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Original Article

Telomere length and variation in telomere biology genes in individuals with osteosarcoma

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Abstract: Osteosarcoma, the most common primary bone tumor, occurs most frequently in adolescents. Chromosomal aneuploidy is common in osteosarcoma cells, suggesting underlying chromosomal instability. Telomeres, located at chromosome ends, are essential for genomic stability; several studies have suggested that germline telomere length (TL) is associated with cancer risk. We hypothesized that TL and/or common genetic variation in telomere biology genes may be associated with risk of osteosarcoma. We investigated TL in peripheral blood DNA and 713 single nucleotide polymorphisms (SNPs) from 39 telomere biology genes in 98 osteosarcoma cases and 69 orthopedic controls. For the genotyping component, we added 1363 controls from the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial. Short TL was not associated with osteosarcoma risk overall (OR 1.39, *P*=0.67), although there was a statistically significant association in females (OR 4.35, 95% CI 1.20-15.74, *P*=0.03). Genotype analyses identified seven SNPs in *TERF1* significantly associated with osteosarcoma risk after Bonferroni correction by gene. These SNPs were highly linked and associated with a reduced risk of osteosarcoma (OR 0.48-0.53, *P*=0.0001-0.0006). We also investigated associations between TL and telomere gene SNPs in osteosarcoma cases and orthopedic controls. Several SNPs were associated with TL prior to Bonferroni correction; one SNP in *NOLA2* and one in *MEN1* were marginally non-significant after correction (P_{adj}=0.057 and 0.066, respectively). This pilot-study suggests that females with short telomeres may be at increased risk of osteosarcoma, and that SNPs in *TERF1* are inversely associated with osteosarcoma risk.

Keywords: Osteosarcoma, telomere, single nucleotide polymorphism, epidemiology, telomere length

Introduction

Osteosarcoma is the most common primary bone tumor; it occurs mainly in adolescents and young adults [1]. The etiology of osteosarcoma is not well understood. Epidemiologic studies suggest that height [2] and/or birth weight [3] may be associated with risk, but the data are inconsistent [4,5]. Osteosarcoma occurs at increased frequency in certain hereditary cancer predisposition syndromes [6], such as Li-Fraumeni syndrome, Werner syndrome, and Rothmund Thomson syndrome, but the genetic contribution to apparently sporadic osteosar-

coma is not well understood.

Studies of common genetic variants in osteosarcoma have identified several potential candidate genetic variants. Positive associations between osteosarcoma and single nucleotide polymorphisms (SNPs) have been noted with the *Fok*I genotype of the vitamin D receptor gene [5], and with SNPs in *IGFR2* [7], *FAS* [8], *MDM2* [9], and *TGFBR1* [10]. An inverse association between osteosarcoma and a *TNF* promoter variant (-238 SNP) was noted [11]. Null, or equivocal studies of the estrogen receptor and collagen Iα1 genes and *TP53* have also been reported [5,12].

Telomere epidemiology is a growing field which seeks to study associations between telomere length (TL) and disease or environmental exposures. Telomeres are comprised of $(TTAGGG)_n$ nucleotide repeats and a protein complex at chromosome ends, and are key components in the maintenance of chromosomal stability [13]. Several studies suggest that blood or buccal cell -derived DNA TL is associated with certain cancers, for example, bladder cancer [14-16], esophageal cancer [17,18], and gastric cancer [19,20]. However, TL was not associated with prostate [21] or colorectal [22] cancer risk.

Telomere dysfunction has been shown to result in numerous chromosomal abnormalities, including aneuploidy and translocations [23]. Somatic osteosarcoma cells often have significant chromosomal aneuploidy suggestive of underlying DNA instability [24]. While most cancer cells overcome cellular crisis through the upregulation of telomerase, the enzyme that extends nucleotide repeats, osteosarcoma cells use the alternative lengthening of telomeres mechanism (ALT) [25,26]. Although a small study did not identify mutations in telomere biology genes in osteosarcoma cell lines [27], no one has examined whether common germline variants influence the risk of developing osteosarcoma.

In this study, we hypothesized that TL and/or common germline genetic variation in telomere biology genes may be associated with risk of osteosarcoma because of the chromosomal instability inherent in osteosarcoma tumors. We conducted a case-control association study of both TL in peripheral blood DNA and common SNPs from telomere biology genes as potential osteosarcoma risk factors.

