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Expression of *TERT* (AS) alternative splicing variants and *TERF2* in osteosarcoma

Indhira Dias Oliveira[1,2], *Antonio Sergio Petrilli*[1], *Carla Renata Pacheco Donato Macedo*[1], *Maria Teresa de Seixas Alves*[1,3], *Reinaldo de Jesus Garcia Filho*[1,4], *Silvia Regina Caminada Toledo*[1,2]

[1] Pediatric Oncology Institute/GRAACC, Department of Pediatrics, Federal University of São Paulo, São Paulo, SP, Brazil; [2] Department of Morphology and Genetics, Division of Genetics, Federal University of São Paulo, São Paulo, SP, Brazil; [3] Department of Pathology, Federal University of São Paulo, São Paulo, SP, Brazil; [4] Department of Orthopedics and Traumatology, Federal University of São Paulo, São Paulo, SP, Brazil

Summary. *Background:* Osteosarcoma (OS) is the most common malignant bone tumor in children and adolescents. Mechanisms of telomere maintenance (TMM) are central features of the tumor cells to maintaining their proliferative capacity. *Aim:* In this study, we investigated the expression of *TERT* (telomerase reverse transcriptase) alternative splicing (AS) variants, *TERC* (telomerase RNA component), *TERF1* (telomeric repeat binding factor 1) and *TERF2* (telomeric repeat binding factor 2) and quantified telomerase enzyme activity in samples of OS, correlating with clinical and pathological aspects. *Methods:* A total of 70 fragments of OS tumor samples and 10 normal bone samples (NB) were used for the analysis of gene expression by nested RT-PCR (reverse transcriptase-polymerase chain reaction) and qPCR (quantitative real time PCR). For the quantification of telomerase by TRAP assay we used 20 OS samples and two NB samples. *Results:* We observed the expression of *TERT* in 44% of the tumors and full length (FL) isoform in only 4%. *TERF2* expression levels were higher in PRECH (pre-chemotherapy) samples than in POSTCH (post-chemotherapy) (p=0.0468). POSTCH samples from patients without relapse showed lower levels of expression of *TERF2* (p=0.0167). *Conclusions:* Despite the high expression of *TERC*, the low prevalence of isoform FL suggested that telomerase may not be the main TMM in OS. Recent studies have correlated *TERF2* overexpression to different mechanisms of resistance to chemotherapeutic drugs. Low levels of *TERF2* gene expression, in POSTCH samples from patients with no relapse of disease suggest a correlation with a better response to treatment.

Key words: pediatric osteosarcoma, telomere maintenance, *TERT,* alternative splicing variants, *TERC*; *TERF1, TERF2*, gene expression, molecular marker

Introduction

Osteosarcoma (OS) is the most common primary bone tumor in children and young adults (1). The presence of metastasis at diagnosis is a prognostic factor, with a strong impact on the overall survival of these patients. Patients without metastasis at diagnosis have a 5-year overall survival rate of 70%, whereas overall survival falls to 32% in patients with metastasis at diagnosis (2). For patients with OS, the use of chemotherapy has improved survival from 11% with surgical resection alone in the 1960s, to 70% by the mid-1980s. However, survival has since plateaued, despite advances in anticancer therapy (3).

Clinical trials that attempted to improve outcome through intensification of therapy or incorporation of new agents have not been widely successful. Therefore, increasing focus has been placed in achieving a greater understanding of basic aspects of OS biology to improve the treatment and overall survival of the patients (3). Among the biological aspects of OS investigated, the telomere biology has been pointed as a potential source of the instability typical of OS, in addition to reducing the likelihood of favorable outcome in patients with the disease (4-9).

Telomeres constitute the ends of eukaryotic chromosomes and are composed of 1000 to 2000 tandem repeats of the hexanucleotide sequence TTAGGG. They progressively shorten in somatic cells during each cell cycle until apoptosis or cell cycle arrest is triggered (10). The stabilization of telomere length plays an important role in tumorigenesis and the maintenance of telomeres is a prerequisite for malignant tumors to preserve their ability to proliferate (11). Cancer cells commonly maintain telomere length by strictly regulating telomerase expression and/or catalytic activity. An alternative mechanism consists of lengthening telomeres by recombination-mediated DNA replication, although the latter mechanism is more prevalent in tumors arising from mesenchymal tissues than in epithelial tumors (12, 13).

Human telomerase consists of a ribonucleoprotein containing a protein catalytic subunit, TERT (telomerase reverse transcriptase), and an RNA component, TERC (telomerase RNA component) (14). *TERT* gene contains 16 exons can be spliced into multiple isoforms (15). To date, 22 isoforms of *TERT* transcripts have been identified (16-18) which can theoretically produce multiple tissue-and disease-specific alternative transcripts (18, 19). Besides FL (full-length) transcript, with all 16 exons, none of the identified AS (alternative splicing) variants has reverse transcriptase activity and they cannot elongate telomeres (20, 21). The alternatively spliced variants within the reverse transcriptase domain of *TERT* include inhibitory α^- (alpha deletion), β^- (nonfunctional beta deletion), or $\alpha^-\beta^-$ (both alpha beta deletions) patterns (20-22).

