Clinical Usefulness of Telomerase Assay in Patients with Ewing's Sarcoma

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Abstract: Purpose: This study was designated to investigate whether telomerase is reactivated in Ewing's sarcoma and whether it has a prognostic significance. **Patients and Methods:** Thirty-two patients with localized Ewing's sarcoma of bone were treated with four VACA blocks, each block lasted 9 weeks and consisted of three courses of treatment each, administered at 3-week intervals. Local therapy was scheduled to be given before the second block and was individually planned for each patient. Telomerase activity was determined using a modified-TRAP assay, hTERT mRNA Expression was performed by quantitative real-time polymerase chain reaction and the telomere length was determined based on the telomere restriction fragment (TRF) – Southern blot analysis. **Results:** Fourteen patients (44%) had a complete response or very good partial response (VGPR). Fifteen patients (47%) had either a partial response or stable disease. Telomerase activity (TA) was detected in 84% of patients. No significant correlation was found between any of the clinical parameters and TA. High expression of human telomerase reverse transcriptase (hTERT) (≥100 copies) was identified in 59% of patients and it was significantly correlated with high TA. Twenty-one of 32 patients exhibited changes in telomere lengths: eight longer and thirteen shorter. No significant correlation was found between telomere length and TA. Nineteen (83%) of 23 patients with high TA relapsed, while none of the nine patients with low TA did. Highly significant correlation was observed between TA and progression free survival (PFS). Low TA patients had 100% PFS, while high-TA patients had 36% PFS ($P < .0001$**).** 58% of patients expressed high hTERT relapsed versus 15% of those expressed low hTERT**.** PFS comparing patients with high and low hTERT expression was statistically significant ($P = .0321$**).** All samples that were tested for hTERT were also analyzed for TA and correlated significantly ($P = 0.0357$**). Conclusion:** Our results showed that telomerase activity could be used as a prognostic factor in patients with Ewing's sarcoma.

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1. Introduction

The treatment of Ewing sarcoma (ES) of bone has evolved dramatically. The rates of long-term success after irradiation of the site of the primary tumor as the only treatment have ranged from 5 to 10 per cent because of the development of metastases **[1]**. The use of intensive multiagent chemotherapy has been the most important advance in the treatment of Ewing sarcoma; in recent reports, the rates of disease-free survival have ranged from 50 to 60 per cent for patients who were managed in this manner **[2].** Nevertheless, among patients with localized tumor at diagnosis, 20% relapse within 4 years and die of the disease despite the most aggressive current protocols **[3]**.

Therefore, in addition to conventional factors such as age, tumor volume and chemotherapy-induced necrosis, new molecular prognostic parameters are needed to risk-stratify patients with localized ES. Furthermore, up to 15% to 25% of patients first present with overt metastases, the strongest adverse conventional prognostic factor, and these patients fare poorly regardless of therapy **[4]**.

Telomeres, the distal end of human chromosomes, consist of thousands of copies of a repeat nucleotide sequence 5′-TTAGGG-3′ that ranges from 5 to 20 kb in length. They display many functional and structural roles during interphase, mitosis, and meiosis and protect chromosomes against exonucleases and ligases, preventing fusion, recombination, and degradation **[5]**. Conventional DNA polymerases replicate DNA only in the 5′ to 3′ direction. Thus, the leading strand can be replicated to the very end, whereas the lagging strand DNA synthesis at sites opposite to the 3′ end of the template DNA cannot be completed, and a gap remains at the newly synthesized 5′ ends. This results in telomeric shortening with each cell division **[6,7]**. Telomerase is an RNA-dependent DNA polymerase that synthesizes TTAGGG telomeric DNA onto chromosome ends to compensate for sequence loss during DNA replication **[8]**. When telomeres become shorter with each cycle of replication to reach a critical length, most cells die

or undergo senescence. However, the rare cells that escape this fate express telomerase activity and have stable telomere lengths. The reactivation of telomerase thus appears to be associated with the immortalization of cells **[9]**. Elevated telomerase activity (TA) is found in the majority of malignancies and telomerase re-activation is believed to play a critical role in tumorigenesis. TA distinguishes between malignant and benign cells and, for a number of tumors, high TA in the primary tumor at diagnosis was associated with unfavorable clinical features. In Ewing's sarcoma, TA is the predominant telomere maintenance mechanism with 70% of tumor specimens and 90% of cell lines expressing TA **[10]**. The current study was designated to investigate whether telomerase is reactivated in Ewing's sarcoma and whether it has a prognostic significance.

2. Patients and Methods

From 2008 to 2013, 32 patients with localized ES of bone were enrolled in study. The study included patients with histologically proven and localized primary ES of bone.