Methods

Study design

The Bone Disease and Injury Study of Osteosarcoma (BDISO) is a hospital-based prospective case-control study which was conducted in the orthopedic surgery departments in 10 United States medical centers between 1994 and 2000 [3]. The study collected blood samples and questionnaire data on patients with osteosarcoma at the time of limb salvage surgery. There were no identified cases of Paget disease of the bone in this study. Orthopedic controls were derived from individuals treated for non-neoplastic conditions including benign tumors (26%) and other non-neoplastic conditions, such as inflammatory diseases, cysts, and trauma, excluding those with hip fracture or osteoporosis. Institutional review boards at each of the medical centers approved the study protocol and informed consent was obtained from all study subjects. The current analysis was limited to individuals who were self-identified whites (98 osteosarcoma cases and 69 orthopedic controls) in order to reduce potential effects of population stratification. The cases included in our study represent 79% of all cases in the BDISO with DNA available to analyze.

For the genotyping component of this study, an additional 1365 cancer-free white control subjects were selected from the Prostate, Lung, Colorectal, Ovarian (PLCO) Cancer Screening Trial [28]. Men and women, ages 55-74 years, were enrolled in the screening trial from 10 different centers in the U.S. between 1993 and 2001. All subjects included for this study were required to have completed a baseline questionnaire, provided a blood specimen, and consented to participate in etiologic studies of cancer and related diseases. Controls were limited to whites living in the continental U.S. without a diagnosis of colon adenoma or cancer at baseline. DNA was extracted from blood specimens using standard procedures. The institutional review boards at the National Cancer Institute and 10 screening centers approved the study.

Telomere length measurement

Genomic DNA was extracted from buffy coat fractions by standard procedures (Gentra Auto-

pure). Relative TL was measured using a multiplexed quantitative polymerase chain reaction (Q-PCR) method previously described [29,30]. Briefly, the average, relative TL was estimated from the ratio of the telomere (T) repeat copy number to a single gene copy number (human β -globin gene; S), expressed as the T/S ratio for each sample using standard curves. All PCR reactions were performed on the Bio-Rad MyiQ Single Color Real-Time PCR detection system. TL in base-pairs (bp) for a T/S ratio of 1.0 is approximately 3.3 kb [29]. Ten blinded quality control samples were included to assess variability, and each sample was run in triplicate. The coefficients of variation (CV) within triplicates of the telomere and single-gene assay were 4.1% and 6.3%, respectively, and the CVs for repeats were 5.1% and 7.9%, respectively.

Genotyping

743 SNPs were derived from genes which code for proteins previously shown to either directly interact with telomeric DNA or to regulate these proteins (*ACD, ATM, BLM, DDX1, DDX11, MCM4, MEN1, MRE11A, MYC, NBN, NOLA1, NOLA2, NOLA3, PARP1, PARP2, PIK3C3, PINX1, POT1, PRKDC, RAD50, RAD51AP1, RAD51C, RAD51L3, RAD54L, RECQL, RECQL4, RECQL5, RTEL1, TEP1, TERC, TERF1, TERF2, TERF2IP, TERT, TINF2, TNKS, TNKS2, WRN, XRCC6*). Genotyping was conducted on a Custom Infinium® BeadChip (iSelect)™ from Illumina, Inc. The iSelect panel was created by investigators in the Division of Cancer Epidemiology and Genetics, National Cancer Institute (NCI) to target genetic variation in genes potentially important in carcinogenesis and cancer risk. Tag SNPs were identified from the HapMap CEU population assuming an r^2 threshold of 0.80, using the Tagzilla module of the GLU software package (http://code.google.com/p/glu-genetics/), across the regions of interest.

The concordance rates between 10 duplicate BDISO and 195 duplicate PLCO samples on the iSelect panel were 99.5% and 99.9%, respectively. SNPs were excluded if they had less than a 90% genotyping rate, or if they failed the Hardy-Weinberg equilibrium test or genotyping validation. Individuals with more than 10% missing genotypes were excluded.

A principal component analysis was performed using a set of 3,843 independent SNPs selected from the iSelect BeadChip (27,905 SNPs) to evaluate population substructure among the BDISO individuals and the PLCO controls. There was no evidence of significant population stratification. However, 6 BDISO individuals and 2 PLCO controls were considered genetic outliers and excluded from the genotyping analyses. Two BDISO individuals were also excluded due to missing genotype data, for a final sample size for the genotyping analyses of: 96 cases, 63 orthopedic controls, and 1363 PLCO controls.

Statistical analyses

Spearman rank correlations and general linear models were used to investigate the association between TL and age and gender in control subjects, adjusting for age or gender. TL was analyzed as a continuous and as a categorical variable. The Wilcoxon rank-sum test was used to compare TL among case and controls as a continuous variable. Logistic regression models were used to obtain the odds ratio (OR) and 95% confidence intervals (CI) for the strength of the association between osteosarcoma and TL, adjusting for age and/or gender. TL was considered as a categorical variable by dichotomizing it at the median according to the distribution in control subjects, with longer length as the referent.