More recently, a host of six telomere binding proteins denominated shelterin, were described critical for assuring that telomeres do not trigger a DDR (DNA-damage response), since an unfolded telomere could be sensed as a double strand DNA break (23). Two of the shelterin components, TERF1 (telomeric repeat binding factor 1) and TERF2 (telomeric repeat binding factor 2), are necessary for the proper formation of the cap structure. Both proteins contain DNA-binding domains and TERF2 has emerged as a major protective factor at chromosome ends. A cell, in response to loss or inactivation of TERF2, typically undergoes rapid senescence or death (24, 25).

Although several investigations have been made of telomerase activity and/or expression in osteosarcoma (4-8), no studies have identified *TERT* alternative splicing mRNA expression in OS, as well as the quantification of the expression of *TERF1* and *TERF2* mRNAs in these tumors. Thus, we here aim to investigate whether or not these events could be associated to OS. Additionally, we quantified the expression of *TERC* mRNA and investigated the telomerase activity by TRAP assay.

Material and methods

For study of gene expression, we selected 70 fresh-frozen OS samples from 30 patients (30 prechemotherapy specimens - PRECH, 30 post-chemotherapy specimens - POSTCH, and 10 postchemotherapy metastasis specimens - META). For the analysis of telomerase activity we selected a subgroup of 23 OS samples (9 PRECH specimens, 8 POSTCH specimens, and 6 META specimens). All samples used in this study were obtained from patients treated at Pediatric Oncology Institute/Grupo de Apoio ao Adolescente e a Criança com Câncer - Federal University of São Paulo (IOP/GRAACC-UNIFESP). This was a retrospective study of samples collected sequentially between 2002 and 2008. Ten healthy bone samples (NB) were used as controls. These samples were obtained from healthy individuals without genetic and/or musculoskeletal diseases who underwent orthopedic surgery due to trauma. Samples from each OS tumor and healthy bone were collected after informed consent was signed by patients/guardians according to the university's institutional review board (IRB/Federal University of São Paulo nº 0050).

Diagnostic Staging and Treatment

All patients with high-grade OS of the extremities (metastatic and non-metastatic) were prospectively enrolled onto Brazilian Osteosarcoma Treatment Group study IV (2). In study IV, patients received carboplatin (500 mg/m^2 intravenous infusion on day 1 of weeks 0, 3, 6, 17, and 26) and cisplatin (100 mg/m^2 intravenous infusion on day 1 of weeks 0, 3, 6, 11, and 20). Doxorubicin was administered either at a dose of 30 mg/m^2 in short-term intravenous infusion on days 1 and 2 of weeks 0, 3, 6, 14, 17, and 23 in the initial phase of the study or at a dose of 35 mg/m^2, following the same schedule administered in a previous group with dexrazoxane. Intravenous infusion of ifosfamide at 3 g/m^2 and mesna, as described (2), were added on days 1, 2, and 3 and on weeks 11, 14, 20, and 26, respectively. The orthopedics team in collaboration with the pediatric oncology team determined the appropriate surgical procedure for each patient. Nonconventional endoprothesis, resection of expendable bones, plaques, and bone graft fixation (autograph or bone bank) were used. Whenever possible, all pulmonary metastases were surgically removed, after resection of the primary tumor.

RNA extraction and cDNA synthesis

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. DNase treatment was used to remove genomic contamination using deoxyribonuclease I Amplification Grade (Invitrogen). First-strand cDNA synthesis was catalyzed with SuperScript® III First-Strand Synthesis System (Invitrogen), from 1 µg of RNA template, following the manufacturer's instructions.

RT-PCR and nested PCR (reverse transcriptase-polymerase chain reaction)

