Short Telomeres: A Novel Potential Predictor of Relapse in Ewing Sarcoma

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Abstract

Purpose: Despite advances in therapy, >50% of patients with Ewing sarcoma will relapse. The current prognostic factors are not optimal for risk prediction. Studies have shown that telomere length could predict outcome in different malignancies. Our aim was to evaluate whether telomere length could be a better prognostic factor in Ewing sarcoma and correlate the results with clinical variables, outcome, and chromosomal instability.

Experimental Design: Telomere length was determined in the primary tumor and peripheral blood of 32 patients with Ewing sarcoma. Chromosomal instability was evaluated by combining classical cytogenetics, comparative genomic hybridization and random aneuploidy. Telomere length was correlated to clinical variables, chromosomal instability, and outcome.

Results: In 75% of the tumors, changes in telomere length, when compared with the corresponding peripheral blood lymphocytes, were noted. The majority of changes consisted of a reduction in telomere length. Patients harboring shorter telomeres had a significantly adverse outcome ($P = 0.015$). Chromosomal instability was identified in 65% of tumors, significantly correlating with short telomeres ($P = 0.0094$). Using multivariate analysis, telomere length remained the only significant prognostic variable ($P = 0.034$). Patients with short telomeres had a 5.3-fold risk of relapse as compared to those with unchanged or longer telomeres.

Conclusion: We have shown that tumors with telomere length reduction result in genomic instability. In addition, telomere length reduction was the only significant predictor of outcome. We suggest that reduction of telomere length in tumor cells at diagnosis could serve as a prognostic marker in Ewing sarcoma.

Ewing sarcoma is the second most common primary malignant bone tumor in children and adolescents, and it is part of a group of neuroectodermal tumors known as Ewing sarcoma family of tumors (EFT; ref. 1). All EFT share one of the following specific translocations t(11;22), t(21;22), t(7;22), t(17;22), and t(2;22) (refs. 2 – 6). These translocations result in the fusion of the *EWS* gene on 22q12 with different members of the ETS family: *FLI-1* (85-90%), *ERG* (5-10%), *ETV-1*, *E1AF* (1%), and rarely, *FEV*. The various EWS rearrangements seem to be pivotal events in EFT tumorigenesis and these genetic alterations are considered to be distinct diagnostic features of these tumors (2). The standard care includes a combination of aggressive chemotherapy, radiotherapy, and surgery. However, despite advances in therapy, >50% of the patients will eventually relapse, even after 5 years (7).

The accepted prognostic factors in EFT are primary site of tumor at diagnosis, presence/absence of metastases at diagnosis, and initial response to therapy, assessed histologically on surgical specimens as the degree of tumor necrosis at the time of definitive surgery.

Telomeres are specialized structures at the ends of eukaryotic chromosomes, consisting of hundreds of repeated hexanucleotides $(TTAGGG)_n$ (ref. 8). Telomeres protect the chromosomes from DNA degradation, end to end fusions, rearrangements, and chromosome loss (9, 10). Due to the inability of DNA polymerase to replicate the ends of double-stranded DNA, known as the "end replication problem," telomeres are progressively shortened with each round of cell division, leading to cellular senescence (11).

Telomerase, a multisubunit ribonucleoprotein, is capable of adding telomeric DNA to the ends of linear chromosomes using its RNA template (12). Telomerase activity has been identified in the majority of human tumors (13). A reduction in telomere lengths has been described in a wide range of human cancers (14 – 18), and progressive telomere shortening has been shown to contribute to chromosomal instability (19 – 21).

Telomere length could also predict outcome in several malignancies including breast carcinoma, prostate cancer,

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Table 1. Clinical features, telomere length, and chromosomal instability in Ewing sarcoma primary tumors