Pretreatment Evaluation

The diagnosis of Ewing's sarcoma was made on representative specimens obtained from an open biopsy for each patient. Two pathologists from the Pathology Department in Zagazig University reviewed the histology of all the cases included in our study and they also reviewed the histologic response to induction chemotherapy. A complete history, thorough physical examination, and several chemical laboratory tests were performed in all patients. Plain x-rays, technetium-99 bone scan, computed tomography (CT), and magnetic resonance imaging were used to stage patients' primary tumors. CT scans of the lungs and bone scintigraphy were used to diagnose the presence of any metastases.

Chemotherapy

Following biopsy, chemotherapy was given in the form of four VACA blocks. Each block was to last 9 weeks and consisted of three courses of treatment each, administered at 3-week intervals. Each VACA block consisting of vincristine at 1.5 mg/m^2/d (days 1, 8, 15, 22), cyclophosphamide at 1,200 mg/m^2/d (days 1, 43) or 400 mg/m^2/d (days 22, 23, 24) both with Mesna as appropriate, Adriamycin (doxorubicin) at 30 mg/m^2/d (days 1, 2, 43, 44), and actinomycin-D at 0.5 mg/m^2/d x 3 (days 22, 23, 24). Cumulative drug doses scheduled were doxorubicin 480 mg/m^2, actinomycin D 6 mg/m^2, vincristine 24 mg/m^2, and cyclophosphamide 14,400 mg/m^2. Table 1 outlines VACA chemotherapy block.

Local Treatment

We scheduled the local therapy to be given prior to the second block; i.e., at week 9. We planned the local therapy individually for each patient. Tumor size and resectability, tumor site, the patient's age, and individual preference were taken into consideration. Complete surgery was preferred wherever possible. Otherwise, 60 Gy of irradiation was delivered to the tumor volume; the tumor-bearing compartment was to receive at least 44.8 Gy. In case of inadequate surgical removal or poor histologic response, postoperative radiotherapy was given.

Histologic Response to Chemotherapy

After chemotherapy, surgical excision or open biopsies were performed in all patients. All specimens were carefully studied, the extent of viable tumor that survived chemotherapy was evaluated and the response to chemotherapy was graded according to the classification of Picci *et al.* **[11]**: grade I, macroscopic foci of viable tumor cells, as identified either by individual nodules that are larger than a x10 magnification field or by smaller scattered nodules that, taken together, occupy an area greater than a x10 field; grade II, isolated microscopic nodules of viable tumor cells that, taken together, occupy an area smaller than a x10 field; and grade III, no viable tumor cells at all.

Telomerase activity

The telomerase activity was determined using a modified-TRAP assay **[12]**. This assay was performed essentially as described by Kim *et al.* **[13]** with only minor modifications. The cells were pelleted and incubated on ice for 30 min in 200 µl of ice-cold lysis buffer (0.5% CHAPS). The lysates were centrifuged at 16,000 g for 20 min at 4°C, and the supernatant was rapidly frozen and stored at -80°C. The protein concentration was measured against bovine serum albumin using a Bio-Rad protein assay reagent, and an aliquot containing 0.05 µg of protein was used. Assay tubes were prepared by sequestering 0.1 µg of 5'-DIG end-labeled CX primer (5'-CCCTTACCCTTACCCTTACCCTAA-3') under a wax barrier (Ampliwax; Perkin Elmer Cetus, Foster City, CA, USA). Each extract was assayed in 50 µl of reaction mixture containing 20 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, 63 mM KCl, 0.05% Tween 20, 1 mM EGTA, 50 µM dNTPs, 0.1 µg of 5'-DIG end-labeled TS oligonucleotide (5'-AATCCGTCGAGCAGAGTT-3') and 2 units of Taq DNA polymerase (Boehringer Mannheim). After a 10-min incubation at 23°C for telomerase-mediated extension of the TS primer, the reaction mixture was heated and then subjected to 31 PCR cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 90 s. The PCR product was electrophoresed on a 12.5% polyacrylamide gel and transferred to a nylon membrane by a semidry electroblotter. TRAP products were detected by the DIG Luminescent Detection Kit (Boehringer Mannheim) according to

the manufacturer's instructions. After color development, the membrane image was photographed and analyzed on a Macintosh computer using NIH image software. Telomerase activity was evaluated by TRAP products/internal telomerase assay standard (ITAS) ratio. (Figure 1)