TERT alternative splicing variants were amplified by nested PCR using primers designed according to GenBank using Primer accession n°AF015950, based in previously published protocol (26). The first round of amplification spanned a region that included α^- and β^- deletion sites with forward primer 5' GCTGCTCAGGTCTTTCTTTTAT 3' and reverse primer 5' GGAGGATCTTGTAGATGTTGGT 3'. PCR was performed in 25 µL of reaction mixture using 1 µL of cDNA and 1U GoTaq polymerase (Promega, Madison, WI, USA) by incubation at 94°C for 2 minutes, followed by 25 amplification cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 90 seconds, and a final extension at 72°C for 5 minutes. This second round of PCR was carried out with 1 µL of the first-round PCR product, nested primer set and Taq. The nested primer set for *TERT* patterns, forward 5'CCGCCTGAGCTGTACTTTGTC3' and reverse 5'CAGAGCAGCGTGGAGAGGAT 3', produced four possible products, FL (418 bp), $\alpha^-\beta^+$ (382 bp), $\alpha^+\beta^-$ (236 bp), and $\alpha^-\beta^-$ (200 bp), respectively. This round was performed by incubation at 94°C for 2 minutes, followed by 35 amplification cycles of 94°C for 20 seconds, 59°C for 20 seconds, and 72°C for 30 seconds, and a final extension at 72°C for 2 minutes. Amplified products were electrophoresed on 2% agarose gel, stained with Gel Red (Biotium, Hayward, CA, USA), for size products identification.

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

We used qPCR methodology to *TERF1*, *TERF2* and *TERC* gene expression quantification. Oligonucleotides for *TERF1*, *TERF2*, *TERC*, and *ACTB* (β actin) were chosen using Primer Express (3.0) Software from Applied Biosystems (Foster City, CA), taking care that the forward and reverse sequences were in different exons. We conducted in NCBI a BLAST (Basic Local Alignment Search Tool) search to confirm the total gene specificity of the nucleotide sequences chosen for primers. Thermal cycling comprised initial steps at 50°C for 2 min and at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. A standard curve was constructed with serial dilutions of a mix of a few samples. The cDNA was amplified and quantified using a Sequence Detection System SDS 7500. To exclude variations arising from different inputs of total mRNA to the reaction, data on *TERF1*, *TERF2* and *TERC* were normalized to an internal housekeeping gene, *ACTB*. All reactions for standard (NB samples) and for patient samples were

performed in triplicate. Relative expression levels were calculated according to comparative C_T method ($2^{-\Delta\Delta C_T}$) (27). The results obtained from relative expression levels were log transformed (log 2) and used for statistical analysis.

Quantification of telomerase activity

For detection of telomerase activity was used a photometric enzyme immunoassay using the telomeric repeat amplification protocol, TeloTAGGG Telomerase PCR ELISA (Roche Diagnostics, Mannheim, Germany). The test was performed according to the manufacturer's instructions. Briefly, it was used 10 μg of cell extract of each sample and ELISA was analyzed within 30 minutes after the addition of stop reagent, with a photometer MikroWin 2000 (Mikrotek Laborsysteme GmbH, NRW, Germany) at 450 nm and a 690 nm reference wavelength. As negative control were used samples with telomerase enzyme inactivated and the control was template supplied by kit. All measurements were performed twice to ensure reliability of the test.

Statistical analyses

Data analysis was performed using GraphPad Prism software, version 4 (San Diego, CA). Clinical and pathological variables that correlated with gene expression were: age at diagnosis, gender, primary site, presence of metastasis at diagnosis, presence of relapse, status in the end of treatment, histological OS subtype and necrosis grade after chemotherapy (Huvos grade). Variables were tested for normality of distribution with Kolmogorov–Smirnov test. Overall survival was defined as the time from diagnosis until the date of either the last follow-up or death. For the event free survival analysis, the duration was defined as the time from diagnosis until the occurrence of metastasis or local relapse. Overall survival and event-free survival curves were generated by applying Kaplan–Meier method, and were then compared by log rank test. Comparisons between the median of the gene expression profile of the tumor samples and normal bone controls were evaluated using Wilcoxon Signed Rank test. Continuous data (gene expression levels) were evaluated and compared using nonparametric tests: Mann-Whitney, Wilcoxon, Kruskal-Wallis, correlation coefficient, Spearman, and Friedman. Categorical data (gender, histological OS subtype, primary site, presence of metastasis at diagnosis, and necrosis grade after chemotherapy) were studied using chi-square or Fisher exact tests. The analysis for high expression and low expression of each group of samples (prechemotherapy, postchemotherapy, and metastasis specimens) used the median relative quantification value of the 10 samples of healthy bones (NB) as the normal reference. Statistical significance was taken as $p<0.05$.

Results

A summary of the clinical and pathological characteristics is detailed in table 1. The clinical and pathological parameters analyzed were age at diagnosis, sex, presence of metastasis at diagnosis, Huvos grade, site of the primary tumor, status in the end of treatment and histological type. We identified the most frequent *TERT* AS variant and performed qPCR to quantify messenger mRNA levels of *TERF1, TERF2* and *TERC* genes in 30 PRECH, 30 POSTCH, and 10 META specimens from 30 patients with OS. Additionally, we investigated the telomerase activity in a subset of 23 OS specimens from 9/30 patients.