Patient no.	Age (y)	Primary site	Metastases at diagnosis	Necrosis (%)	Progression (mo)
1	24	Femur	-	0	+ (48)
2	20	Ilium	-	100	+ (58)
3	7	Cranium	-	ND	+ (29)
5	11	Tibia	-	100	- (209)
6	17	Pelvis	-	80	+ (10)
7	18	Ilium	-	100	- (114)
8	18	Ilium	+ (Lung)	80	+ (22)
9	13	Radius	-	30	- (102)
10	15	Sacrum	-	100	- (118)
11	19	Femur	-	90	+ (31)
12	21	Ilium	-	60	+ (5)
13	15	Sacrum	-	ND	- (118)
14	15	Pelvis	-	100	- (141)
16	12	Sacrum	-	100	+ (29)
17	12	Humerus	-	80	- (51)
18	13	Femur	-	ND	+ (8)
19	16	Pelvis		ND	- (10)
20	6	Ilium	+ (Lung)	100	+ (45)
21	27	Femur	-	99	+ (61)
22	16	Femur	-	100	- (28)
23	14	Femur	+ (Lung)	100	+ (31)
24	2	Chest	-	100	- (34)
25	18	Spine	-	100	- (45)
26	0.3	Spine	-	ND	+ (32)
27	14	Femur	-	100	+ (60)
28	14	Chest	-	100	- (36)
29	18	Clavicle	-	100	- (144)
30	18	Ilium	+ (Lung)	95	+ (14)
31	11	Fibula	-	97	- (20)
32	12	Tibia	-	99	- (102)
33	10	Ilium	-	100	- (33)
34	11		Multifocal	ND	+ (19)

NOTE: Patient nos. 1 to 16 were the same as in Amiel et al. (29). Necrosis (%), as defined at the time of definitive surgery; Chromosomal instability, two cytogenetic changes (identified by cytogenetics/CGH or random aneuploidy) in addition to the classical translocation.
Abbreviations: FISH, fluorescence *in situ* hybridization; T, tumor; NK, normal karyotype; ND, not done; NM, no mitoses; C, group C chromosomes (6, 7, 8, 9, 10, 11, 12); N, normal.

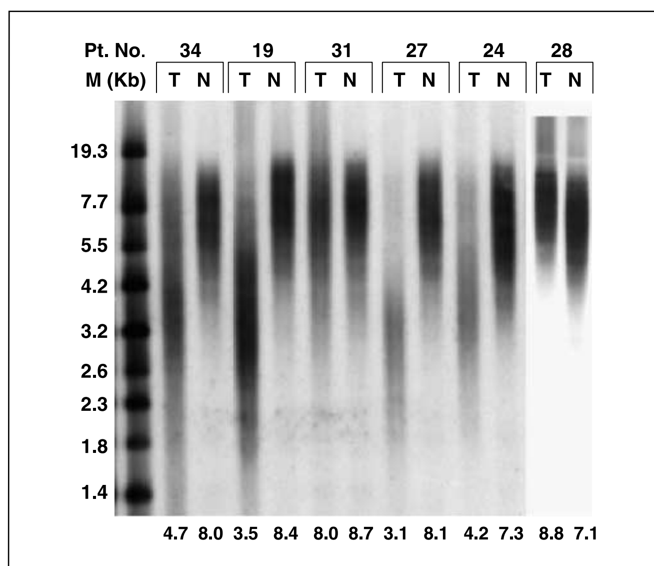

Fig. 1. Representative gel from TRF analysis. Pt. No., patient number; M, marker; T, tumor; N, normal PBL. Bottom, telomere lengths in kilobase.

colorectal carcinoma, multiple myeloma, chronic lymphocytic leukemia, and neuroblastoma (22–27).

In this retrospective study, we measured the telomere length of Ewing primary tumors compared with the corresponding

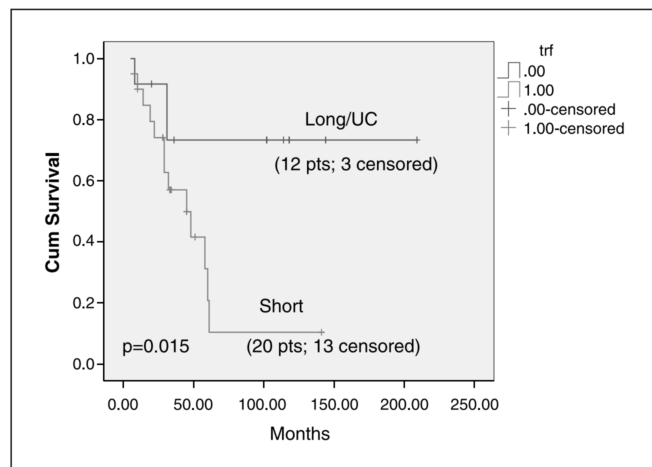

Fig. 2. Kaplan-Meier plot for progression-free survival by telomere length of Ewing sarcoma primary tumors and paired PBL. UC, unchanged.