Logistic regression models were used to estimate the OR and 95% CI for the association between osteosarcoma risk independently for each SNP, adjusting for gender. The common allele or the homozygote of the common allele was used as the referent category for the log-additive or dominant model, respectively. We evaluated the log-additive genetic model (log-additive effect of each minor allele) and a dominant inheritance model for each SNP in relationship to osteosarcoma case status. For rare SNPs, we also used the Fisher's Exact Test to evaluate the significance of the allelic associations. We conducted gene-level and pathway-level analyses based on Yu et al [31]. The gene-level analysis is a global test for the association between the outcome and a subset of SNPs within a given gene or region. The pathway-level analysis is a global test for the association between the outcome and any subset of genes within a given pathway. *P*-values for these analyses were estimated with 20,000 permutation steps according to the algorithm given in Yu et al [31].

Table 1. Characteristics of study subjects

	n (%) Male	*n* (%) Female	Mean age (SD)	Total *n*
Telomere Length and Osteosarcoma Risk Analysis				
Osteosarcoma Cases	56 (57.1)	42 (42.9)	26.7 (16.5)	98
Orthopedic Controls	38 (55.1)	31 (44.9)	24.4 (14.4)	69
Genotype Analyses				
Osteosarcoma Cases	54 (56.3)	42 (43.7)	26.6 (16.5)	96
All Controls	904 (63.4)	522 (36.6)	60.9 (9.9)	1426
Orthopedic Controls	34 (54.0)	29 (46.0)	24.7 (15.1)	63
PLCO Controls	870 (63.8)	493 (36.2)	62.6 (5.2)	1363

Abbreviations: *n* = number of individuals; SD = standard deviation; PLCO = Prostate, Lung, Colon, Ovarian Cancer Cohort.

Linear regression models were used to estimate the association between TL as a continuous variable and each SNP independently, adjusting for age and gender. The common allele was used as the referent category using an additive model to evaluate the additive effect of each minor allele. Bonferroni adjustments (P_{adj}) were conducted by gene (for all SNPs in a gene) for correction of multiple tests.

Statistical power was calculated with Quanto [32] using the log-additive and dominant models, 96 cases and 1426 controls, baseline population risk of 0.0000001, and type 1 error of 0.05. For the log-additive model, power was greater than 80% for the following minor allele frequencies (MAF): MAF of 0.1 could detect an OR of 1.82 and MAF of 0.3 could detect an OR of 1.53 or higher.

We evaluated the correlation between SNPs [linkage disequilibrium (LD)] with Haploview version 4.1 [33]. Statistical analyses were performed using SAS software, version 9.1 (SAS Institute, Cary, NC), R language, and PLINK software, version 1.06 (http://pngu.mgh.harvard.edu/purcell/plink/).

Results

Characteristics of study subjects

The characteristics of all study participants are shown in **Table 1**. Subjects evaluated in the TL only component of this study consisted of 98 osteosarcoma cases, median age was 19.5 years (range 7-76), and 69 orthopedic controls, median age was 18.5 (range 7-68). Osteosarcoma cases and orthopedic controls had nearly equal numbers of males and females. For the genotyping component of the study we augmented the sample size through the addition of 1,363 controls from PLCO. These individuals were older than the BDISO participants with a median age of 62.6 (range 55-75). There were more males (63.8%) than females (36.2%) in the PLCO controls. All participants were self-identified whites from the continental United States.

Telomere length in osteosarcoma cases and controls

We measured relative TL in buffy coat DNA derived from osteosarcoma cases and orthopedic controls in BDISO to test the association between TL and osteosarcoma risk. The mean TL for osteosarcoma cases (1.997, standard deviation [SD] 0.32) and controls (2.012, SD 0.33) were not different ($P_{wilcoxon}$ = 0.42). As expected, TL declined with increasing age (correlation coefficient = -0.489, $P < 0.0001$). TL was significantly different between male and female controls (1.93 *vs.* 2.11, $P = 0.012$), after adjusting for age, with females having longer telomeres. Due to the small sample size, we evaluated TL dichotomized at the median and compared long (above the median) to short (below the median) TL. TL was not associated with risk of osteosarcoma overall or when subjects were grouped by age (**Table 2**). When males and females were evaluated separately, a statistically significant association between short telomeres and osteosarcoma was noted only in females, with an OR of 4.35 (95% CI 1.20-15.74, $P = 0.03$).