TERT AS variants and FL patterns in OS tumors

TERT gene expression was detected in 31 of the 70 (44%) tumors. In total, only 3/70 (4%) of samples exhibited exclusively FL pattern. The presence of inhibitory α^- deletion, nonfunctional β^-, and $\alpha^-\beta^-$deletion variants were detected in 28/70 (40%) and 39/70 (56%) did not show any of the patterns. Representative gels are shown in figure 1. *TERT* AS variants and FL patterns present in OS and control samples are demonstrated in table 2 and supplementary data. The presence of full-length *TERT* gene expression was observed in PRECH (7%) and META (10%) groups and not in POSTCH group. Half of NB specimens expressed *TERT* patterns with deletions and the other half did not express *TERT* mRNA. No NB exhibited a full-length pattern.

Table 1. Clinical and pathological characteristics of Osteosarcoma patients.

Parameters	Classes	N (%)
Gender	Male	19 (37)
	Female	11 (63)
Age (years)	<10	2 (7)
	≥10≤20	25 (83)
	>20	3 (10)
Metastasis at diagnosis	Yes	15 (50)
	No	15 (50)
Primary Tumor	Femur	16 (53)
	Tibia	8 (27)
	Humerus	4 (13)
	Fibula	2 (7)
Relapse	Yes	7 (23)
	No	21 (70)
	Progression	2 (7)
Surgery	Conservative	22 (73)
	Amputation	7 (24)
	Treatment drop out	1 (3)
Final treatment status	Treatment drop out	2 (7)
	Disease progression	4 (13)
	Complete Remission	24 (80)
Last contact status	Stable disease	1 (3)
	Death	10 (34)
	Complete Remission	18 (60)
	Treatment of relapse	1 (3)
Tumor necrosis	≤ 90%	17 (57)
	> 90%	10 (33)
	Uninformed	3 (10)
Histology	Chondroblastic	5 (17)
	Osteoblastic	9 (30)
	Others	16 (53)

TERF1 mRNA expression in osteosarcoma patients

Of the 70 tumors analyzed, 64 (81%) showed high expression of *TERF1* compared to NB ($p<0.05$). A comparison of *TERF1* expression levels in PRECH *versus* POSTCH or metastatic samples showed no significant differences ($p>0.05$ and $p> 0.05$, respectively) (figure 2). *TERF1* overexpression or downregulation did not correlate with any of clinical and pathological parameters and survival.

TERF2 mRNA expression in osteosarcoma patients

TERF2 gene was overexpressed in 42 (60%) of 70 tumor specimens compared to NB ($p<0.001$) (figure 2). A comparison of *TERF2* expression levels in PRECH and POSTCH specimens showed significant differences. *TERF2* expression was significantly lower in POSTCH group ($p=0.0468$) (figure 3a). Additionally, *TERF2* downregulation was also associated in POSTCH specimens from patients with no relapse or disease progression ($p=0.0167$) (figure 3b).

TERC mRNA expression in osteosarcoma patients

Forty-seven (68%) of 70 tumor samples overexpressed *TERC* compared to controls ($p<0.05$) (figure 2). A comparison of *TERC* expression levels in PRECH *versus* POSTCH or metastatic samples showed no significant differences ($p>0.05$ and $p>0.05$, respectively). *TERC* overexpression or downregulation did not correlate with any of clinical and pathological parameters and survival.

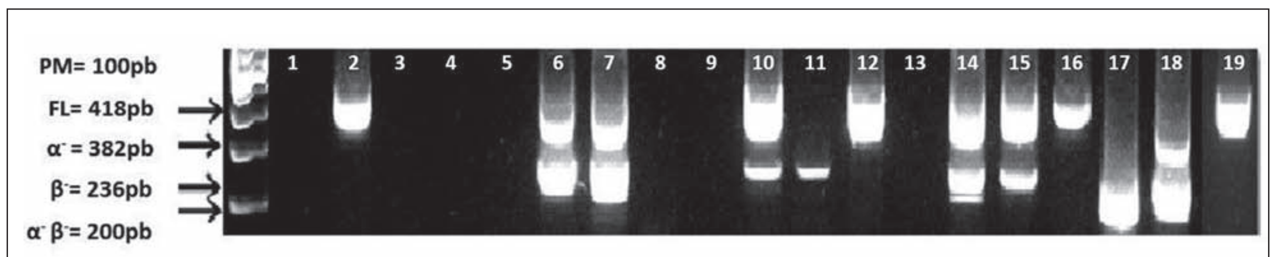

Figure 1. Gel demonstrating *TERT* AS variants and FL patterns present in representative OS specimens.