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Table 1. Clinical features, telomere length, and chromosomal instability in Ewing sarcoma primary tumors (Cont'd)

TRF (T; PBL)	Chromosomal instability	Classical cytogenetics/ FISH	CGH	Aneuploidy
Short (3.9;7.0)	+	ND	Gain 1q; Loss 19q	Yes
Short (6.2;7.3)	+	NM	Gain 8q,12q	No
Short (3.3;6.6)	+	Inv 9p	Gain 12q	No
Unchanged (4.7;5.4)	+	ND	Gain 12q,13q	No
Short (3.6;8.3)	+	NK	Gain 8,7q, 18	Yes
Unchanged (11.3;10.8)	+	ND	Gain 1q, 4, 8q, 9q, 12, 13q; Loss 1p, 10q, 14q, 19q	ND
Short (7.4;8.8)	+	NK	Gain 5p	Yes
Long (8.1;7.1)	-	NM	N	No
Unchanged (7.3;7.9)	-	Gain 8	Gain 8	No
Unchanged (7.9;7.0)	+	Gain 2, 3, 5, 15, 20	ND	Yes
Short (4.2;6.8)	+	ND	Gain 3q, 5p, 6q, 8q, 17q; Loss 5p, 10, 12q, 19	Yes
Unchanged (9.7;9.1)	-	ND	N	No
Short (5.1;7.3)	+	ND	Gain 2, 4q	No
Short (7.7;8.7)	+	t(11;22)	ND	Yes
Short (2.3;4.3)	+	Gain 1q, 4, 8, 21	ND	ND
Unchanged (7.2;6.7)	-	t(11;22;13)	ND	ND
Short (3.5;8.4)	ND	NM	ND	ND
Short (7.1;9.1)	ND	NK	ND	ND
Short(5.8;10.4)	ND	NK	ND	ND
Short (4;10.5)	ND	ND	ND	ND
Unchanged (5.9;6.6)	ND	NK	ND	ND
Short (4.2;7.3)	+	ND	Gain 3, 8q	ND
Short (3.3;5.1)	ND	NK	ND	ND
Short (3.2;5.3)	ND	NK	ND	ND
Short (3.1;8.1)	ND	NM	ND	ND
Long (8.8;7.1)	-	Gain 8	ND	ND
Long (9.3;7.8)	-	t(11;22)	ND	ND
Short (2.9;7.7)	-	Gain 8	ND	ND
Unchanged (8.0;8.7)	-	ND	N	ND
Long (9.6;7.5)	+	Gain C, X	ND	ND
Short (2.2;6.3)	ND	ND	ND	ND
Short (4.7;8.0)	+	ND	Gain 1q,4q,18q; Loss 16	ND

normal peripheral blood lymphocyte (PBL) samples of each patient and correlated the results with clinical variables, outcome, and chromosomal instability.

Materials and Methods

Patients

Primary tumor specimens were obtained from 32 EFT patients who were admitted to the Pediatric Hematology Oncology Department at Schneider Children's Medical Center, Petach Tikva, Israel. Informed consent was obtained from the patients or their guardians, and the local and national ethics committees approved the research project. All patients were treated according to the protocol for EFT, including a combination of aggressive chemotherapy based on the VACA and IV-VACA protocols (vincristine, actinomycin-D, cyclophosphamide, adriamycin, ifosphamide, etoposide) radiotherapy, and surgery. Median age at diagnosis was 14 years (range, 0.3-27). Thirteen patients were females and 19 were males. Primary sites were: 14 pelvis, 14 limbs, and 4 others. Metastasis at diagnosis was present in five patients. Response to therapy was defined by histopathology and assessed by the percentage of tumor necrosis at the time of surgery (limb salvage procedure) following neoadjuvant chemotherapy and radiotherapy. Twenty patients were good responders (≥90% tumor necrosis at the time of resection), 6 were poor responders (<90% tumor necrosis), and in 6 cases, the results were not known (inoperable tumors and prior to surgery in one patient). Sixteen patients (50%) progressed, 5 locally, 4 in the lungs, and 7 in the distal bony sites. Median follow-up was 47 months (range, 7-209). All tissue samples were snap-frozen in liquid nitrogen immediately after surgery and were stored at -80°C. All tumors harbored the *EWS-FLI1* chimeric transcript.

