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Original Article

Upregulated human telomerase reverse transcriptase (hTERT) expression is associated with spinal chordoma growth, invasion and poor prognosis

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Abstract: Altered expression or activity of human telomerase reverse transcriptase (hTERT) has been associated with human carcinogenesis. This study detected hTERT expression in spinal chordoma tissues and associated the level of hTERT expression with clinicopathological data and patient survival. Tissue samples from 54 patients and 20 controls were subjected to immunohistochemical analysis of hTERT protein levels. hTERT expression levels were then analyzed for associations with patient survival rates and clinicopathological parameters (such as age, gender, tumor size, location, tumor grade, tumor stage, muscle invasion, recurrence or not, type of resection, tumor hemorrhage, tumor necrosis, levels of tumor-infiltrating lymphocytes (TILs) and Ki-67 expression). hTERT expression was detected in all 54 spinal chordomas. Expression levels were weak in 7, moderate in 17 and strong in 30 spinal chordoma tissue samples. In contrast, hTERT was rarely expressed in nucleus pulposus tissues (20 samples). hTERT expression was significantly associated with the Ki-67-staining index ($t = -6.616$, $p < 0.001$), TIL levels ($F = 5.27$, $p = 0.008$) and tumor invasion of the surrounding muscle tissue ($t = -4.49$, $p < 0.001$). Kaplan-Meier curves indicated that high hTERT expression was significantly associated with poor local recurrence-free survival of patients ($\chi^2 = 19.07$, $p < 0.001$ *via* the log-rank test), but not associated with overall patient survival. Multivariate analysis of local recurrence-free survival demonstrated that hTERT expression was an independent prognostic factor among spinal chordoma patients (HR = 1.013, 95% CI: 1.002-1.024, $p = 0.016$). High hTERT expression was associated with spinal chordoma growth, invasion and poor patient prognosis. Future studies will investigate the use of hTERT as a biomarker to predict patient prognosis and disease progression or as a potential spinal chordoma therapy target.

Keywords: Chordoma, human telomerase reverse transcriptase (hTERT), biomarker, prognosis

Introduction

Chordoma is a very rare, slow growing and locally invasive malignant mesenchymal tumor with a low to intermediate grade of malignancy that originates from remnants of the embryonic notochord [1-3]. The etiology and risk factors of chordoma remain unknown; however, multiple family cases and genetic predispositions have been reported in association with chordoma development [4, 5]. Chordoma occurs in approximately one case per million in the worldwide population [6]. Chordoma occurs preferentially in the axial skeleton, including the sacrum (50-60%), the spheno-occipital region (25-30%) and the cervical (10%) and thoracolumbar vertebrae (5%) [7]. Chordoma therapy usually consists of complete surgical resection followed by radiation therapy, and this strategy promises the best long-term control of the disease [8, 9]; however, incomplete resection plus radiotherapy in patients with late stage disease may result in tumor recurrence because standard chemotherapy and conventional radiotherapy have poor sensitivity [10-12]. Therefore, it has been suggested that tumor free resection margins are likely the most important factor determining chordoma recurrence and long-term patient prognosis [13-16]. However, achieving wide resection margins with chordoma lesions is often technically difficult due to the proximity of the lesion to vital neurovascular structures and invasion of the tumor into surrounding soft tissues [17]. Therefore, better understanding of chordoma biology [18] and biomarkers capable of predicting patient prognosis and treatment

Table 1. Association of hTERT expression with clinicopathological data of patients