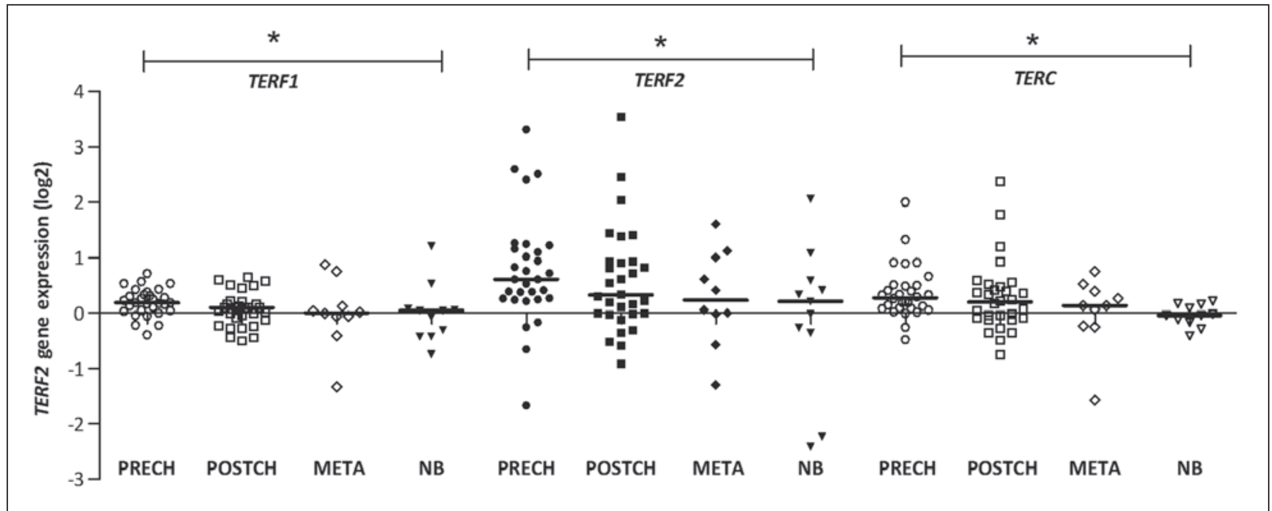

Figure 2. *TERF1*, *TERF2* and *TERC* gene expression profiles in OS and normal bone samples.

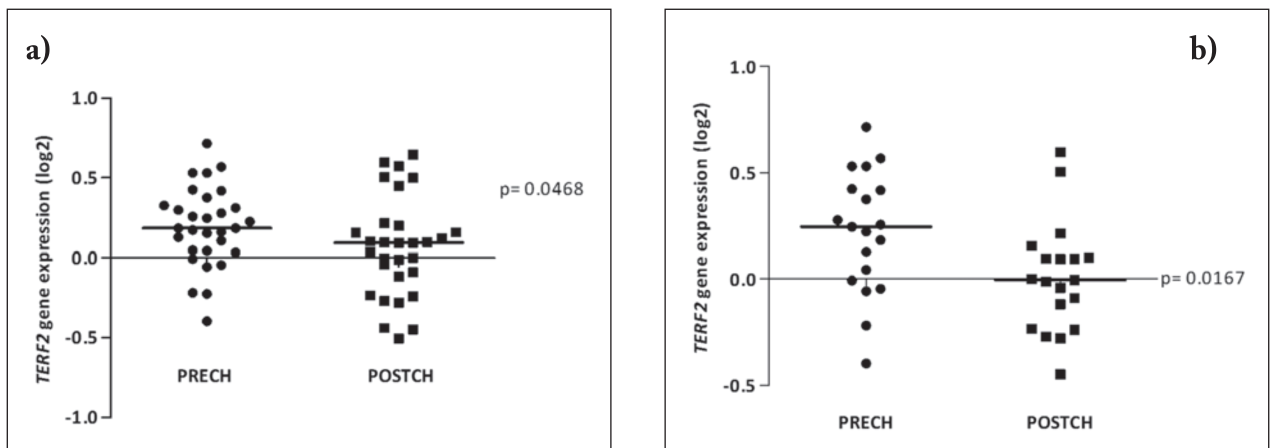

Figure 3. a) Comparison of *TERF2* expression levels in PRECH and POSTCH specimens. b) *TERF2* expression levels in PRECH and POSTCH specimens from patients with no relapse or disease progression

Telomerase enzyme activity

We tested a subset of 23 OS specimens and two normal bone control for functional telomerase activity. The tumors did not show significantly difference median of telomerase enzyme activity levels compared to NB controls (p=0.5743). No TERT AS variants and FL patterns were significantly associated with different levels of telomerase enzyme activity (p=0.3942). Furthermore the telomerase enzyme activity levels were not significantly different between PRECH, POSTCH and META groups (p=0.1553). However, a paired analysis of 9 PRECH and POSTCH samples, demonstrated that the telomerase enzyme activity levels were significantly higher in PRECH group (p=0.0078) (figure 4).