Telomere length determination

Genomic DNA was extracted from tumor specimens and from 10 to 15 million peripheral blood mononuclear cells using PUREGENE DNA isolation kit (Gentra). Mean telomere length was measured using the TeloTAGGG telomere length assay (Roche Diagnostics GmbH). Briefly, 5 μg of genomic DNA was digested with a *Hin*fI/*Rsa*I mixture (5 units/1 μg DNA) and separated on 0.8% agarose gels. Fractionated DNA fragments were transferred onto a nylon membrane (Roche Diagnostics) and hybridized with a digoxigenin-labeled telomeric probe $(TTAGGG)_3$. The hybridized probe was incubated with a digoxigenin-specific antibody covalently coupled to alkaline phosphatase. The immobilized telomere probe was visualized by a highly sensitive chemiluminescent substrate for alkaline phosphatase, and telomere DNA detection was made on Lumi-Film Chemiluminescent Detection Film (Roche Diagnostics). The intensity of the hybridization was evaluated by densitometric analysis with Quantity One software (PDI) and terminal restriction fragments (TRF), an indicator of mean telomere length of a sample, was estimated according to $\Sigma(OD_i)/\ \Sigma(OD_i/L_i)$, where OD is the intensity of hybridization signal and L is the length in kilobase (Kb), at the gel point i. L_i represents the mean molecular size of 30 equal intervals of the telomeric smears in the range of 2 to 23 Kb (28). The cutoff of 1 Kb was determined using receiver operating characteristic analysis to discriminate telomere shortening or elongation of each tumor in comparison to the mean TRF of its PBL counterpart sample. Unchanged length was determined up to 1 Kb.

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Chromosomal instability

Chromosomal analysis was done in samples from 30 patients and included classical cytogenetics, comparative genomic hybridization (CGH), and random aneuploidy for chromosomes 9 and 18 by fluorescence *in situ* hybridization. The results of CGH and random aneuploidy from 13 patients have previously been reported and are numbered the same as in the prior publication (patients 1-16; ref. 29).

Classical cytogenetics. Fresh, finely minced, and collagenase-disaggregated tumor tissues were briefly cultured and processed for cytogenetic analysis on G-banded chromosomes. Karyotypes were described according to the International System for Human Cytogenetic Nomenclature (30).

CGH. DNA was isolated from tissue tumors with a Qiagen kit (Qiagen GmbH). Tumor and control DNA were labeled by nick translation with Spectrum green-dUTP and Spectrum orange-dUTP (Vysis, Abbott Laboratories), respectively. Mixture probe with 60 μL of Cot-1-DNA (Vysis) was denatured at 76°C for 7 min, and re-annealed at 37°C for 1 h. Metaphase chromosomes were denatured by applying 150 μL of 70% formamide in 2× SSC (pH 7.0) on a hot plate at 72°C for 1.5 min. Probes and slides were hybridized and incubated in a moist chamber at 37°C for 3 days. Posthybridization washes were done with 4× SSC at 50°C for 2 min, followed by a second wash of 2× SSC 0.1% NP40 at room temperature for 1 min. Chromosomes were counterstained with 4′,6-diamidino-2-phenylindole.

Results were analyzed with Applied Imaging System (Applied Imaging Corporation) software, attached to a fluorescent Olympus BX50 microscope. Approximately 12 to 15 metaphases were chosen for image analysis on the basis of their high fluorescence intensity and uniform hybridization. Trisomies or partial chromosome gains, and monosomies or partial chromosome losses were defined as having a green-to-red ratio of >1.20 and <0.80, respectively (as recommended by the software; Applied Imaging version 2.03).

Statistical analyses

The cutoff value of telomere length was determined by receiver operating characteristic analysis, which evaluates the specificity and sensitivity of different cutoff values for a given set of variables. The cutoff of 1 Kb was the optimal regarding sensitivity of 80% and specificity of 66%. Telomere length was assessed for potential association with a number of clinical and molecular variables, including patients' age, primary site, tumor necrosis, and chromosomal instability. The associations were evaluated using Fisher exact test and $P < 0.05$ was considered to be statistically significant. Distribution of progression-free survival based on telomere length were estimated by the Kaplan-Meier analysis (using log rank). Results were considered significant for $P < 0.05$. Univariate and multivariate analyses were done using Cox regression study.

Results

TRF measurements were done on 32 Ewing sarcoma primary tumors and compared with the measurement obtained from the normal PBL counterpart sample. The mean average TRF of the tumors was 4.9 versus 7.5 Kb of the PBLs. Changes in TRF, i.e., shortening or lengthening, as compared with the corresponding PBL, were defined if at least a 1 Kb difference in TRFs was observed between the tumor and the matched normal PBL of the same patient. A representative Southern blot gel is presented in Fig. 1. Changes in TRF were observed in 24 tumors (75%). In the majority of tumors (20 of 24) the telomeres were shorter and in only 4 tumors, longer, than in the corresponding PBL. In eight patients, the TRF was determined as unchanged, as it was within the range of 1 Kb, compared with PBL. Progression-free survival evaluated by Kaplan-Meier curves revealed a highly significant correlation between TRF and outcome. Patients harboring shorter telomeres had a 10% progression-free survival as compared with those with longer or unchanged TRFs with 73% progression-free survival ($P = 0.015$; Fig. 2). Table 1 summarizes the clinical variables of the patients and TRF results.

