Absence of a Telomere Maintenance Mechanism as a Favorable Prognostic Factor in Patients with Osteosarcoma

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Abstract
There are two telomere maintenance mechanisms (TMMs) in human tumors, telomerase activation (TA) and, more rarely, the process termed alternative lengthening of telomeres (ALT). Unlike most carcinomas, sarcomas, including osteosarcomas (OS), have been reported to display TA and ALT in more balanced proportions and, thus, present an opportunity to examine the impact of different TMMs on clinical tumor behavior. We studied OS samples from 62 patients for molecular evidence of TA and ALT. Kaplan-Meier analysis demonstrated that the absence of both TA and ALT (in 18%) was more strongly associated with improved survival ($P = 0.05$) than were stage ($P = 0.16$) or chemotherapeutic response ($P = 0.18$) in this group of patients with OS. Subsets of OS cases with either TA or ALT did not differ significantly from each other in clinical outcome. There were no significant associations of presence, absence, or type of TMM with patient age, stage, or chemotherapeutic response. Thus, the absence of a detectable TMM may identify a favorable clinical subset of OS patients. Our study also suggests that the likelihood of detecting correlations between TMMs and clinical outcome in studies of certain other tumor types might be improved if, in addition to TA, ALT is included in future analyses. Finally, we note that OS cases with a TA−/ALT− phenotype seem to be as clinically aggressive as TA+ cases in terms of stage and clinical outcome.

Introduction
Telomeres, the repetitive sequences at the ends of linear chromosomes, shorten with each cell division. Activation of a TMM is a central feature of cancer cells that allows them to replace lost telomeric DNA, thereby evading a checkpoint triggered by critical telomere shortening and maintaining the ability to divide indefinitely (reviewed in Refs. 1 and 2). In humans, ~85% of carcinomas maintain their telomeres with telomerase, an enzyme that synthesizes new telomeric DNA (1). This holoenzyme includes a template RNA, hTERT, and a catalytic protein, hTERT (1). The presence of TA has been reported to have prognostic value in several cancer types (1). In some tumors, telomeres are maintained in an elongated and heterogeneous state despite the absence of TA (Ref. 3; reviewed in Ref. 4). This has been referred to as ALT and may result from one or more mechanisms. Homologous recombination is a leading candidate mechanism for ALT (4). The ALT telomere phenotype is recognized by the presence of heterogeneous telomeres ranging from less than 3 kb to 50 kb in length, a range notably higher than the maximum length in nonneoplastic somatic tissues (~10–15 kb in humans; Refs. 3, 4). Unlike most epithelial cancers, which show TA in the great majority of cases, OS cell lines have been reported to display TA and ALT in more even proportions (3, 5). Thus, we hypothesized that patients with OS might present an opportunity to examine, for the first time to our knowledge, the impact of different TMMs on clinical tumor behavior. We studied telomerase gene expression, TA, and telomere lengths in 71 OS samples from 62 patients and analyzed the clinical outcomes of these patients to determine the prognostic significance of these molecular tumor parameters.

Materials and Methods
Tumor Samples and Clinical Data. Tumor samples and follow-up data for 71 OS samples (61 primary tumors, 1 metachronous primary tumor, 3 local recurrences, 6 metastases) from 62 patients were procured at Memorial Sloan-Kettering Cancer Center under an Institutional Review Board-approved protocol. Tissue obtained at the time of surgery was confirmed by pathological examination, and samples were flash-frozen and stored at −70°C. Patients in this single-institution retrospective analysis were diagnosed between 1992 and 1998 and their tumors were similarly managed on protocols including preoperative chemotherapy followed by surgery with limb-sparing intent (6). Because the aggregate event-free survival for these protocols was constant throughout the period under analysis (6), this factor was unlikely to have had a significant impact on the probability of survival. Tumor response to chemotherapy was graded histologically using the Huvos grading system (6). Grades 3 and 4 were considered good chemotherapy responses (~90% necrosis), whereas Grades 1 and 2 were grouped as poor responses (<90% necrosis).

