

Different expression of alternative lengthening of telomere (ALT)-associated proteins/mRNAs in osteosarcoma cell lines

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Abstract. Tumors, including osteosarcoma (OS), are capable of evading senescence and cell death, which is caused by telomere loss with cell division. Alternative lengthening of telomeres (ALT) is considered as the main telomere maintenance mechanism in OS. In this study, we investigated the expression of ALT-associated proteins and mRNAs in human OS cell lines. Western blotting was used to detect the protein expression in OS cell lines, while the expression of mRNA was determined by reverse-transcriptase PCR and quantitative real-time PCR analysis. Whole-genome expression arrays were used to analyze the expression of all the mRNAs involved in telomere maintenance mechanisms (TMMs) including human telomerase reverse transcriptase, promyelocytic leukemia proteins and other related proteins. OS and normal cell lines do not express telomerase reverse transcriptase (hTERT) as a key subunit of telomerase, although they show varying levels of ALT-associated proteins and mRNAs such as PML, Rad52, MRE11 and FEN1 by Western blotting and quantitative real-time PCR analysis. A number of mRNAs that play essential roles in ALT are expressed more in OS cell lines than in the osteoblast cell line, as shown by whole-genome expression arrays. In conclusion, OS cell lines maintain their telomere length primarily through the ALT mechanism. There are numerous other proteins that regulate this process in OS; therefore, anti-ALT therapy may be a more effective method to treat OS than anti-telomerase therapy.

Introduction

Osteosarcoma (OS) in which the neoplastic osteoid is produced by the proliferating spindle cell stroma of mesenchymal derivation is the most common primary malignant bone tumor in children and adolescents. A total of 10-15% of OS patients present distant metastases at diagnosis

(90% lung and 10% other locations) (1). Despite significant clinical improvements over the past few decades through the use of combination chemotherapy and surgery, patients with metastasis or who respond poorly to adjuvant chemotherapy continue to have an extremely poor prognosis (2). Therefore, the identification of specific molecular targets is crucial in devising more targeted and appropriate therapeutic strategies to improve patient survival.

Tumors have unlimited proliferative capacity, which is closely linked to the maintenance of telomeres (3). The tumor cell prevents critical telomere dysfunction by specific telomere maintenance mechanisms (TMMs) referred to as telomerase activity (TA), alternative lengthening of telomeres (ALT), and other unknown mechanisms used to evade senescence and cell death (4). Similarly, OS cells need to maintain telomeres stable by specific mechanisms. In this study, we investigated the expression of two TMM-related components in human OS cell lines, and showed the level of expression of mRNA and proteins. Therefore, we may treat OS with molecular immunology methods targeting ALT.

Materials and methods

Cell culture and antibodies. Human OS cell lines (MG-63, U2-OS and SAOS-2) and an osteoblast cell line (hFOB 1.19) were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). U2-OS, MG-63 and SAOS-2 were cultured in RPMI-1640 (HyClone, USA) containing 10% heat-inactivated fetal bovine serum (FBS; HyClone). hFOB 1.19 was cultured in DMEM/F12 (v/v) containing 10% heat-inactivated FBS and 0.3 mg/ml G418 (Sigma, St. Louis, MO, USA). The OS cell lines were cultured at 37°C in a 5% CO₂ water-saturated atmosphere and hFOB 1.19 was cultured at 34°C.

The primary antibodies used were: mouse monoclonal anti-hTERT (ab5181, Abcam, Boston, MA, USA); mouse monoclonal anti-PML (sc-966), anti-FEN1 (sc-28355), anti-MRE11 (sc-135992) and anti-Rad52 (sc-365341) (all three purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA); and mouse monoclonal anti-β-actin (Fermentas, Burlington, Canada).

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Whole-genome expression arrays. We used commercially available Illumina HumanWG-6 Expression BeadChips for whole genome expression analysis. Purified cells were

Table I. Human oligonucleotide sequences used in the PCR experiments.