Human telomerase reverse transcriptase (hTERT) mRNA Expression

Total RNA was extracted by Tri-reagent (Molecular Research Center, Cincinnati, OH) and cDNA was synthesized using 2 μg of total RNA, random hexamer, and Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA). Analysis of TERT mRNA expression was performed by quantitative real-time polymerase chain reaction (PCR), using primers that detect all TERT alternative splice forms **[14]**. Standard curves were generated from serial dilutions of cDNA derived from the TERT-positive neuroblastoma cell line SY5Y. Tumors were considered to have detectable TERT mRNA if the Ct value (the number of PCR cycles required for the fluorescence level to exceed a predefined threshold level) was less than 39 cycles. This cutoff was based on TERT mRNA expression data obtained in our laboratory from normal human fibroblasts and kidney tissue, which are considered to be TERT-negative **[15]**. (Figure 2)

Telomere length

The telomere length was determined based on the telomere restriction fragment (TRF) – Southern blot analysis as previously described **[16,17]**. The cells were digested with 400 μl of DNA extraction buffer (0.25 % NP-40, 10 μg/ml RNase I-A, 1 mM EDTA.2Na, 5 mM Tris bolic acid [pH 8.0]) and proteinase K (1 mg/ml). After extraction using phenol/chloroform, DNA was precipitated with ethanol and then was dissolved in distilled water. The DNA concentration was measured, and 5 μg of extracted DNA was digested with 10 units of HinfI (Wako) for 1 h at 37°C. Electrophoresis of digested genomic DNA was performed in 1% agarose gel in 1 × TAE buffer for 75 min at 50 V. After electrophoresis, the separated DNA was denatured in 0.5 M NaOH/1.5 M NaCl at room temperature for 15 min, neutralized twice in 1 M Tris/1.5 M NaCl (pH 7.5) at room temperature for 5 min, and transferred to a nylon membrane (Immobilon S; Millipore, Tokyo) using 10 × SSC by a semidry electroblotter (Horizeblot, AE-6675: P/N type; ATTO, Tokyo) for 1 h. After prehybridization in hybridization buffer (1 × SSC, 1% milk blocking solution, 0.5% SDS solution) at 42°C for 1 h, the membrane was hybridized to a 5'-DIG end-labeled telomeric probe (TTAGGG)4 in hybridization buffer at 42°C for 16 h. The membrane was washed in 1 × SSC/0.1% SDS solution and 0.1 × SSC/0.1% SDS solution. Telomeric smears were detected by the DIG Luminescent Detection Kit (Boehringer Mannheim, Tokyo) according to the instruction manual. After color development, the membrane image was photographed, and the peak and mean TRF lengths were analyzed on a Macintosh computer using NIH image software (version 1.60). (Figure 3)

Statistical analysis

Data were entered, checked and analyzed using the SPSS Software system (version 11.0; Chicago, IL).

3. Results

Patient characteristics

We analyzed the results of treatment of 32 patients with Ewing's sarcoma. The median age was 9.8 years (range 6.7–15.9). Eighteen patients were male (56%). The location of the primary tumor was located in the extremity in 25 patients and in axial skeleton in seven patients. The clinical characteristics of the patients are summarized in table 2.

Local Therapy

Local therapy was surgery in 4 patients (13%), surgery and radiotherapy in 17 patients (53%), or radiotherapy alone in 11 patients (34%). Types of local treatment according to the tumor site are presented in table 3.

Pathologic Response to Chemotherapy

Pathologic response data are available for 29 patients (91%) who underwent a definitive surgical procedure after the third cycle of chemotherapy. In addition, three patients had unresectable disease. These data are summarized in Table 4. Fourteen patients (44%) had a CR or VGPR. Fifteen patients (47%) had either a partial response or stable disease.

Telomerase activity (TA)

TA was detected in 84% (27 of 32). The telomerase positive tissue extracts produced a characteristic 6-bp ladder as shown in Figure 1. High TA (HTA) was demonstrated in 72% (23 of 32) of patients. By Fisher's exact test, TA was correlated to known clinical parameters such as age at diagnosis, primary site of tumor, sex, and response to therapy (Table 5). No significant correlation was found between any of these clinical parameters and TA.

***hTERT* expression**

hTERT expression. Thirty-two tumor samples were tested for expression of hTERT mRNA. High expression of hTERT (≥100 copies) was identified in 59% (19 of 32), and it significantly correlated with high TA as nineteen of the 23 samples over-expressing hTERT mRNA, also presented high TA (P = 0.0357, Table 5).

Telomere length

Twenty-one of 32 tumor exhibited changes in telomere lengths: eight longer and thirteen shorter

than the median benign samples. Alteration in telomere length was associated with high TA in fifteen (71%) of twenty-one tumor samples. No significant correlation was found between telomere length and TA.