In 30 of the tumors, samples for cytogenetic analysis were available. Samples from 21 patients were processed for classical cytogenetics. Karyotypes from 17 patients were described, but results could only be considered from 10 patients because there was no mitosis in 4 patients and only a normal karyotype without any of the specific translocations was detected in 7 patients. In 3 of these 10 patients, 2 or more changes were identified. CGH was done on 15 samples (12 from our previous analysis and 3 from this study), and in 9 samples, multiple changes were detected. Analysis of aneuploidy was done in a previous study (29) on 13 cases and was identified in 6 of them. Altogether, a cytogenetic result could be obtained from 23 samples.

We defined chromosomal instability when a sample harbored more than two chromosomal aberrations and/or harbored random aneuploidy in addition to the t(11;21). Altogether, we identified chromosomal instability in 15 out of 23 (65%) samples. Table 1 also summarizes the chromosomal aberrations. In 11 out of these 15 cases with chromosomal instability, a reduction in telomere length was evident. This indicates a highly significant correlation between short telomeres and chromosomal instability ($P = 0.0094$; Table 2). No significant correlation was observed between clinical variables as age, primary site, or necrosis at the time of definitive surgery and telomere length (Table 2). Neither the clinical variables nor chromosomal instability were significant predictors of outcome (data not shown).

We did univariate and multivariate analyses including age, primary tumor site, percentage of necrosis, and telomere length (Table 3). The only significant variable found was telomere length ($P = 0.034$). Patients with short telomeres had a 5.3-fold risk of relapse (95% confidence interval, 1.135-25.188) compared to those with unchanged or longer telomeres (Table 3).

Discussion

Telomeres in tumors are altered when compared with normal tissue and cause genomic abnormalities, including chromosomal

Table 2. Association between telomere length and clinical/biological variables

Variable	Short TRF	Unchanged/ Long TRF	*P*
Age (y)			
≥12	14	10	0.67
<12	6	2	
Primary site			
Pelvis	11	3	0.14
Other	9	9	
Necrosis (%)			
<90	5	1	0.35
>90	11	9	
Chromosomal instability			
+	11	4	0.0094
-	1	7	

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Table 3. Cox regression analysis of known clinical variables and TRF in Ewing sarcoma

Covariables	Univariate *P* value	Multivariate *P* value	Relative risk (95% confidence interval)
Age (y): ≤12 vs. >12	0.34	0.39	
Primary: pelvis vs. nonpelvis	0.10	0.24	
Necrosis (%): ≥90 vs. <90	0.13	0.16	
TRF: short vs. unchanged/long	0.019	0.034	5.34 (1.13-25.18)

NOTE: Necrosis (%), at the time of definitive surgery.

instability, aneuploidy, and loss of heterozygosity. These properties predispose the tumor to increased aggressive behavior with the ability to cause recurrence of the disease (21). We have studied telomere length in 32 Ewing sarcoma primary tumors and correlated the results with clinical outcome and chromosomal instability.

It has been shown that in sarcomas lacking specific translocations, telomeres are dramatically long and heterogeneous. In contrast, in those sarcomas harboring specific translocation, telomere lengths were similar or reduced when compared with nonneoplastic tissue (31, 32). The mean average TRF observed by Ulaner et al. (32) in Ewing sarcoma tumor samples was similar to the lengths identified in our study—5 Kb in contrast to a mean average of 7.5 Kb in the corresponding PBLs. TRF alterations were identified in 23 out of the 32 tumors studied. Reduction in telomere length was detected in the majority of them and significantly correlated with a progression-free survival of only 10% as compared with 73% in those with longer or unchanged TRFs ($P = 0.015$). No significant clinical correlation between TRF and age, primary site, or percentage of necrosis was detected.