Gene	Forward	Reverse	Accession no.	Length (bp)
hTERT	AGGCTCACGGAGGTCATCGC	CAACAAGAAATCATCCACCAAACG	NM_198253	447
PML	ACCAACAACATCTTCTGCTCCAACC	CCGAGGCGTAGCACTTCATCC	NM_033244	475
FEN-1	TCAGGCGAGCTGGCCAAACG	GTCGCCGCACGATCTCCTCG	NM_004111	508
MRE11A	TTCAGGTTTACGGCCCTGCGG	TGCTGGACTCTTCGAAGCCCCA	NM_005590	133
Rad52 ^a	AGACCTCTGACACATTAGCCTTGAA	AAGATCCAGATTTTGCTTGTGGTT	NM_134424	99
β -actin	GTCCACCGCAAATGCTTCTA	TGCTGTCACCTTCACCGTTC	NM_001101	190

^aThe primers are designed by NCBI primer-blast or other literature (6).

lysed in a TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions using 1 ml TRIzol reagent per 10⁶ cells. Isolated total RNA was then purified further using an Illumina Total Prep RNA amplification kit (Ambion, Austin, TX, USA). Each purified RNA sample was assessed for RNA concentration and purity (A260:280) using the Nano Drop 2000 Spectrophotometer. All information on RNA processing and quality assessment is available. Total RNA (500 ng) was amplified using the Illumina Total Prep RNA amplification kit according to the manufacturer's instructions (7). The biotinylated cRNA (1500 ng per sample) was applied to Illumina HumanWG-6 v3 Expression BeadChips, which provided whole genome transcriptomic coverage, and was hybridized overnight at 58°C. Chips were washed and detected according to the manufacturer's instructions. The HumanWG-6 v3 Expression BeadChips were scanned on an Illumina BeadArray™ reader. The BeadStudio software package included with the Illumina® BeadStation 500GX system extracted gene expression data from the images collected from the Illumina BeadArray Reader.

Western blotting assays. Cell lines were washed with phosphate-buffered saline (PBS) and lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 50 μ g/ml leupeptin, 30 μ g/ml aprotinin and 1 mM PMSF). Following centrifugation at 15,000 x g at 4°C for 10 min, the supernatants were separated on sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel and transferred onto a nitrocellulose membrane (Millipore, Billerica, MA, USA) in the standard transfer buffer. After being blocked with 5% skim milk in blocking solution (Tris-buffered saline containing 0.1% Tween-20, TBST) at room temperature, the membrane was incubated with primary antibodies diluted in blocking solution overnight at 4°C. The antibodies and dilutions used were: anti-hTERT (1:1500), anti-PML (1:200), anti-FEN-1 (1:200), anti-MRE11 (1:100), anti-Rad52 (1:200) and anti- β -actin (1:1000). Following washing in TBST 3 times, the secondary antibodies (1:3000-5000, Santa Cruz Biotechnology), conjugated with HRP, were applied for 2 h at room temperature. Following extensive washing in TBST, specific immunoreactivity was visualized using enhanced chemiluminescence (ECL) Western blotting substrate (Pierce, Rockford, IL, USA) and the ECL system (Fusion FX7, French). The relative levels were normalized to β -actin expression.

Complementary DNA (cDNA) synthesis. TRIzol reagent (Invitrogen) was used to extract total RNA from tissue samples and cell lines in accordance with the manufacturer's instructions. cDNA was synthesized using 0.5 μ g of total RNA and the FirstStrand synthesis kit (Fermentas). cDNA was incubated for 60 min at 42°C, and the reaction was terminated by heating at 70°C for 5 min.

Quantitative real-time PCR (qRT-PCR) analysis. Relative mRNA expression was evaluated by qRT-PCR performed with the Mx-3000P Real-time PCR system (Stratagene, La Jolla, CA, USA) and real-time PCR kit (SYBR® Premix Ex Taq™, Takara, Japan) according to the manufacturer's instructions. The primers (β -actin as the control gene) in qRT-PCR were synthesized by Invitrogen. The reaction conditions were initial denaturation at 95°C for 1 min followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 56°C for 15 sec and extension at 72°C for 45 sec. The final extension was performed at 72°C for 10 min. The total procedure was repeated nine times for each reaction and the average value was calculated. In this experiment, β -actin was used as the control gene. Levels of mRNA expression were calculated based on the method of 2^{- $\Delta\Delta$ CT} (5).

