telomeres (the canonical function of TERT) it contributes to cancer formation and progression. There are many regulatory mechanisms involved in telomerase reactivation and adjustment of *TERT* expression, among which *TERTp* mutation is perhaps the most important. Other major TERT regulation mechanisms (also known as telomere maintenance mechanisms) are: chromosome rearrangements, methylation, miRNA interference, binding of transcription factors, genetic polymorphism, and alternative splicing. Some of these mechanisms may interact with each other, having a synergistic effect on TERT expression. Aside from the better-known telomere lengthening function, TERT also has many secondary, telomere-independent roles (non-canonical functions of TERT). Taking in to account its major importance in cancer, TERT has become a target of various therapeutic strategies in cancer treatment and continues to be an interesting object of research.

The following features of TERT described in this manuscript can be highlighted:

- TERT is a functional catalytic protein subunit of telomerase, which lengthens telomeres by adding short DNA repeats, consequently averting chromosomal instability;
- Its regulation is a multifarious process where both transcriptional and posttranscriptional mechanisms are involved;
- TERT is also a major component of various oncogenic signaling pathways, and its overexpression often contributes to tumorigenesis;
- *TERT* gene is often overexpressed in cancers, and this overexpression can be induced by a variety of mechanisms, such as: *TERT* gene amplification, *TERT* gene polymorphism, *TERTp* mutation and methylation, and miRNA interference, alternative splicing of the *TERT*;
- Aside from its primary nuclear localization, TERT can also be transported to cytoplasm and mitochondria;
- It has many non-canonical, i.e. telomere-unrelated, functions these include: interaction with signaling pathways, stress protection, regulation of chromatin structure, binding to and protection of mitochondrial DNA;
- TERT and its gene may also act as an attractive target for therapeutic interventions with a diagnostic and prognostic impact.

AUTHOR CONTRIBUTIONS

MD and KB-K contributed to the conception and design of the review, drafted, and finalized the manuscript. BW contributed to the conception and design of the review and its draft version. PŁ contributed to the writing, reviewing, and editing of the final version of the manuscript. TK contributed to the final version of the manuscript and drew all the figures. All authors contributed to the article and approved the submitted version.

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CONCLUSIONS

1. The haematological cell lines were characterized by higher expression of the *TERT* gene compared to solid tumour cell lines, in the absence of the *TERT*p mutation (Dratwa et al., 2020).
2. The presence of somatic mutations *C228T* and *C250T* located in *TERT*p affects the length of telomeres in solid tumour cell lines (Dratwa et al., 2020).
3. The *in vitro* research model used showed significant correlations between *TERT* gene expression and telomerase activity, *TERT* gene expression and telomere length (only in haematological lines), and between telomerase activity and telomere length (Dratwa et al., 2020).
4. Genetic variation in *TERT* is related to telomere length, overall survival, and disease severity in patients with haematological malignancies (Dratwa et al., 2021; Wysoczanska et al. 2019).
5. There are statistically significant associations between single nucleotide polymorphisms of the *TERT* gene, the variable number of tandem repeats - VNTR-MNS16A and telomere length, tumour invasiveness and *HER2* gene amplification in women with breast cancer (Dratwa et al., 2022).
6. Important differences in expression level of *TERT, MYC*, *SP1*, *TP53* and their relationships between cells cultured from tumour tissue (organoids) and cells from whole blood of breast cancer patients were found (Dratwa et al., 2022).
7. Telomere length depends on age, presence of mutations and single nucleotide polymorphisms, stage of the disease, and type of cancer (Wysoczanska et al. 2019; Dratwa et al., 2021; Dratwa et al., 2022).
8. Results of these studies suggest that genetic variability and expression of *TERT* gene, as well as telomere length can be used as potential biomarkers in haematological and solid cancers.