Telomerase activity (TA) and patient's outcome

High TA was observed in 23 patients (72%). Nineteen (83%) of 23 patients with high TA relapsed, while none of the nine patients with low TA did. Highly significant correlation was observed between TA and progression free survival (PFS). Low TA patients had 100% PFS, while high-TA patients had 36% PFS ($P < .0001$). Age older than 12 years at diagnosis ($P = 0.0126$), and more than 90% tumor necrosis at the time of tumor resection ($P < .0001$) were favorable prognostic factors for predicting PFS. In an attempt to identify which independent factor had a significant influence on survival. The Cox multivariate study could not be established mathematically to evaluate telomerase activity because one subset of events was empty (no cases of relapse within the group of low telomerase samples). Our data show that for patients with more than 90% tumor necrosis, low TA was a significant favorable parameter and high TA was an adverse prognostic parameter regardless of their initial good histopathologic response ($P = .00043$), and therefore, TA is a better prognostic indicator. When we performed a multivariate analysis with high TA cases only, tumor necrosis was a significant protective parameter ($P = .0325$). According to our data, TA turned to be a significant prognostic factor in ES patients.

hTERT expression and patient's outcome

Nineteen patients expressed high hTERT (>100 copies), eleven of them relapsed (58%), while of the 13 patients whose samples expressed low hTERT (<100 copies), only two relapsed (15%). PFS comparing patients with high and low hTERT expression was statistically significant ($P = .0321$). All these samples that were tested for hTERT were also analyzed for TA and correlated significantly ($P = 0.0357$, Table 5).

Table 1, VACA chemotherapy block

Cyclophosphamide 1200 mg/m^2 I			**Cyclophosphamide 400 mg/m^2 III**			**Cyclophosphamide 1200 mg/m^2 I**		
Adriamycin 30 mg/m^2 II			**Actinomycin 0.5 mg/m^2 III**			**Adriamycin 30 mg/m^2 II**		
Vincristine 1.5 mg/m^2								
I	**I**	**I**	**I**					
Week								
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**

Table 2. Patient Characteristics

Characteristics	No. of Patients	%
Sex		
Male	18	56
Female	14	44
Age at diagnosis		
Median	9.8	
Range	6.7–15.9	
Primary site		
Extremity	25	78
Axial skeleton	7	22
Pelvis	4	57
Spine	2	29
Chest wall	1	14

Table 3. Type of Local Treatment, by Tumor Site

Tumor Site	No. of Cases	Type of Local Treatment					
		Radiation Therapy		Surgery		Surgery + Radiation Therapy	
		No.	%	No.	%	No.	%
Extremities	25	7	28	3	12	15	60
Femur	7	2	29	1	14	4	57
Tibia	3	1	33	-	-	2	67
Humerus	10	3	30	-	-	7	70
Fibula	5	1	20	2	40	2	40
Axial Skeleton	7	4	57	1	14	2	29
Pelvis	4	2	50	-	-	2	50
Spine	2	2	100	-	-	-	-
Ribs	1	-	-	1	100	-	-

Table 4. Pathologic response after 3 cycles

Response	No. of Patients	%
VGPR or CR	14	44
PR or SD	15	47
Not evaluated	3	9

VGPR, very good partial response; CR, complete response; PR, partial response; SD, stable disease

Table 4. Telomerase activity and clinical parameters

	Telomerase Activity				***P***
	High		**Low**		
	No.	**%**	**No.**	**%**	
Age					
≤12 years	5	63	3	37	0.5285
> 12 years	18	75	6	25	
Site of primary tumor					
Extremities	18	72	7	28	0.2054
Axial skeleton	5	71	2	29	
Sex					
Male	13	72	5	28	0.501
Female	10	71	4	29	
Response to treatment					
VGPR or CR	11	79	3	21	0.0792
PR or SD*	12	67	6	33	
hTERT					
< 100 copies	9	69	4	31	0.0357
≥ 100 copies	14	74	5	26	

* Included 3 patients an unresectable tumor after 3 cycles of chemotherapy but with clinical PR & not evaluated pathologically