Statistical analysis. Quantitative data were expressed as the mean \pm standard deviation (SD). P<0.05 was considered to be statistically significant. Analysis was carried out using SPSS 13.0.

Results

Whole-genome expression arrays. The mRNA expression of the proteins related to the maintenance of telomere length were listed (Table II) as reported in the literature. Possible different genes including TRF1, TRF2, TERC, PML, RAD50, MRE11A, FEN1 and FANCA were primarily identified, since there is a 2-fold difference in signal intensities (ratio >2 or <0.5 in the Tables) between hFOB 1.19, which served as a control, and OS cell lines such as MG-63, SAOS-2 and U2-OS.

Western blot analysis. Western blot analysis shows that hTERT is expressed in the MG-63 cell line, whereas it is negatively expressed in the SAOS-2, U2-OS and hFOB 1.19 cell lines (Fig. 1A). Fig. 1B shows the varying levels of PML

Table II. Expression of different telomere-associated proteins/mRNAs.

mRNAs	hFOB 1.19	MG-63	U2-OS	SAOS-2
Shelterin protein complex				
TRF1	6.4	15.8	7.4	-2.3
TRF2	-16.8	-3.2	-10.1	-6.1
TPP1	15.4	94.3	174.1	138.5
TINF2	2074.3	1963.7	2204.3	1826.5
TERF2IP	1485.7	1806	1974.9	2069
POT1	-10.1	21.2	43.7	34
Telomerase-associated proteins				
TERC	40.9	0.025	152.6	0.0013
TERT	11.4	0.9	-0.8	-0.1
TEP1	-7.6	5.8	11.6	13.7
ALT-associated proteins				
PML	18.8	23.7	45.6	30.7
RAD50	74.6	143.5	192.7	203.1
RAD51	200.4	193.6	151.3	125.1
RAD52	20	37.7	14.5	33.6
SMC5	-14.8	-12.7	-17.5	-23.8
SMC6	117	74.5	35.5	58.5
BLM	459.7	396.1	388.6	532.2
TOP3A	630.3	730.2	846.4	783.9
BLAP75	344.3	653.1	587.1	492.5
MMS21	1788.4	3720.1	2370.4	1450.6
MRE11A	101	198.7	226.4	153.1
FEN1	5856.5	8893	7145.3	6782.1
MUS81	454.2	582.3	712.8	821.3
FANCD2	436	433.7	324.6	398.2
FANCA	18.9	174.5	111.6	133.5

Values are the corresponding average score of all probes. '-' means the signal of this probe is less than the background of the chip.

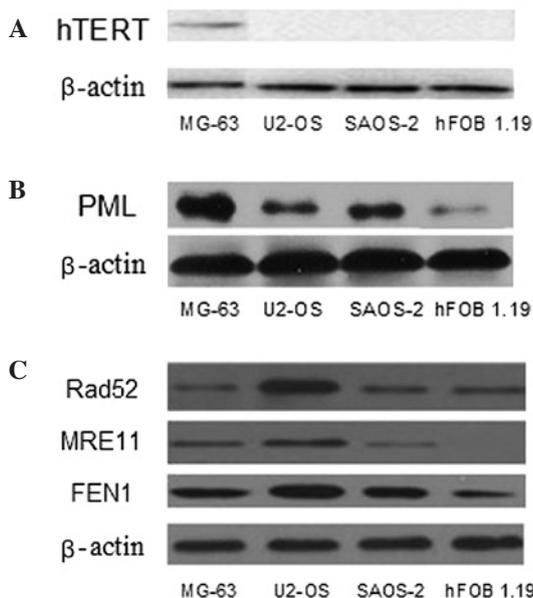


Figure 1. Expression of hTERT and PML by Western blot analysis in the different cell lines. Expression of (A) hTERT, (B) PML, and (C) Rad52, MRE11, FEN-1 and β -actin (as internal control) in different cell lines.