Association of genetic variation in telomere biology genes in osteosarcoma

We evaluated associations between individual SNPs in telomere biology genes and risk of os-

Table 2. Association of osteosarcoma risk with relative leukocyte telomere length dichotomized at the median

		Case *n* (%)	Control* *n* (%)	OR† (95% CI)	*P*
Overall‡	Short	58 (59.2)	35 (50.7)	1.39 (0.70-2.76)	0.67
	Long	40 (40.8)	34 (49.3)	(ref)	
Age§					
≤15	Short	8 (33.3)	8 (47.1)	0.55 (0.15-1.98)	0.36
	Long	16 (66.7)	9 (52.9)	(ref)	
16-30	Short	22 (51.2)	11 (33.3)	2.15 (0.83-5.54)	0.11
	Long	21 (48.8)	22 (66.7)	(ref)	
31-45	Short	14 (93.3)	9 (81.8)	2.80 (0.20-40.06)	0.45
	Long	1 (6.7)	2 (18.2)	(ref)	
46-77	Short	14 (87.5)	7 (87.5)	1.42 (0.09-23.36)	0.81
	Long	2 (12.5)	1 (12.5)	(ref)	
Gender¶					
Male	Short	31 (55.4)	22 (57.9)	0.77 (0.32-1.87)	0.56
	Long	25 (44.6)	16 (42.1)	(ref)	
Female	Short	27 (64.3)	13 (41.9)	4.35 (1.20-15.74)	0.03
	Long	15 (35.7)	18 (58.1)	(ref)	

† Odds ratio (95% confidence intervals); * includes orthopedic controls only from BDISO; ref = referent group; ‡ adjusted for age and gender; § adjusted for gender; ¶ adjusted for age.

teosarcoma in the BDISO subjects, and included 1,363 controls from PLCO in order to improve statistical power. In total, 713 SNPs from 39 telomere biology genes were analyzed. These genes are described in Supplemental Table 1. Forty-one SNPs were significantly ($P < 0.05$) associated with osteosarcoma risk before correction for multiple tests by gene (Supplemental Table 2). There were 6 significant SNPs in *PARP2*, and 9 in *TERF1* and *TNKS*. Only 7 SNPs in *TERF1* remained significant after Bonferonni correction (by gene) (**Table 3**). They had an inverse association with osteosarcoma (OR 0.48-0.53, $P = 0.0001$-0.0006). These SNPs were highly correlated in our controls ($r^2 = 0.9$-0.99).

We also conducted global tests by gene and functional pathway (including all telomere biology genes). Three genes were significantly associated with risk of osteosarcoma (**Table 3** and Supplemental Table 2): *TERF1* (Gene $P = 0.0009$), *PARP2* (Gene $P = 0.034$), and *TNKS* (Gene $P = 0.043$). However, if we corrected for multiple tests (39 genes), only *TERF1* remained significant ($P_{adj} = 0.035$). As a whole, the telomere biology pathway was not significantly associated with osteosarcoma ($P = 0.152$).

Relative telomere length and genetic variation in telomere biology genes

Potential associations between TL in the BDISO subjects ($n = 159$) and genetic variation in the 39 telomere biology genes were also evaluated in this study. For this analysis, we combined osteosarcoma cases and BDISO orthopedic controls, because there was no primary association between TL and osteosarcoma. Linear regression models were used to estimate the effect of each SNP, and the direction of the regression coefficient corresponded to each minor allele increasing or decreasing TL. There were 20 SNPs significantly associated with TL before correction for multiple tests ($P < 0.05$; **Table 4**), including multiple SNPs in *BLM*, *NOLA2*, *POT1*, *TEP1*, and *TERC*. No associations remained significant after Bonferroni correction by gene; one SNP in *NOLA2* and *MEN1* were marginally non-significant (P_{adj}=0.057 and 0.066, respectively).

Discussion

Our study had three primary goals, to: 1) determine if germline TL was associated with risk of osteosarcoma, 2) identify associations between SNPs in telomere biology genes and osteosarcoma risk, and 3) determine if those SNPs were associated with TL. We hypothesized that since osteosarcoma somatic cells typically have significant chromosomal abnormalities and often use the alternative lengthening of telomeres pathway for telomere maintenance aberrations in telomere biology could contribute to osteosarcoma risk. Overall, we found that short TL was associated with osteosarcoma risk in females,

Table 3. Significant SNPs associated with risk of osteosarcoma after Bonferroni correction by gene (P_{adj}) using a log-additive genetic model

Gene	SNP	Genomic position		Minor allele	MAF (%) Controls	MAF (%) Cases	OR†	95% CI	*P*	P_{adj}	Gene *P*
TERF1	rs2929593	Chr8: 74076067	upstream	T	31.2	19.3	0.52	(0.36, 0.75)	0.00051	0.0101	0.0009
	rs9298211	Chr8: 74079372	upstream	T	31.1	18.4	0.50	(0.34, 0.72)	0.00026	0.0052	
	rs2929586	Chr8: 74087966	IVS1-718	G	30.8	18.8	0.52	(0.36, 0.75)	0.00047	0.0095	
	rs2929585	Chr8: 74089419	IVS2+640	G	30.9	18.8	0.52	(0.36, 0.75)	0.00045	0.0091	
	rs2306494	Chr8: 74113781	IVS8-124	G	31.5	18.9	0.51	(0.35, 0.74)	0.00033	0.0065	
	rs2306492	Chr8: 74114456	IVS9+448	A	31.6	18.1	0.48	(0.33, 0.69)	0.00012	0.0025	
	rs7001277	Chr8: 74128713	downstream	A	31.6	19.8	0.53	(0.37, 0.76)	0.00063	0.0125	

† Odds ratios and 95% confidence intervals were estimated using logistic regression models with the most common allele as the referent, adjusted for gender; MAF = minor allele frequency.