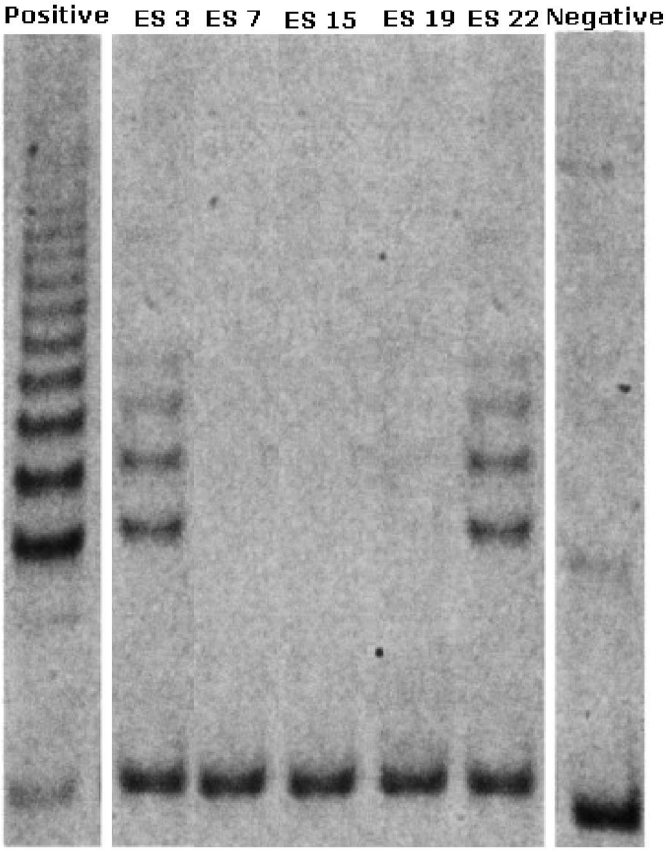

Fig. (1) Telomeric repeat amplification protocol (TRAP) assay for telomerase enzyme activity. Patients (ES 3 and ES 22) demonstrated telomerase activity in this example, as manifested by the 6-bp telomeric repeat ladder.

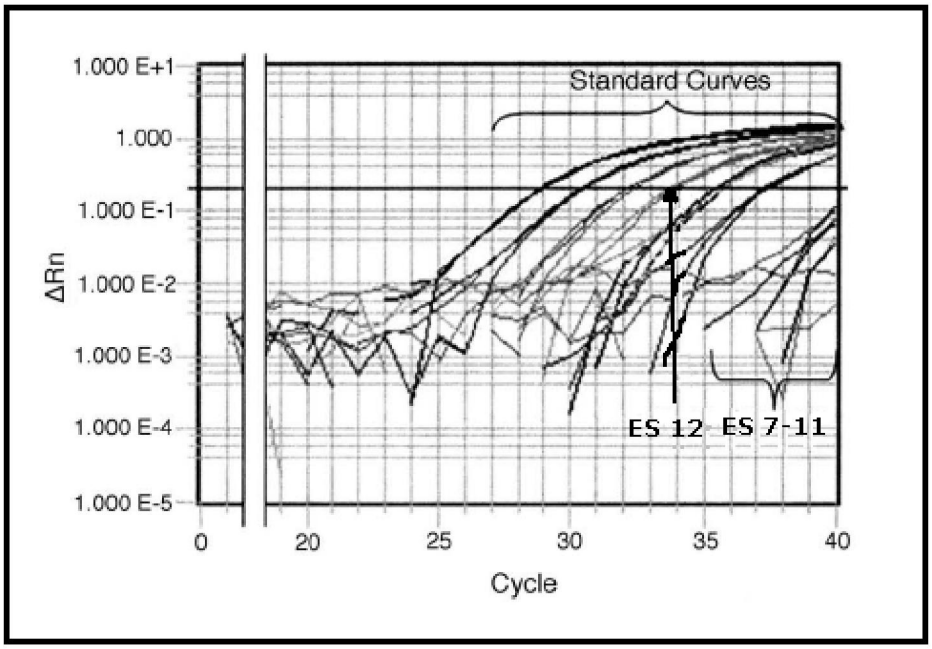

Fig. (2). hTERT mRNA expression by quantitative real-time polymerase chain reaction. ES 12 was hTERT-positive, ES 7 to 11 were hTERT-negative.