expression in all of the cell lines, particularly in the MG-63 cell line, whereas PML is less expressed in hFOB 1.19. Fig. 1C shows the different expression levels of Rad52, MRE11 and FEN1 between the OS cell lines (MG-63, U2-OS and SAOS-2) and the osteoblast cell line (hFOB 1.19).

qRT-PCR analysis. The mRNA expression of hTERT was detected in the MG-63 cell line, but was almost negative in U2-OS, SAOS-2 and hFOB 1.19. As for the mRNA level of PML, Fig. 2A shows varying levels of PML mRNA in all four cell lines, particularly MG-63. The differences of PML, MRE11A and FEN-1 between MG-63 and hFOB 1.19 achieved statistical significance ($P < 0.05$), as did Rad52, MRE11A and FEN-1 in U2-OS, and MRE11A and FEN-1 in SAOS-2, compared to hFOB 1.19.

Discussion

Telomeres prevent chromosome ends from inducing a DNA damage response and end-to-end fusions, which result in chromosome breakage and recombination (7-9). Telomeres shorten by 50-200 bp with each cell division, leading to cell

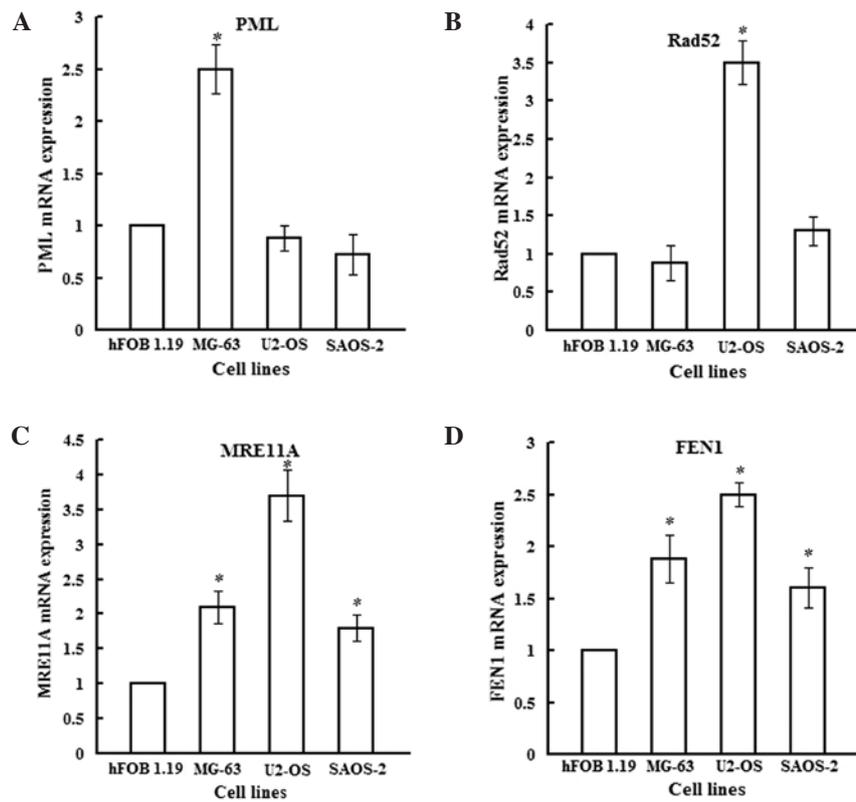


Figure 2. The mRNA expression of PML, Rad52, MRE11 and FEN-1 by qRT-PCR in the different cell lines. The CT value is the fractional cycle number at which the fluorescence passes the fixed threshold. The value of $2^{-\Delta\Delta CT}$ is the relative expression of mRNA in each cell line compared with hFOB 1.19. * $P < 0.05$.

senescence and eventually death, as their ends cannot be completely replicated by the DNA-replication machinery (the end-replication problem) (10).