Discussion

Numerous studies in the past two decades have revealed that alterations in TMM (Telomere Maintenance Mechanisms) are among the most important mechanisms in carcinogenesis and tumor progression

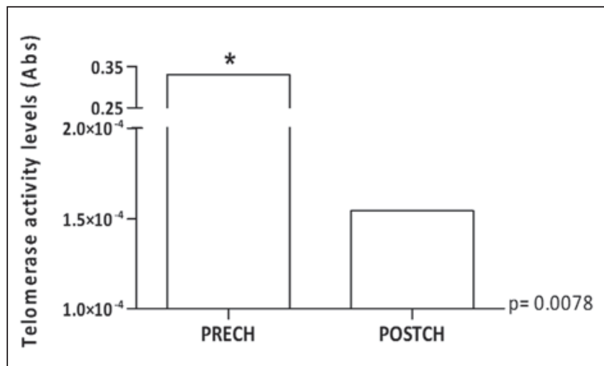

Figure 4. Comparison of telomerase enzyme activity levels in PRECH and POSTCH OS specimens.

(15, 23, 26). In this study, we investigated the patterns of *TERT* AS variants and quantified the mRNA levels of *TERF1*, *TERF2* and *TERC* genes in 70 OS samples divided into three groups: PRECH, POSTCH and META. Additionally, we measured telomerase activity in a subgroup of OS samples.

Our results demonstrated *TERT* mRNA expression in 44% of tumors. However, only 4% of these samples exhibited exclusively FL pattern, while 96% exhibited α^-, β^- and $\alpha^-\beta^-$ deletion variants or did not show any of the patterns (Table 2 and supplementary data). None AS variants and FL patterns were significantly associated with levels of telomerase enzyme activity. Previous studies have been demonstrating TERT expression in 32-44% of OS samples (4-8). However, neither of these studies investigated *TERT* AS variants and FL patterns. As FL pattern is the only associated with active telomerase enzyme, these results suggest that the prevalence of TMM by telomerase activity in OS could be lower than the studies have been reported.

Additionally, the establishment of associations between *TERT* FL and AS variants and tumor clinical-biological behavior becomes even more difficult because of evidence that telomerase has non-canonical functions that are unrelated to telomere lengthening. These in turn can be divided into the functions that still require the integrity of the catalytic site of *TERT* and/or *TERC* and the ones that do not (28). Among other functions, both enzymatically active and inactive, *TERT* modulates Wnt pathway in positive e negative telomerase cells, indicating that this extratelomeric function can be partially preserved in variants with deletion (28-31). Also, *TERT* protects normal and cancer cells from apoptosis independently of catalytic activity (32-34); so it is still unknown precisely the parts of *TERT* responsible for these effects and which specific variants retain these characteristics (17, 21, 28, 34, 35).

The regulation of telomerase activity is a complex process involving several steps, operating at both the transcriptional and post-transcriptional levels. The two principal mechanisms regulating telomerase activity are the transcriptional regulation of *TERT* gene and alternative splicing of *TERT* transcripts (36). In this study we observed the expression of *TERT* spliced transcripts, but no significant association with telomerase activity levels in OS samples. Besides, the telomerase activity levels were significantly lower in POSTCH samples than in PRECH samples. The low telomerase activity levels post-chemotherapy and the difficulty of association with *TERT* spliced transcripts could be attributed to the effect of chemotherapy on the tissue, causing necrosis and inactivating the enzymatic activity.

Table 2. Distribution of *TERT* gene expression patterns in OS and NB controls specimens

Samples/TERT pattern	FL* N (%)	α^-del/ β^-del/ $\alpha^-\beta^-$del** N (%)	NE N (%)	Total N
PRECH OS	2 (7%)	13 (43%)	15 (50%)	30
POSTCH OS	0	8 (27%)	22 (73%)	30
META OS	1 (10%)	7 (70%)	2 (20%)	10
TOTAL	3 (4%)	28 (40%)	39 (56%)	70
NB control	0	5 (50%)	5 (50%)	10

FL: full length, α^-del: α^-deletion, β^-del: β^-deletion, $\alpha^-\beta^-$del: $\alpha^-\beta^-$deletions, NE: No *TERT* expression. *Specimens carrying exclusively *TERT* FL pattern. **Specimens carrying *TERT* variants with deletion, including samples carrying both, FL and variants with deletion patterns

The use of different qualitative and quantitative methodologies to measure *TERT* mRNA and telomerase activity in studies make it difficult to directly compare interpretation of the results (21, 37, 38). Splicing variants of several proteins in tumor cells have been proposed as diagnostic or prognostic biomarkers and may provide potential drug targets. The prospective use of more sensitive and refined methodologies, such as digital PCR, could collaborate to identify and quantify more precisely the splicing of low-abundance *TERT* transcripts (15, 36-38).