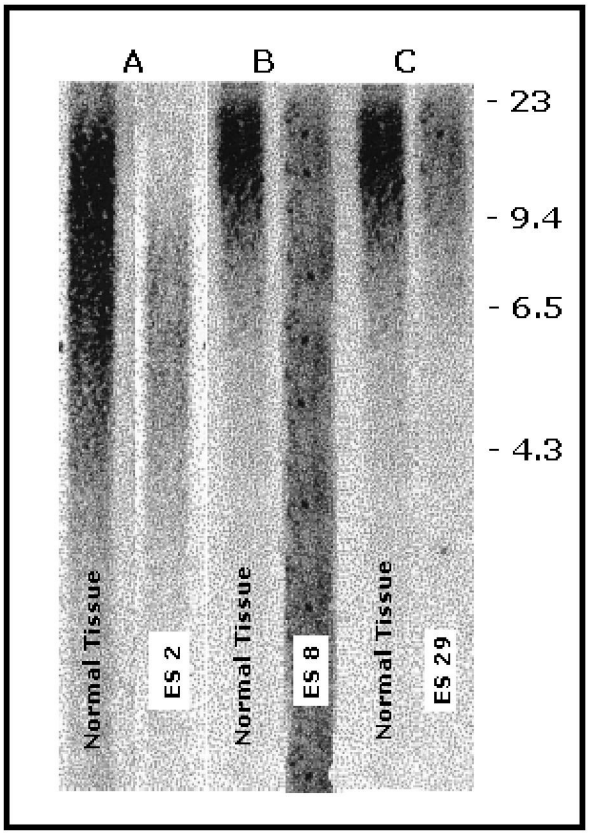

Fig. (3). Telomere length determined by Southern blot in three patients. Each tumor sample was compared with normal tissue from the same patient. Telomere is short in (A), long in (B) and unchanged in (C).

4. Discussion

Ewing's sarcoma is a small round-cell tumor of bone in children or adolescents. Up to 30% of patients have clinically evident metastatic disease at diagnosis, and their outcome remains poor, despite treatment with the most aggressive current therapeutic protocols **[18,19]**. The presence of metastases at diagnosis is the main negative prognostic factor for this type of tumor. Operative treatment for the local control of Ewing sarcoma provides an opportunity to examine the histological response of the primary tumor to preoperative chemotherapy and the relationship between the histological response and the oncological outcome. We have found that chemotherapy-induced tumor necrosis is an important indicator of progression-free survival for patients who have had operative treatment of Ewing sarcoma. Patients who had more than 90% tumor necrosis were considered to have had a good response to chemotherapy. At four years, the rate of progression-free survival for these patients was superior to that for patients who had had less than 90% tumor necrosis (85 per cent compared with zero per cent, respectively) in accord with other published reports **[20,21]**.

Telomerase, an enzyme involved in cell immortalization, is considered a new diagnostic marker of malignancy. This enzyme is a ribonucleoprotein reverse transcriptase, and its template RNA component is complementary to the telomere region that exists in chromosome ends **[22]**. Telomeres consist of repetitive DNA sequences and the binding proteins that protect the chromosome ends from being treated as broken DNA molecules **[23]**. Mammalian telomeres consist of tandem arrays of TTAGGG repeats. In the present study, we reported on TA and its possible prognostic significance in patients with ES over a relatively long period of follow-up (8 to 55 months; median, 43 months). Our results showed that 84% of Ewing's sarcomas had some telomerase activity confirming previous studies in neuroblastomas **[24]**, hepatocellular cancers **[25]** and gastrointestinal cancers **[26]**. It is known that activation of telomerase by tumor cells can ensure survival with genomic instability. Therefore, although telomerase might not directly act on the carcinogenetic process it certainly is a promoter **[27]**. Many studies in various other malignancies showed that high TA in primary tumors at diagnosis was associated with unfavorable clinical features, and some of them revealed a significant correlation between TA and survival in cancer patients **[28-30]**. In some of these studies, hTERT expression also correlated with TA.

In the present study, a highly significant correlation between high TA and poor outcome was observed ($P < .0001$), and TA could distinguish between patients with a high- or low-risk of relapse. While 83% of the patients with high TA relapsed, none of the low TA group did. Tumor necrosis, which is considered a strong clinical prognostic factor, was also highly correlated with outcome ($P < .0001$).

Numerous studies have shown that changes in telomere lengths are associated with unfavorable outcome. A reduction in telomere length has been reported in several malignancies: colorectal carcinoma, renal cell carcinoma, and childhood leukemia **[31-33]**. A correlation between shortened telomeres and TA was also established for ovarian and gastric cancer **[28,25]**. However, telomeres can be stabilized at virtually any length. Indeed some cancers, such as basal cell carcinomas, may have unchanged or elongated telomere restriction fragments (TRFs), whereas liposarcomas show a characteristically large variation in telomeric length, shortened or elongated in comparison with normal tissues **[34,35]**. TRFs in ES revealed a heterogeneous pattern. In our small cohort study, we found changes in TRF in 66%; thirteen had shortened TRFs, eight had elongated ones, and the rest were unchanged. Seventy-one

percent of the tumor samples with changes in TRF also expressed high TA. The eight patients whose tumors showed elongated TRFs are well with no evidence of disease, while six of the thirteen patients whose tumors exhibited shortened TRFs had relapsed. Our preliminary data suggest that elongated TRFs may be associated with a more favorable outcome in ES patients, but the study should be extended to a much larger cohort. In our study high expression of hTERT (≥100 copies) was identified in 59% (19 of 32), and it significantly correlated with high TA as nineteen of the 23 samples over-expressing hTERT mRNA. Although the number of patients in our study is small, our results suggest that TA could be used as a prognostic factor in ES patients. This study should be extended to a larger cohort of patients to further validate our observations.