RT-PCR Assays. Tri-reagent (Sigma, St. Louis, MO) was used to extract total RNA from tissue samples. cDNA was synthesized using 0.4 µg of total RNA, random hexamer, and reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD). The cDNA samples were amplified as described previously, incorporating [γ-32P]dCTP into PCR products (7). The following primers were used in this study: β-actin-sense, 5′-CAGGTCTACCATGTTGGCAATGAGC-3′; β-actin-antisense, 5′-CGGATGCCACCTCAGCCTTCTGTA-3′; hTERT-sense, 5′-CTAACCTCATTGGAGAGGCTAG-3′; hTERT-antisense, 5′-GAAGGCGGCAGGCCGGAAT-3′; hTERT-1784-sense, 5′-CGGAAAGGCTTCTGGAGCAA-3′; hTERT-1928-antisense, 5′-GGGATTGCAGGCTGTGCA-3′; and hTERT-1928-antisense, 5′-GGATGGGCTGCTGCA-3′. PCR amplification of hTERT cDNA was performed with an initial heating at 95°C for 60 s, followed by 28 cycles of 95°C for 20 s, 68°C for 40 s, and 72°C for 40 s. β-actin primers were added at 72°C of cycle no. 7 of the hTERT PCR for the multiplexed β-actin internal control. PCR amplification of hTERT cDNA was performed using identical conditions, but proceeded for 33 cycles. Amplified products were electrophoresed on 5% polyacrylamide-urea gel. PCR products were scored as present (+) or absent (−). hTERT RT-PCR reactions were performed in duplicate with consistent results.
TA Measurements. The TRAPEze telomerase detection kit (Sero- logicals Corporation, Norcross, GA) was used to produce protein homogenates and to assess TA. Protein concentrations in the homogenates were diluted to ~1 µg/µl for analysis as determined by Coomassie Protein Assay (Bio-Rad, Hercules, CA). Analyses included a telomerase positive control cell line and a homogenate with no cells as a telomerase negative control. Homogenates of each sample were assayed both with and without heat inactivation of the telomerase enzyme (75°C for 10 min). Radiolabeled TRAP products were electrophoresed on 5% polyacrylamide-urea gel and visualized using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). TRAP assay products were scored as present (+) or absent (−). TRAP assays were performed in duplicate with consistent results.

Telomere Length Measurements. Total nucleic acid was collected from tissues using an extraction solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate, 1% 2-mercaptoethanol, and 0.5% Sarkosyl. The homogenate was extracted with phenol/chloroform and then was precipitated with 2-propanol. The pellet was washed with ethanol and was dissolved in distilled water for restriction endonuclease digestion. Ten µg of total nucleic acid from each tissue sample was restriction-digested with 12 units of Rsal and 12 units of Alul for 6 h at 37°C to produce TRFs. TRFs were resolved on 0.35% agarose gels containing ethidium bromide. Resolved DNA was visualized under UV light to document complete digestion of DNA, and then Southern blotting was used to transfer digested DNA to nylon membranes. Membranes were incubated for 20 min at 42°C in Rapid-hyb buffer (Amersham Pharmacia Biotech, Piscataway, NJ), then hybridized with 5′-end32 P-labeled (TTAGGG)4 for 1 h at 42°C in Rapid-hyb buffer, and then washed for 30 min at 42°C in 4× SSC (0.6 M sodium chloride/0.06 M sodium citrate). TRFs were visualized using a PhosphorImager and compared with radiolabeled DNA molecular weight ladders to determine the range of TRF lengths. Samples with elongated and heterogeneous telomeres with maximum TRF lengths exceeding those found in germ-line tissues (15 kb) were considered as having the ALT telomere phenotype (ALT+).

Data Analysis. Molecular studies (RT-PCR analyses, TA measurement, telomere length measurements) were performed by personnel blinded to patient data and clinical outcome (G. A. U., J. O.). Clinical data collection was conducted by personnel blinded to molecular results (H. Y. H., M. L.). Kaplan-Meier estimates of overall survival time in the various groups were compared using the log-rank test. Age was treated as a continuous variable, and the effect of age on survival time was evaluated using a Cox proportional hazards model. The ages of patients in the various telomerase maintenance groups were compared using a Wilcoxon test. Associations between telomerase maintenance phenotypes and other factors were examined using Fisher’s exact test. TRF lengths were compared between groups using unpaired t tests.