TERF2 participates in t-loop formation and is essential for protecting telomeres from being repaired by nonhomologous end-joining and recognized as DNA damage by ATM (ATM serine/threonine kinase) (39, 40). Functional inactivation of *TERF2* results in a high frequency of end-to-end fusions, which arise from the inability of cells to distinguish natural telomeric ends from broken DNA. In addition to these fusions, cells without a protective telomere cap are also vulnerable to chromosomal rearrangements and aneuploidy (23-25).

In telomeric sequences, the presence of one chelate of cisplatin causes a marked decrease of TERF2 affinity (41). Moreover, cisplatin inhibits the stimulation of invasion that is caused by TERF2 through its deleterious effect on TERF2 binding to DNA and increases protein-independent invasion by destabilizing the double helix. All these results suggest that platination of telomeric sequences by cisplatin in cancerous cells could impact on TERF2 binding and TERF2-mediated telomeric invasion and consequently could affect the integrity of telomeres (42-46). In our study we observed lower expression of *TERF2* gene in POSTCH OS samples than in PRECH samples (p=0.0468). As cisplatin is one of most important chemotherapeutic drug used in OS treatment, *TERF2* downregulation in POSTCH samples observed in our results could have been induced by chelation of cisplatin in telomeric DNA, since platination of telomeric DNA strongly decrease TERF2 affinity.

Currently, studies have demonstrated a possible role of *TERF2* in mediating drug resistance of cancer cells. RNAi-mediated inhibition of *TERF2* expression partially reversed the phenotype of multidrug-resistant variant SGC7901 gastric cancer cells (46). In our study we observed that, patients with absence of relapse or disease progression showed *TERF2* gene expression levels significantly lower in POSTCH specimen tumors (p=0.0167). Previous studies demonstrated that inhibition of *TERF2* results in apoptosis mediated by p53 and ATM and the apoptosis was not due to fusions of chromosome ends, but to activation of a DNA damage checkpoint (46-47). Thus, *TERF2* downregulation observed in this group of tumor cells could be activating ATM-dependent DDR and consequently trigger senescence/apoptosis signaling, contributing to the good response to treatment.

We would like to acknowledge the limitations of our study: no significant association between *TERT* AS variants and the clinical-pathological parameters and the absence of *in vitro* functional tests to support the role of *TERF2* expression in response to treatment in OS. Despite this, our results strongly suggest, for the first time, the presence of *TERT* post transcriptional mechanism in OS and a possible link between *TERF2* expression, response to chemotherapy treatment and OS sarcomatogenesis.

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Address: Silvia Regina Caminada de Toledo
Pediatrics Oncology Institute/GRAACC (Grupo de Apoio ao Adolescente e à Criança com Câncer)-UNIFESP (Federal University of São Paulo). Rua Botucatu, n° 743,
Floor 8 - Genetics Laboratory, Vila Clementino, São Paulo-SP, Brazil - Zip Code: 04023-062
Tel. +55 11 5080-8582
Fax +55 11 5080-8480.
E-mail: silviatoledo@graacc.org.br

Supplementary data

Patients	Gender	Age (years)	Metastasis at diagnosis	Primary tumor	Histology	Surgery	Necrosis grade	Relapse	Final treatment status
1	Male	20	No	Tibia	Others	Conservative	≥90%	No	Complete remission
2	Male	16	Yes	Tibia	Telangiectatic	Amputation	≤90%	Yes	Complete remission
3	Male	16	No	Fibula	Osteoblastic	Conservative	≤90%	Yes	Complete remission
4	Male	29	Yes	Femur	Osteoblastic	Amputation	≤90%	No	Complete remission
5	Male	19	Yes	Femur	Osteoblastic	Amputation	≤90%	Yes	Disease progression
6	Female	18	Yes	Femur	Others	Conservative	≤90%	Yes	Treatment drop out
7	Female	14	Yes	Tibia	Osteo/Chondroblastic	Treatment drop out	No information	Yes	Treatment drop out
8	Female	23	Yes	Humerus	Others	Conservative	≤90%	No	Complete remission
9	Male	34	Yes	Femur	Osteo/Chondroblastic	Conservative	≤90%	Yes	Complete remission
10	Male	19	No	Tibia	Mixed	Conservative	≤90%	Yes	Complete remission
11	Male	15	Yes	Humerus	Chondroblastic	Conservative	≤90%	Yes	Complete remission
12	Male	19	Yes	Femur	Chondroblastic	Conservative	≥90%	No	Treatment drop out
13	Male	38	No	Tibia	Osteoblastic	Conservative	No information	No	Complete remission
14	Male	22	No	Fibula	Chondroblastic	Conservative	≤90%	Yes	Complete remission
15	Female	9	No	Femur	Chondroblastic	Amputation	≤90%	No	Disease progression
16	Male	26	Yes	Humerus	Chondroblastic	Conservative	≤90%	Yes	Complete remission
17	Female	13	No	Femur	Telangiectatic	Amputation	≥90%	No	Complete remission
18	Female	21	Yes	Humerus	Osteo/Chondroblastic	Conservative	No information	No	Disease progression
19	Male	13	No	Tibia	Osteoblastic	Conservative	≥90%	No	Complete remission
20	Female	16	Yes	Femur	Others	Conservative	≥90%	No	Complete remission
21	Male	12	Yes	Tibia	Mixed	Amputation	≤90%	No	Complete remission
22	Male	10	No	Femur	Osteoblastic	Conservative	≥90%	No	Complete remission
23	Male	11	Yes	Femur	Others	Conservative	≤90%	Yes	Complete remission
24	Male	17	No	Femur	Telangiectatic	Conservative	≥90%	No	Complete remission
25	Male	11	Yes	Femur	Osteoblastic	Conservative	≤90%	No	Complete remission
26	Male	11	No	Femur	Osteoblastic	Conservative	≥90%	No	Complete remission
27	Female	27	Yes	Femur	Osteoblastic	Amputation	≤90%	No	Disease progression
28	Male	11	No	Tibia	Others	Conservative	≤90%	No	Complete remission
29	Female	9	No	Femur	Osteo/Chondroblastic	Conservative	≥90%	No	Complete remission
30	Male	16	No	Femur	Others	Conservative	≥90%	No	Complete remission