References

1. Jürgens H, Dirksen U. Ewing sarcoma treatment. Eur J Cancer. 2011 Sep;47 Suppl 3:S366-7.
2. Potratz J, Dirksen U, Jürgens H, Craft A.: Ewing sarcoma: clinical state-of-the-art. Pediatr Hematol Oncol. 2012 Feb;29(1):1-11.
3. Stahl M, Ranft A, Paulussen M, Bölling T, Vieth V, Bielack S, Görtitz I, Braun-Munzinger G, Hardes J, Jürgens H, Dirksen U.: Risk of recurrence and survival after relapse in patients with Ewing sarcoma. Pediatr Blood Cancer. 2011 Oct;57(4):549-53.
4. Meyers PA, Krailo MD, Ladanyi M, Chan KW, Sailer SL, Dickman PS, Baker DL, Davis JH, Gerbing RB, Grovas A, Herzog CE, Lindsley KL, Liu-Mares W, Nachman JB, Sieger L, Wadman J, Gorlick RG.: High-dose melphalan, etoposide, total-body irradiation, and autologous stem-cell reconstitution as consolidation therapy for high-risk Ewing's sarcoma does not improve prognosis. J Clin Oncol 19:2812-2820, 2001.
5. Zakian V.A.: Telomeres: beginning to understand the end. Science; 270:1601–1607, 1995.
6. Trusina A. Stress induced telomere shortening: longer life with less mutations? BMC Syst Biol. 2014 Mar 1;8:27.
7. Kong CM, Lee XW, Wang X. Telomere shortening in human diseases. FEBS J. 2013 Jul; 280(14):3180-93.
8. Allsopp R.C., Chang E., Kashefi-Aazam M., Rogaev E.I., Piatyszek M.A., Shay J.W. and Harley C.B.: Telomere shortening is associated with cell division in vitro and in vivo. Exp Cell Res; 220: 194–200, 1995.
9. Shay J.W. and Wright W.E.: Telomerase activity in human cancer. Curr Opin Oncol 8: 66-71, 1996.
10. Mazumdar M, Gorlick R, Meyers P, Healey JH and Ladanyi M: Divergent patterns of telomere maintenance mechanisms among human sarcomas: sharply contrasting prevalence of the alternative lengthening of telomeres mechanism in Ewing's sarcomas and osteosarcomas. Genes Chromosomes Cancer 41: 155-162, 2004.
11. Picci P, Rougraff BT, Bacci G, Neff JR, Sangiorgi L, Cazzola A, Baldini N, Ferrari S, Mercuri M, Ruggieri P: Prognostic significance of histopathologic response to chemotherapy in non-metastatic Ewing's sarcoma of the extremities. J Clin Oncol 11:1763-1769, 1993.
12. Okusa Y, Shinomiya N, Ichikura T, Mochizuki H: Correlation between telomerase activity and DNA ploidy in gastric cancer. Oncology 1998, 55:258-264.
13. Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL, Shay JW: Specific association of human telomerase activity with immortal cells and cancer. Science, 266: 2011-2015, 1994.
14. Counter C.M., Gupta J., Harley C.B., Leber B. and Bacchetti S.: Telomerase activity in normal leukocytes and in hematologic malignancies. Blood 85: 2315-2320, 1995.
15. Dome JS, Chung S, Bergemann T, Umbricht CB, Saji M, Carey LA, Grundy PE, Perlman EJ, Breslow NE, Sukumar S.: High telomerase reverse transcriptase (hTERT) messenger RNA level correlates with tumor recurrence in patients with favorable histology Wilms' tumor. Cancer Res 59:4301-4307, 1999.
16. Counter CM, Avilion AA, LeFeuvre CE, Stewart NG, Greider CW, Harley CB, Bacchetti S: Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity.EMBO J, 11: 1921-1929, 1992.
17. Engelhardt M, Ozkaynak MF, Drullinsky P, Sandoval C, Tugal O, Jayabose S, Moore MA: Telomerase activity and telomere length in pediatric patients with malignancies undergoing chemotherapy. Leukemia, 12:13-24, 1998.
18. Cangir A, Vietti TJ, Gehan EA, Burgert EO Jr, Thomas P, Tefft M, Nesbit ME, Kissane J and Pritchard D.: Ewing's sarcoma meta-static at diagnosis: Results and comparison of two intergroup Ewing's sarcoma studies. Cancer 66:887-893, 1990.
19. Paulussen M, Ahrens S, Burdach S, Craft A, Dockhorn-Dworniczak B, Dunst J, Frohlich B, Winkelmann W, Zoubek A, Jurgens H.: Primary metastatic (stage IV) Ewing tumor: Survival analysis of 171 patients from the EICESS studies—European Intergroup Cooperative