Results

TMMs. Evidence of TA by the TRAP assay was found in 31 (44%) of 71 OS samples. RT-PCR for hTERT was positive in all of the 31 cases that were also positive by TRAP. In addition, RT-PCR for hTERT was positive in three additional cases that were not positive by TRAP. No cases were TRAP-positive but negative for hTERT. Actin and hTR were detected by RT-PCR in all of the samples. The assignment of TA status was based on the TRAP results. Evidence of the ALT phenotype (TRFs >15 kb by Southern blotting) was present in 47 (66%) samples. These samples all had heterogeneous telomeres with an average minimum TRF length of 4.3 kb (range, 0.5–7.2 kb), as part of the ALT phenotype. Surprisingly, 19 (27%) OS samples were both TA+ and had an ALT phenotype. Twelve samples had no evidence of either TA or ALT. By definition, maximum and average TRF lengths were greater in ALT+ tumors compared with ALT− tumors. Minimum TRF lengths were, likewise, significantly longer in the former group (means, 4.3 kb versus 2.4 kb; P < 0.0001). Comparisons of minimum, maximum, and average TRF lengths showed no significant differences between TA+/ALT− samples and TA−/ALT+ samples, or between TA−/ALT− samples and TA+/ALT+ samples (unpaired t tests; analysis not shown), suggesting that TA alone had no impact on TRF lengths. Representative molecular data are shown in Fig. 1.

More than one tumor sample was obtained from eight patients (three samples from one patient, two from each of seven patients), including three matched pairs of primary and metastatic samples. In six of these eight patients, the telomere maintenance phenotype was stable over time, including one pair of primary and metastatic samples that both showed a TA−/ALT+ phenotype. In the remaining two patients, there was acquisition of a TMM in the metastasis relative to the primary tumor, going from TA−/ALT+ to TA+/ALT+ in one patient (4-month interval between primary and metastasis) and from TA−/ALT− to TA−/ALT+ in the other (4-year interval between primary and metastasis). The assays on the samples from these two patients were repeated with consistent results.

Fig. 1. TA/ALT categories in OS. Results of gene expression analysis by RT-PCR, TA measurement by TRAP assay, and TRF length measurement by Southern blotting for four OS samples (OS71, OS98, OS130, OS107). These four samples are representative of the four TA/ALT categories in Table 1, namely TA−/ALT−, TA+/ALT−, TA+/ALT+, and TA−/ALT+. TA was scored as positive when the TRAP assay produced a ladder of telomerase activities with maximum nonneoplastic somatic length (15 kb) were considered as evidence of ALT. The Actin RT-PCR product is shown as a control for RNA adequacy. Unique tumor sizes are indicated in kilobases.
Because several groups have recently described heterogeneity of TA within the same tumor specimen (8, 9), we repeated the hTERT RT-PCR, TRAP assay, and TRF length measurements in 15 cases (TA+/ALT+ tumors OS42, 91, 141, 144, 153, 154, and 181; TA+/ALT− tumors OS134, 158, 169, and 178; TA+/ALT+ tumors OS99 and 155; TA−/ALT− tumors OS107 and 161) using an additional frozen sample from the same surgery, to evaluate for topographic heterogeneity in TMMs. In only one case (OS158) was one tumor piece TA+ whereas another portion of the same resection was TA−. This sample was classified as TA+ for statistical analysis. Repeat maximum and minimum TRF measurements were within 1–3 kb of each other, and ALT TRF phenotypes were concordent in these 15 cases. Thus, there was only limited evidence for intratumoral heterogeneity of TMMs in OS.

Survival Analysis and Associations between Factors. Median survival for the entire study group (n = 62) was 89 months (95% CI, 65–120) with a median follow-up of 55 months. Sixty patients were categorized by their TA/ALT status into four groups: TA−/ALT+ (n = 21); TA+/ALT− (n = 11); TA+/ALT+ (n = 17); or TA−/ALT− (n = 11). The two patients in whom there was a change in TA/ALT status between primary and metastatic tumors were excluded from this analysis. Examples of the molecular data in representative cases of each group are shown in Fig. 1.

Kaplan-Meier analyses of overall survival were performed according to the telomere maintenance phenotype, and the subgroups were compared using the log-rank test. When the four above groups were considered individually, the TA−/ALT− group had lower mortality than the ALT+, TA+, or TA+/ALT+ groups, although this did not reach statistical significance (P = 0.29; not shown). However, when the three patient groups with at least one TMM were combined and compared with the TA−/ALT− group, patients in the latter group showed a better overall survival (P = 0.05; Fig. 2A), with 90% (95% CI, 71–100%) 5-year survival compared with 60% (95% CI, 45–76%) for patients whose tumors showed evidence of one or both TMMs. The finding of better overall survival in the TA−/ALT− group analysis was maintained in the subset of 53 patients with localized disease at diagnosis (P = 0.05; not shown), despite the smaller number of patients in the analysis.

Because most previous studies of TMM in human tumors have examined only TA, we repeated the analysis of the above 60 patients grouping them solely according to TA status. Thus, there were 28 patients in the TA+ group and 32 in the TA− group. There was no significant difference in survival between the two groups (P = 0.33; Fig. 2B).