Table 4. Significant SNPs ($P < 0.05$) in telomere biology genes associated with telomere length before correction for multiple tests

Gene	SNP	Genomic position		Minor allele	Beta†	SE	*P*	P_{adj}
ATM	rs1800056	Chr11: 107643213	Ex17-67 (F858L)	C	0.478	0.164	0.0041	0.122
BLM	rs7183841	Chr15: 89095901	IVS3-120	C	0.106	0.050	0.0351	1.000
BLM	rs4932363	Chr15: 89124105	IVS12-2951	A	0.177	0.086	0.0416	1.000
MEN1	rs670358	Chr11: 64348255	downstream	A	0.140	0.052	0.0083	0.066
MYC	rs4645946	Chr8: 128817567	Ex1+70	A	0.432	0.168	0.0110	0.274
NOLA1	rs10516559	Chr4: 110966179	downstream	C	0.138	0.066	0.0394	0.433
NOLA2	rs6601217	Chr5: 177501445	upstream (in *RMND5B*)	G	-0.114	0.043	0.0094	0.057
NOLA2	rs6873523	Chr5: 177505533	upstream (in *RMND5B*)	C	-0.108	0.042	0.0118	0.071
NOLA2	rs13189047	Chr5: 177511481	IVS2-881	A	-0.089	0.044	0.0422	0.253
NOLA3	rs2169480	Chr15: 32422661	downstream	G	-0.093	0.043	0.0304	0.455
POT1	rs4360236	Chr7: 124313975	IVS2+5581	T	0.124	0.054	0.0244	0.170
POT1	rs727505	Chr7: 124249317	upstream	A	-0.091	0.043	0.0373	0.261
RAD50	rs6884762	Chr5: 131966629	IVS13-262	T	0.286	0.115	0.0140	0.238
RAD51L3	rs9915078	Chr17: 30467328	IVS3+2305	G	0.173	0.065	0.0080	0.144
TEP1	rs2678685	Chr14: 19949151	IVS1+2253	G	0.103	0.037	0.0061	0.250
TEP1	rs4246977	Chr14: 19952431	downstream	C	-0.080	0.037	0.0316	1.000
TERC	rs9860874	Chr3: 170968965	downstream (in *ARPM1*)	A	-0.114	0.046	0.0145	0.087
TERC	rs12638862	Chr3: 170960200	upstream	G	-0.114	0.046	0.0152	0.091
TERC	rs12696304	Chr3: 170963965	upstream	G	-0.105	0.049	0.0343	0.206
WRN	rs11574212	Chr8: 31046197	IVS7+812	T	0.223	0.107	0.0385	1.000

† Represents the effect of each minor allele on telomere length from a linear regression model adjusting for age and gender; SE = standard error; P_{adj} = Bonferroni corrected *P* by gene.

SNPs in *TERF1* were associated with decreased osteosarcoma risk, and that telomere biology gene SNPs were not strongly associated with TL.

TL in surrogate tissues (*e.g.,* blood or buccal cells) has been postulated to be a biomarker of cancer risk. Several case-control studies have found statistically significant associations between shorter telomeres and risk of cancers such as bladder [14-16], esophageal [17,18], gastric [19,20], head and neck [16], lung [16,34], ovarian [35], and renal [16,36]. A few studies have also suggested that longer telomeres are associated with risk of melanoma [37], non-Hodgkin lymphoma [38], and breast cancer [39,40], although the breast cancer TL association studies have been inconsistent [39,41-43]. Null associations with TL were reported in prospective studies of prostate [21] and colorectal cancers [22]. Overall, significant differences in TL between osteosarcoma cases and controls were not identified.

Our study and others suggest that healthy females have longer telomeres than males [44-47]. We also found a statistically significant association between shorter TL and risk of osteosarcoma in females. This association was not noted in males or in the combined male-female dataset. This gender difference might reflect the effects of estrogen on telomere dynamics, possibly through the activation of the *hTERT* gene promoter [48], posttranslational regulation of *hTERT* [49], or through its antioxidative capacity [50]. It is also possible that this finding is a false positive due to small sample size. Alternatively, one could theorize that females with telomeres that are shorter than expected for their gender might be at even higher risk of cancer related to telomere shortening than males, as others have observed for other cancers [16,44]. It is also possible that females could have different osteosarcoma risk factors than males. A recent study of the Pro72Arg *TP53* polymorphism in osteosarcoma found that the variant allele was associated with osteosarcoma only in females [9].