There are a number of adjuvant structures that protect the telomere DNA, which is mostly double-stranded and has a single-stranded terminus with 130-210 bases on average in human cells (11). The telomeric DNA is bound by the shelterin protein complex, which includes six subunit proteins as follows: telomeric repeat-binding factor 1 (TRF1 or TERF1), telomeric repeat-binding factor 2 (TRF2 or TERF2), protection of telomeres 1 (POT1), tripeptidyl peptidase I (TPP1 or PIP1, TINT1), TRF1-interacting nuclear protein 2 (TIN2 or TIN2) and TRF2-interacting telomeric protein (RAP1 or TERF2IP). This nucleoprotein complex prevents the chromosome end from being folded as a DNA double-strand break (DSB) (12). In the present study, TRF1, TRF2, TPP1 and POT1 were differentially expressed between OS and osteoblast cell lines (Table II), indicating that they may play a significant role in OS; particularly TPP1 and POT1, which revealed insignificant differences in the relative level of mRNAs compared to hFOB 1.19.

In humans, almost 85% of carcinomas maintain their telomeres with telomerase, which synthesizes new telomeric DNA to repair the ends of the chromosomes, whereas in normal tissues and cells expression is almost negative. Thus, telomerase is a classic target for developing an anti-tumor therapy (13). However, in 10-15% of tumors, DNA replication is achieved through a mechanism known as ALT, which is dependent on homologous recombination or other molecular mechanisms. Therefore, ALT may be a significant new target for tumor therapy. Tumors maintain the extreme heterogeneity

of telomere lengths using distinct nuclear structures called ALT-associated promyelocytic leukemia (PML) bodies (APBs) (14). The PML protein is a zinc finger transcription factor expressed as three major transcription products due to alternative splicing. PML was first found in acute promyelocytic leukemia (APL) and plays a significant role in genome stability (15).

Besides the hTERT and PML, there are a number of other related protein components of these two TMMs, which are essential for telomere elongation or telomere loss prevention. As is well known, human telomerase is composed of an RNA component (hTR), a catalytic protein subunit (hTERT) and the telomerase-associated protein (TEP1) (16,17). However, hTR has a lack of specificity as it is widely expressed in a number of tissues, even in those tissues without telomerase activity (TA) (18,19). Few studies exist regarding TEP1 compared with the other two components of telomerase. Certain findings indicate that the reverse transcriptase domain of hTERT interacts with TEP1 (20). TEP1 also binds a small RNA (vault RNA, vRNA) within the cytoplasmic vault complex (21). Inhibition of TEP1 leads to greatly reduced vRNA levels, as well as a loss of TEP1 and vRNA from the vault caps, but no reduction in TA or telomere length (22,23). These results suggest that TEP1 is a multifunctional RNA-binding protein and may not be essential for TA and telomere length. Results of the present study have shown that hTR (also known as TERC) and TEP1 were differentially expressed between the two cell lines, whereas hTERT was negative in the normal and OS cell lines. Therefore, to identify whether TEP1 plays a physiological role in telomerase mechanisms, further experiments should be conducted.

Findings of previous studies have shown that 31-87% of OS tissues use the TA mechanism to maintain their telomere length (24-27). On the other hand, numerous reports in the available literature describe the absence of telomerase activity or hTERT mRNA/protein expression in a number of OS cell lines. The different extracellular matrix and detection methods used may result in this diversity (28,29). In OS, hTERT is a predictive indicator of worse prognosis, with a trend in favor of shorter progression-free survival in patients whose tumors expressed telomerase, and promotes the invasion ability of telomerase-negative tumor cells *in vitro* (28,30). Due to the different expression between normal and malignant tissue, hTERT has been considered as a significant potential target for tumor therapeutics. Therefore, various telomerase inhibition therapies are currently in progress including a lipid-modified thio-phosphoramidate oligonucleotide (GRN163), which is the furthest along in clinical development, a derivative of benzoic acid (BIBR1532) and a bisphosphonate (31,32). However, there may be a number of adverse effects of telomerase inhibition therapy on patients, particularly in growing children, as certain normal cells express telomerase, including hematopoietic stem, germ, immune and other progenitor cells. Therefore, more investigations regarding the role of telomerase are required.