Altered telomeres (reduction or elongation) have been associated with prognosis in a variety of malignancies. Reduction of telomere length which correlated with an adverse outcome has been identified in breast carcinomas (22, 33), prostate cancer (23, 34), multiple myeloma (25), and in hematologic malignancies (26). In other tumor types, elongation of telomeres has been associated with adverse outcome; in colorectal carcinoma (24), head and neck cancer (35), and in our study on neuroblastoma (27). It is interesting that, in our lab, two pediatric small round cell tumors, neuroblastoma and Ewing sarcoma, exhibit an opposite pattern of telomere dysfunction in correlation with prognosis. The differences in the mode of telomere change in association with outcome in various malignances might imply a tissue-specific pattern of telomere dysfunction (21).

In patients with Ewing sarcoma, we have previously shown a significant correlation between telomerase activity during follow-up and outcome, but not at diagnosis (36). Nineteen samples from this previous cohort were also studied for telomere length in this study. Sixty-seven percent and 50% of the samples with short and unchanged/long telomeres exhibited high telomerase activity, respectively. Thus, no correlation between TRF and telomerase activity could be detected and this is in agreement with other studies in soft tissue sarcoma (37), childhood neuroectodermal brain tumors (38), and colorectal carcinoma (39).

Because short telomeres are associated with genetic instability such as chromosomal instability, aneuploidy, allelic imbalance, and loss of heterozygosity, we compared the chromosomal aberrations with telomere length. A highly significant correlation was identified between short telomeres and chromosomal instability ($P = 0.0094$). The majority of cytogenetic changes were gains and losses of whole or parts of chromosomes or aneuploidy. The most recurrent chromosomal changes were gain of chromosome 8 (27%), 1q (18%), and 12q (14%). These changes have been identified in other studies in Ewing sarcoma (40–42). Hattinger et al. (40)

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Table 4. Correlation between telomere length and risk group as defined by microarray analysis

Patient no.	Telomere length	Microarray definition	Clinical outcome
1	Short	High risk	R (48)
2	Short	High risk	R (58)
3	Short	High risk	R (29)
5	Unchanged	Low risk	NED (209)
6	Short	High risk	R (10)
8	Short	High risk	R (22)
9	Long	Low risk	NED (102)
10	Unchanged	Low risk	NED (118)
12	Short	High risk	R (5)
13	Unchanged	Low risk	NED (118)
14	Short	High risk	NED (141)
16	Short	High risk	R (29)

NOTE: χ^2 analysis between telomere length and microarray definition was highly significant ($P = 0.005$). Values in brackets indicate number of months from diagnosis. Microarray definition according to Ohali et al. (48).
Abbreviations: R, relapse; NED, no evidence of disease.

detected a highly significant correlation with adverse outcome between gain of 1q and loss of 16q. Kullendorff et al. (41) suggested that Ewing tumors harboring aneuploidy may be associated with poor prognosis. Tarkkanen et al. (42) showed a trend towards worse outcome and increase in copy numbers of 1q, gain of chromosomes 8 and 12, and a significant association between an increase of 6p and worse relapse-free and overall survival. We have previously identified a trend toward an adverse prognosis in Ewing sarcoma tumors exhibiting high genomic instability (43). Several published studies have identified a correlation between telomere length and chromosomal abnormalities. Chromosomal instability is related to telomere shortening in Barrett's esophagus (44). In multiple myeloma, a strong correlation was observed between high telomerase activity, short telomeres, and cytogenetic abnormalities (25). Telomere length was significantly reduced in patients with acute myeloid leukemia, and it correlated with chromosomal abnormalities (45). Another study on acute myeloid leukemia has shown a critical significant shortening of telomeric DNA in samples with loss or gain of chromosome pairs, much shorter than in the group with reciprocal translocations/inversion and than in the group with no chromosomal aberrations (46). Reduced telomere DNA content was correlated with aneuploidy and allelic imbalance in breast cancer (33, 47). Furthermore, telomere shortening combined with allelic imbalance have also been identified in normal tissue adjacent to prostate and breast tumors (34, 47).

In addition, we correlated the telomere length results with the results obtained from our previous gene expression profiling study (48). Interestingly, we observed a perfect correlation between both results (Table 4; $P = 0.005$). All patients that were defined as high-risk by the microarray analysis also harbored short telomeres. There was also a high correlation (in 11 of 12 patients) between the results of both studies and clinical outcome. This suggests that analysis of telomere length of the primary tumor at diagnosis is as predictive as the expression signature (48) of the patients' risk to relapse, at a much lower cost.

We have shown that tumors with telomere length reduction result in genomic instability. In addition, in a multivariate analysis, telomere length was the only significant predictor of outcome. We suggest that reduction of telomere length in tumor cells at diagnosis could serve as a prognostic marker in Ewing sarcoma.

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