This pilot study was the first to explore the association between SNPs in telomere biology genes and osteosarcoma risk. We were able to augment our statistical power through the addition of controls from the PLCO study. With the addition of these controls, there was 80% power to detect an OR of 1.82 for SNPs with MAFs of at least 0.1. We chose to interpret the SNP data conservatively, by using the Bonferroni correction based on the number of SNPs per gene, because of the study's small sample size, and we used global gene- and pathway-level analyses to comprehensively evaluate our data.

This approach identified seven statistically significant SNPs in *TERF1* after Bonferroni correction for the number of SNPs per gene. However, no associations remained significant if corrected for all 713 SNPs in the study. The SNPs in *TERF1* were all inversely associated with osteosarcoma risk and were strongly correlated with each other. At the gene-level, *TERF1* was also significantly associated with osteosarcoma after correction for multiple tests. *TERF1* encodes TRF1, a member of the shelterin telomere protection complex which protects telomeres from degradation and inappropriate DNA repair [51]. The role of *TERF1* in osteosarcoma pathogenesis is not known. One small study did not find *TERF1* mutations in osteosarcoma cell lines [52].

We also evaluated the association between TL and SNPs in telomere biology genes in the BDISO participants, to better understand the role of common SNPs in TL regulation. A total of 20 SNPs in 13 genes were statistically significantly associated with TL before Bonferroni correction, but none remained significant ($P < 0.05$) after this conservative statistical correction (**Table 4**). A recent genome-wide association study (GWAS) identified a SNP in the *TERC* locus, rs12696304, that was inversely associated with TL [53]. This SNP was also associated with a reduction in TL in our dataset which was significant before correction (Beta -0.105, SE 0.05, $P = 0.034$). Two other SNPs in our dataset in this region were also significant before Bonferroni correction. These three *TERC* SNPs were all highly correlated in our dataset ($r^2 = 0.8$-0.98). Recent genome-wide association studies have found variants in the *TERT-CLPTM1L* locus associated with cancer risk [54,55]. We evaluated 16 SNPs in the *TERT* locus and did not find associations with osteosarcoma or TL.

Other studies have mapped loci influencing TL to chromosome 14q23.2 [56], and to variants in the *BICD1* [57], *DDX11* [56], and *VPS34/PIKC3C* [58] genes. Of these genes, only *DDX11* was in our data set and its SNPs were

not associated with TL. Another candidate gene study of TL and SNPs in 43 telomere biology genes found that SNPs in *MEN1* were associated with TL [59]. A SNP in *MEN1* that was in both studies, rs670358, was significantly associated with TL before Bonferroni correction (P = 0.008) in our study. In the current study this SNP was associated with an increase in TL, but the converse was true in the other study. This discrepancy may be due in part to differences in the age of the study populations (median age of 19 years in this study compared with 62 years in the other).

In summary, this pilot-study explored the potential role of telomere biology in osteosarcoma etiology. The results were very conservatively interpreted using Bonferroni correction which reduces the potential for false positive findings, but may be too stringent. The role of SNPs in TL regulation is an area of active investigation. This study confirms some of those associations, including an association between TL and SNPs in *MEN1* and *TERC*. Common variants in *TERF1* were inversely associated with risk of osteosarcoma. Additional studies of the role of *TERF1* and other components of shelterin in osteosarcoma are warranted. Lastly, we found that females with shorter telomeres had higher risks of osteosarcoma than males. The sample size was small and larger studies are required to better understand this gender difference.

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Supplemental Table 1. Description of the 39 telomere genes included in our study