Patients	Last contact status	High levels of TERF2 gene on POSTCHEMO specimens	TERT pattern on PRECHEMO specimens	Tested for telomerase activity on PRECHEMO specimens	TERT pattern on POSTCHEMO specimens	Tested for telomerase activity on POSTCHEMO specimens	TERT pattern on POSTCHEMO Metastasis specimens	Tested for telomerase activity on POSTCHEMO METASTASIS specimens
1	Complete Remission	No	NE	NA	NA	NA	NA	NA
2	Death	No	FL	Yes	NA	Yes	NA	Yes
3	Death	Yes	NE	Yes	NA	Yes	β^{-}del	Yes
4	Complete Remission	No	NE	NA	NA	NA	NA	NA
5	Death	No	α^{-}del/β^{-}del	NA	α^{-}del/β^{-}del	NA	NA	NA
6	Death	Yes	α^{-}del/β^{-}del/$\alpha\beta^{-}$del	Yes	NA	Yes	NA	NA
7	Death	No	NE	NA	NA	NA	NA	NA
8	Complete Remission	Yes	NE	NA	NA	NA	NA	NA
9	Death	No	NE	NA	β^{-}del	NA	α^{-}del	NA
10	Death	Yes	FL/β^{-}del	Yes	NA	Yes	$\alpha\beta^{-}$del	Yes
11	Death	No	β^{-}del	NA	NA	NA	$\alpha\beta^{-}$del	NA
12	Stable disease	Yes	FL	Yes	NA	Yes	NA	NA
13	Death	No	NE	NA	NA	NA	NA	NA
14	Complete Remission	Yes	NE	NA	β^{-}del/$\alpha\beta^{-}$del	NA	$\alpha\beta^{-}$del	NA
15	Death	No	α^{-}del/ β^{-}del/ $\alpha\beta^{-}$del	NA	NA	NA	$\alpha\beta^{-}$del	Yes
16	Complete Remission	Yes	FL/α^{-}del/β^{-}del	Yes	α^{-}del/β^{-}del	Yes	α^{-}del/ β^{-}del	Yes
17	Complete Remission	No	NE	Yes	NA	Yes	NA	NA
18	Death	Yes	$\alpha\beta^{-}$del	NA	β^{-}del	NA	NA	NA
19	Complete Remission	No	α^{-}del	NA	NA	NA	NA	NA
20	Complete Remission	No	$\alpha\beta^{-}$del	NA	NA	NA	NA	NA
21	Complete Remission	No	NE	Yes	β^{-}del	Yes	FL	Yes
22	Complete Remission	No	NE	NA	NA	NA	NA	NA
23	Complete Remission	No	α^{-}del/$\alpha\beta^{-}$del	NA	β^{-}del	NA	NA	NA
24	Complete Remission	Yes	NE	NA	NA	NA	NA	NA
25	Complete Remission	No	NE	NA	NA	NA	NA	NA
26	Complete Remission	No	NE	NA	NA	NA	NA	NA
27	Death	No	$\alpha\beta^{-}$del	NA	β^{-}del/$\alpha\beta^{-}$del	NA	NA	NA
28	Complete Remission	No	β^{-}del	NA	NA	NA	NA	NA
29	Complete Remission	No	NE	NA	NA	NA	NA	NA
30	Complete Remission	No	$\alpha\beta^{-}$del	NA	NA	NA	NA	NA