Findings of certain studies have shown that TA and ALT may elongate the telomere by various mechanisms, and even coexist in the same immortal cells by transfection (33). Such findings suggest a significant anti-tumor target, particularly for tumors that maintain the telomere stability by ALT. Moreover, one hallmark of the ALT mechanism is the presence of APBs, which contain PML bodies and some telomere-related components.

The ALT mechanism is reportedly rare in epithelial tumors but more common in tumors of neuroepithelial (e.g., astrocytoma) or mesenchymal origin (e.g. OS and liposarcoma). However, although certain hypotheses have been put forward to explain it, the basis of this tissue specificity has yet to be elucidated (34).

Numerous proteins have been identified in APBs that may be involved in ALT mechanisms, such as POT1, TRF1, TRF2, as well as other proteins involved in homologous recombination repair, including DNA repair protein RAD50, RAD51, RAD52, the structural maintenance of chromosomes SMC5-SMC6 complex and the MRN complex (35), and protein complexes that include the BLM helicase, topoisomerase 3 α (TOP3A) and BLAP75 (BLM-associated polypeptide 75) (36,37). The (SMC5)-SMC6 complex, which is involved in telomere elongation, is composed of SMC5, SMC6, and methyl methanesulfonate-sensitivity 21 (MMS21). MMS21 may sumoylate TRF1, TRF2, TIN2 and RAP1 to elongate telomeres based on SMC5 and SMC6. The MRN complex, which is composed of meiotic recombination 11 (MRE11), Rad50 and Nijmegen breakage syndrome protein (NBS1), is the first protein complex to be identified as necessary for ALT-mediated telomere maintenance (38).

MRE11 is a nuclear 3'-5' exonuclease/endonuclease that associates with Rad50 and affects homologous recombination, telomere maintenance and DNA double-strand break repair. MRE11 is not detected in osteoblast cell lines by Western blot analysis. Rad52, which interacts with Rad51, forms a heptameric ring that binds single-stranded DNA ends and cata-

lyzes the DNA-DNA interaction necessary for the annealing of complementary strands (39,40). Recent studies have demonstrated that other proteins, including flap endonuclease 1 (FEN1), MUS81, the Fanconi anaemia group D2 (FANCD2) and Fanconi anaemia group A (FANCA) are also significant for ALT mechanisms (41-43). These proteins are significant in telomere maintenance, as shown by previous studies (44,45). This difference in Rad52, MRE11 and FEN1 protein levels indicates that these cell lines are dependent on ALT to different extents. MRN, MUS81, FEN1 and TOP3A all bind TRF2, suggesting that reducing relative TRF2 saturation limits control over these proteins at chromosomal telomeres (36).

In this study, the expression of hTERT, PML, Rad52, MRE11 and FEN1 at mRNA and protein levels show the difference between the OS cell lines and the osteoblast cell line. On the other hand, other related proteins including RAD50, BLAP75, MRE11A, FEN1, MUS81 and FANCA are expressed more in OS cell lines than in hFOB 1.19 in whole-genome expression arrays. RAD50 and MRE11A have been shown to play a role in telomere elongation, whereas BLAP75, FEN1, MUS81 and FANCA are considered to be significant proteins that prevent telomere loss in an ALT mechanism. Therefore, we suggest that there are more proteins in OS cell lines that prevent telomere loss rather than telomere elongation, and these may become significant therapeutic targets in the treatment of OS in the future.

In conclusion, OS cell lines maintain their telomere length primarily through the ALT mechanism. A number of other proteins regulate this process in OS. Therefore, anti-ALT therapy may be a significant method used treat OS, which requires in-depth study of the ALT mechanism.

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