Among other factors, age was inversely related to survival, as reported in most studies of OS. The hazard ratio for age was 1.4 (95% CI, 1.13–1.79; P = 0.002). Also consistent with previous studies of OS (6), patients whose tumors showed >90% necrosis after chemotherapy had a better overall survival than those who did not, but this trend did not reach statistical significance (P = 0.18). Stage at diagnosis was not significantly associated with survival (P = 0.16), possibly because of the small number of patients who presented with metastatic disease in this series (n = 8). There were no significant associations of presence, absence, or type of TMM with chemotherapy response, stage at diagnosis, or age (Table 1).

Discussion

OS are notable for their complex karyotypes that show the cytogenetic hallmarks of marked telomeric dysfunction (10). Thus, it seems unlikely that OS arises from TA+ osteoblasts. Rather, their highly complex karyotypes suggest that the acquisition of a TMM must be a late event in the development of these tumors, but this event has thus far been generally assumed to occur at a preclinical time point. The present results indicate that this assumption does not hold in all cases. The absence of a TMM, either TA or ALT, was a feature of 12 (19%) of the OS samples. This has been previously reported in sporadic cases of other sarcomas (9). Likewise, at least some TA− retinoblastomas show short telomeres consistent with a TA−/ALT− phenotype (11). Recent experimental data suggest that the acquisition of a TMM is not strictly required for tumorigenesis in human fibroblasts (12) but may occur as a later event in tumor progression. Indeed, this may have been observed in one of our patients, whose primary tumor showed TA−/ALT− and subsequent metastasis showed TA−/ALT+. Another patient with a TA−/ALT− primary tumor had metastases at presentation (Table 1), but the latter were not available for analysis. Thus, we cannot comment on whether TA−/ALT− OS cells can form metastases without acquiring a TMM. Although these data may suggest that TA−/ALT− tumors would be smaller, lower-stage tumors, this was not evident in the present series but might become apparent in a larger series. Likewise, elucidating the possible impact of the lack of a TMM on cellular parameters, such as proliferative rate, karyo-
Although TA has been found to correlate with worse prognosis in some cancers (1), many studies have shown no correlation. It is possible that some negative studies might have reached different conclusions if ALT had been included in the analysis. Indeed, to simulate the situation in which ALT data are not available, we reanalyzed the survival data in the present series based on TA status alone and found no significant difference (Fig. 2B). Analyzing for the ALT mechanism in addition to TA would be expected to have the greatest effect on studies of tumors with a relatively low incidence of TA, such as sarcomas and papillary thyroid carcinomas (4).

In summary, whereas ~85% of carcinomas maintain their telomeres by expressing telomerase, only 44% of OSs in this study used this mechanism. The predominant TMM in the present series of OS was ALT. It has been proposed that ALT may be more frequent in sarcomas than in carcinomas because the lower cell turnover in mesenchymal tissues may be associated with stronger physiological repression of telomerase expression (4). We found evidence that the absence of both of the known TMMs, TA and ALT, may define a subset of OS patients with increased overall survival, despite the somewhat heterogeneous nature of the present retrospective study group. These intriguing initial data on TMMs and survival in OS provide a rationale to examine this biological variable in a larger and more uniform series of OS and in other mesenchymal tumors, ideally on a prospective basis.

Finally, our data on TA/~ALT+ OS are of particular interest given the recent publication of two studies suggesting that ALT+ tumor cells may be less tumorigenic than TA+ tumor cells. In the first study, also mentioned above, expression of oncogenic H-Ras in the ALT+ human fibroblast cell line GM847 conferred anchorage-independent growth but not metastatic potential, whereas the subsequent introduction of hTERT did confer metastatic potential (19). In the second study, mouse embryonic fibroblast cultures, derived from mice lacking both TERT and INK4/ARF that had acquired ALT, were unable to generate metastases in immunocompromised mice, whereas TERT reintroduction readily generated metastasizing tumors (20). Together, these data from model systems might suggest that TA is more potent than ALT at generating malignant tumors. In contrast, our data show that human TA/~ALT+ OSs are potentially lethal tumors that can metastasize. Indeed, two of the TA/~ALT+ samples in the present series were lung metastases, and the stage at diagnosis and the survival of patients with TA/~ALT+ primary tumors did not differ significantly from their TA+/ALT− counterparts (Table 1 and results not shown). Hence, the relevance of the above-mentioned model-systems observations to spontaneous human tumors remains to be determined.

References
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