Gene	No. SNPs	Chr.	Start of gene	End of gene	Gene Name; aka	Functional group
ACD	4	16	66248934	66252214	Adrenocortical dysplasia homolog (mouse); PTOP, Pip1, TINT1, Tpp1	shelterin
ATM	36	11	107598769	107745036	Ataxia-Telangiectasia Mutated; TEL1	DNA repair
BLM	31	15	89061606	89159602	Bloom syndrome, RecQ helicase-like; RECQ3	helicase
DDX1	14	2	15649221	15688676	DEAD (Asp-Glu-Ala-Asp) box polypeptide 1	helicase
DDX11	5	12	31118077	31148992	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 11 (CHL1-like helicase homolog, *S. cerevisiae*)	helicase
MCM4	3	8	49036047	49052621	minichromosome maintenance complex component 4	DNA repair
MEN1	8	11	64327572	64335342	Multiple endocrine neoplasia I; menin	other telomere
MRE11A	34	11	93790115	9386688	MRE11 meiotic recombination 11 homolog A (*S. cerevisiae*)	DNA repair
MYC	26	8	128817498	128822856	v-myc myelocytomatosis viral oncogene homolog (avian); MRTL	other telomere
NBN	21	9	91014740	91066075	Nibrin; p95 protein of the MRE11/RAD50 complex	DNA repair
NOLA1	11	4	110956115	110965342	Nucleolar protein family A, member 1; NOLA1, GAR1	other telomere
NOLA2	6	5	177509070	177513567	Nucleolar protein family A, member 2; NOLA2, NHP	telomerase associated
NOLA3	15	15	32421209	32422654	Nucleolar protein family A, member 3; NOP10	telomerase associated
PARP1	25	1	224615129	224662414	Poly(ADP-ribose) polymerase-1	other telomere
PARP2	31	14	19881639	19895903	Poly(ADP-ribose) polymerase-2; ADPRTL2	other telomere
PIK3C3	12	18	37789197	37915442	Phosphoinositide-3-kinase, class 3	other telomere
PINX1	39	8	10659883	10734796	PIN2-interacting protein 1	other telomere
POT1	7	7	124250549	124324486	Protection of telomeres 1	shelterin
PRKDC	15	8	48848222	49035296	Protein kinase, DNA-activated, catalytic polypeptide; XRCC7	other telomere
RAD50	29	5	131920529	132007498	RAD50 homolog (*S. cerevisiae*)	DNA repair
RAD51AP1	15	12	4518317	4539475	RAD51 associated protein 1	DNA repair
RAD51C	8	17	54124962	54166691	RAD51 homolog C (*S. cerevisiae*)	DNA repair
RAD51L3	21	17	30451514	30471001	RAD51-like 3 (*S. cerevisiae*)	DNA repair
RAD54L	14	1	46486004	46516732	RAD54-like (*S. cerevisiae*): RAD54A	DNA repair
RECQL	30	12	21513965	21545796	RecQ protein-like (DNA helicase Q1-like); RECQL1	helicase
RECQL4	9	8	145707622	145713976	RecQ protein-like 4	helicase
RECQL5	7	17	71134520	71174864	RECQ protein-like 5	helicase
RTEL1	16	20	61760091	61800495	Regulator of Telomere elongation helicase 1	other telomere
TEP1	41	14	19905766	19951420	Telomerase protein component 1	telomerase associated
TERC	7	3	170965092	170965542	Telomerase RNA component	telomerase associated
TERF1	22	8	74083661	74122281	Telomeric repeat binding factor (NIMA-interacting) 1; TRF1	shelterin
TERF2	9	16	67947032	67977374	Telomeric repeat binding factor 2	shelterin
TERF2IP	9	16	74239185	74248829	Telomeric repeat binding factor 2, interacting protein; hRap1	shelterin
TERT	16	5	1306282	1348159	Telomerase	telomerase associated
TINF2	14	14	23778693	23781640	TERF1 (TRF1)-interacting nuclear factor 2; TIN2	shelterin
TNKS	52	8	9450855	9671801	Tankyrase, TRF1-interacting ankyrin-related ADP-ribose polymerase; TANK1, PARP5A	other telomere
TNKS2	8	10	93548049	93615012	TRF1-interacting ankyrin-related ADP-ribose polymerase 2; TANK2, PARP5B	other telomere
WRN	28	8	31010320	31150819	Werner syndrome, RecQ helicase-like; RECQL2	helicase
XRCC6	15	22	40347241	40389998	X-ray repair complementing defective repair in Chinese hamster cells 6; KU70	DNA repair

Chr = chromosome; aka = also known as.

Supplemental Table 2. Significant SNPs ($P < 0.05$) in telomere biology genes associated with osteosarcoma before correction for multiple tests