Ewing Sarcoma Studies. Ann Oncol 9:275-281, 1998.
20. Arpaci E, Yetisyigit T, Seker M, Uncu D, Uyeturk U, Oksuzoglu B, Demirci U, Coskun U, Kucukoner M, Isıkdogan A, Inanc M, Alkis N, Ozkan M.: Prognostic factors and clinical outcome of patients with Ewing's sarcoma family of tumors in adults: multicentric study of the Anatolian Society of Medical Oncology. Med Oncol. 2013 Mar;30(1):469.
21. Elomaa I, Blomqvist CP, Saeter G, Akerman M, Stenwig E, Wiebe T, Bjork O, Alvegard TA: Five-year results in Ewing's sarcoma: The Scandinavian Sarcoma Group experience with the SSG IX protocol. Eur J Cancer 36: 875-880, 2000.
22. Morin GB. The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. *Cell*; 59: 521–529, 1989.
23. Raynaud CM, Sabatier L, Philipot O, Olaussen KA, Soria JC. Telomere length, telomeric proteins and genomic instability during the multistep carcinogenic process. Crit Rev Oncol Hematol. 2008 May;66(2):99-117.
24. Hiyama E, Hiyama K, Yokoyama T, Matsuura Y, Piatyszek MA and Shay JW: Correlating telomerase activity levels with human neuroblastoma outcomes. Nat Med 1: 249-255, 1995.
25. Tahara H, Nakanishi T, Kitamoto M, Nakashio R, Shay JW, Tahara E, Kajiyama : Telomerase activity in human liver tissues: comparison between chronic liver disease and hepatocellular carcinomas. Cancer Res 55(13): 2734-6, 1995.
26. Chadeneau C, Hay K, Hirte HW, Gallinger S, Bacchetti S: Telomerase activity associated with acquisition of malignancy in human colorectal cancer. Cancer Res 55(12):2533-6, 1995.
27. Campisi J, Kim Sh, Lim Ch S, Rubio M: Cellular senescence, cancer and aging: the telomere connection. Exp Geront, 36:1619-1637, 2000.
28. Kuhn E, Meeker AK, Visvanathan K, Gross AL, Wang TL, Kurman RJ, Shih IeM. Telomere length in different histologic types of ovarian carcinoma with emphasis on clear cell carcinoma. Mod Pathol. 2011 Aug;24(8):1139-45.
29. Poremba C, Willenbring H, Hero B, Christiansen H, Schafer KL, Brinkschmidt C, Jurgens H, Bocker W, Dockhorn-Dworniczak B.: Telomerase activity distinguishes between neuroblastomas with good and poor prognosis. Ann Oncol 10:715–721, 1999.
30. Yoshida K, Sugino T, Tahara H, Woodman A, Bolodeoku J, Nargund V, Fellows G, Goodison S, Tahara E, Tarin D.: Telomerase activity in bladder carcinoma and its implication for noninvasive diagnosis by detection of exfoliated cancer cells in urine. Cancer 79:362–369, 1997.
31. Franco S, Ozkaynak MF, Sandoval C, Tugal O, Jayabose S, Engelhardt M, Moore MA. Telomere dynamics in childhood leukemia and solid tumors: a follow-up study. Leukemia. 2003 Feb;17(2):401-10.
32. Svenson U, Grönlund E, Söderström I, Sitaram RT, Ljungberg B, Roos G. Telomere length in relation to immunological parameters in patients with renal cell carcinoma. PLoS One. 2013;8(2):e55543.
33. Feng TB, Cai LM, Qian KQ, Qi CJ. Reduced telomere length in colorectal carcinomas. Asian Pac J Cancer Prev. 2012;13(2):443-6.
34. Johnson JE, Varkonyi RJ, Schwalm J, Cragle R, Klein-Szanto A, Patchefsky A, Cukierman E, von Mehren M, Broccoli D. Multiple mechanisms of telomere maintenance exist in liposarcomas. Clin Cancer Res. 2005 Aug 1;11(15):5347-55.
35. Anic GM, Sondak VK, Messina JL, Fenske NA, Zager JS, Cherpelis BS, Lee JH, Fulp WJ, Epling-Burnette PK, Park JY, Rollison DE. Telomere length and risk of melanoma, squamous cell carcinoma, and basal cell carcinoma. Cancer Epidemiol. 2013 Aug;37(4):434-9.

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