Gene	SNP	Minor allele	MAF (%) Controls*	MAF (%) Cases	OR†	95% CI	*P*	P_{adj}	Model or Test§	Gene *P*
ATM	rs1800889	T	5.4	1.6	0.27	(0.08, 0.87)	0.028	0.833	Dominant	0.251
ATM	rs228606	T	40.3	47.9	1.39	(1.03, 1.88)	0.029	0.870	Log-additive	
ATM	rs618499	A	43.2	36.3	0.73	(0.54, 0.99)	0.049	1.000	Log-additive	
BLM	rs2532105	A	15.1	19.8	1.58	(1.03, 2.42)	0.037	1.000	Dominant	0.428
BLM	rs2518968	C	45.1	52.6	1.37	(1.01, 1.85)	0.042	1.000	Log-additive	
DDX1	rs2890489	G	38.1	47.4	1.49	(1.11, 2.01)	0.009	0.114	Log-additive	0.071
DDX1	rs10169288	G	38.3	47.4	1.49	(1.10, 2.01)	0.010	0.125	Log-additive	
DDX1	rs4668944	A	40.8	48.9	1.41	(1.05, 1.89)	0.024	0.309	Log-additive	
DDX1	rs807629	G	33.4	40.6	1.38	(1.02, 1.87)	0.036	0.462	Log-additive	
NOLA3	rs17236875	C	11.2	15.6	1.62	(1.03, 2.56)	0.036	0.544	Dominant	0.321
NOLA3	rs2279686	C	48.5	56.3	1.36	(1.02, 1.83)	0.037	0.559	Log-additive	
NOLA3	rs7162607	A	45.3	37.5	0.74	(0.55, 0.99)	0.043	0.647	Log-additive	
PARP1	rs3219123	A	5.3	1.6	0.28	(0.09, 0.89)	0.032	0.773	Dominant	0.276
PARP2	rs3093938	G	0.00	1.04	$2.4x10^{10}$	(0, inf)	0.004	0.083	Fishers Exact Test	**0.034**
PARP2	rs3093919	G	0.04	1.04	31.07	(2.78, 347.8)	0.011	0.238	Fishers Exact Test	
PARP2	rs11622655	G	25.8	32.8	1.40	(1.02, 1.92)	0.034	0.716	Log-additive	
PARP2‡	rs10147163	C	26.7	33.9	1.41	(1.03, 1.92)	0.033	0.298	Log-additive	
PARP2‡	rs3093942	C	21.4	27.6	1.53	(1.01, 2.31)	0.045	0.407	Dominant	
PARP2‡	rs4981998	T	24.4	30.7	1.38	(1.00, 1.90)	0.047	0.422	Log-additive	
POT1	rs727505	A	29.3	22.4	0.70	(0.49, 0.99)	0.047	0.331	Log-additive	0.217
TEP1	rs2104977	A	15.2	21.4	1.56	(1.02, 2.40)	0.041	1.000	Dominant	0.674
TERF1	rs2306492	A	31.6	18.1	0.48	(0.33, 0.69)	0.0001	**0.0025**	Log-additive	**0.0009**
TERF1	rs9298211	T	31.1	18.4	0.50	(0.34, 0.72)	0.0003	**0.0052**	Log-additive	
TERF1	rs2306494	G	31.5	18.9	0.51	(0.35, 0.74)	0.0003	**0.0065**	Log-additive	
TERF1	rs2929585	G	30.9	18.8	0.52	(0.36, 0.75)	0.0005	**0.0091**	Log-additive	
TERF1	rs2929586	G	30.8	18.8	0.52	(0.36, 0.75)	0.0005	**0.0095**	Log-additive	
TERF1	rs2929593	T	31.2	19.3	0.52	(0.36, 0.75)	0.0005	**0.0101**	Log-additive	
TERF1	rs7001277	A	31.6	19.8	0.53	(0.37, 0.76)	0.0006	**0.0125**	Log-additive	
TERF1	rs3116136	C	23.8	30.7	1.42	(1.04, 1.94)	0.028	0.559	Log-additive	
TERF1	rs6990223	T	0.95	2.6	2.72	(1.02, 7.27)	0.047	0.940	Fishers Exact Test	
TERT	rs4073918	C	21.9	30.2	1.51	(1.11, 2.07)	0.010	0.145	Log-additive	0.102
TINF2	rs2748516	A	5.7	9.9	2.01	(1.18, 3.41)	0.010	0.137	Dominant	0.074
TNKS	rs6985140	G	7.4	13.5	2.09	(1.29, 3.38)	0.003	0.129	Dominant	**0.043**
TNKS	rs4474027	G	6.3	10.9	1.96	(1.18, 3.26)	0.010	0.484	Dominant	
TNKS	rs6984737	G	6.1	10.4	1.91	(1.14, 3.21)	0.014	0.713	Dominant	
TNKS	rs10090277	G	6.2	10.4	1.86	(1.11, 3.12)	0.019	0.937	Dominant	
TNKS	rs11249944	A	5.5	9.3	1.88	(1.08, 3.27)	0.025	1.000	Dominant	
TNKS	rs5002815	T	6.5	10.4	1.80	(1.07, 3.03)	0.025	1.000	Dominant	
TNKS	rs5002814	G	6.5	10.4	1.79	(1.07, 3.01)	0.027	1.000	Dominant	
TNKS	rs10093972	C	6.6	10.4	1.76	(1.05, 2.95)	0.032	1.000	Dominant	
TNKS	rs11787443	T	6.9	10.5	1.72	(1.02, 2.88)	0.040	1.000	Dominant	

† Odds ratio (95% confidence intervals) using a log-additive genetic model, adjusted for gender; MAF = minor allele frequency; * includes orthopedic controls and controls from PLCO; P_{adj} = Bonferroni corrected *P* by gene; § results are shown for the model or test with the best fit for the data and significant *P* value; ‡ these SNPs are located downstream of *PARP2* and upstream of *TEP1*.