Clinicopathological factors		Number of patients	The mean hTERT level (%)	*p*-value
Age (years)	≤ 50	23	180.87 ± 75.03	0.38
	> 50	31	197.13 ± 61.11	
Sex	Male	35	201.83 ± 65.88	0.084
	Female	19	168.79 ± 65.99	
Tumor size (cm)	≤ 5	21	195.86 ± 71.46	0.62
	> 5	33	186.61 ± 65.23	
Tumor location	Sacral vertebra	42	188.55 ± 66.54	0.73
	Cervical or thoracic or lumbar vertebra	12	190.00 ± 72.19	
Tumor invasion into the Surrounding muscle	Yes	35	215.17 ± 61.68	0.000[a]
	No	19	144.21 ± 51.65	
Preoperative recurrence	Yes	11	189.18 ± 65.76	0.95
	No	43	190.47 ± 68.34	
Grade	High	38	186.24 ± 66.57	0.50
	Low	16	199.63 ± 69.96	
Stage	IA	13	188.31 ± 71.28	0.76
	IB	6	223.00 ± 65.32	
	IIA	4	200.75 ± 71.78	
	IIB	27	184.07 ± 67.78	
	III	4	178.00 ± 67.43	
Type of resection	Enneking inappropriate	17	201.12 ± 65.30	0.42
	Enneking appropriate	37	185.19 ± 68.36	
Tumor hemorrhage	No	10	185.30 ± 77.98	0.80
	Yes	44	191.32 ± 65.48	
Tumor necrosis	Absent	13	192.08 ± 60.42	0.42
	Mild	18	207.06 ± 65.61	
	Moderate	15	167.53 ± 66.92	
	Severe	8	191.75 ± 81.78	
Level of tumor-infiltrating lymphocytes	Absent	0	0.00 ± 0.00	0.008[b]
	Rare/few	23	159.17 ± 63.44	
	Moderate	15	204.00 ± 56.76	
	Prominent	16	221.88 ± 65.67	
Ki-67 index	Low	25	142.24± 43.00	0.000[a]
	High	29	231.55 ± 56.04	

[a]by Student's *t* test and [b]by One-Way ANOVA test.

responses [3] are needed to improve treatment outcomes, provide new therapeutic targets and ensure that chordoma patients receive optimal treatment.

Human telomerase reverse transcriptase (hTERT) is a 127-kDa protein with a reverse transcriptase motif in its C-terminal [19, 20]. hTERT is a rate-limiting component during the formation of the telomerase complex and helps form the structure of telomeric DNA at the ends of human chromosomes by synthesizing telomeric sequences *de novo via* an internal RNA template [19, 21]. hTERT expression is undetectable in normal somatic cells, but is essential for unlimited cell growth [22, 23]. Previous studies have reported high levels of hTERT expression in various human cancers, including gastric and colorectal cancers, ampullary carcinoma, glioma and oral squamous cell carcinoma [20-22, 24-26]. Increased hTERT expression has also been associated with tumor recurrence and poor prognosis in some of these cancer types [21, 22, 26]. Few studies have investigated hTERT expression in spinal chordoma [3, 27]. Therefore, we investigated hTERT expression in spinal chordoma using immunohistochemistry to associate telomerase expression with the clinicopathological features of chordoma tumors and patient survival.

Materials and methods

Patients and tissue samples

This study enrolled 54 patients with spinal chordoma who were admitted for surgical treat-

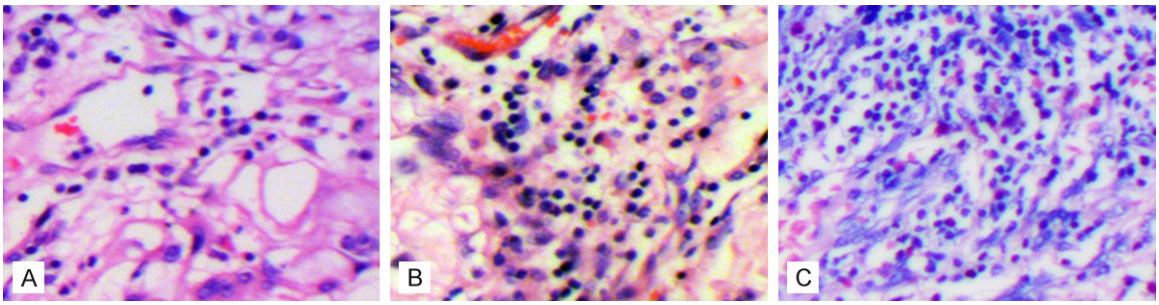

Figure 1. Representative illustration of tumor-infiltrating lymphocytes (TILs) in chordoma tissues (×200) that have been stained with hematoxylin and eosin. (A) Rare/few TILs, (B) Moderate TILs, (C) Prominent TILs.

ment in The Department of Spine Surgery, The Second Xiangya Hospital, Central South University between June 2002 and April 2015. All patients were diagnosed using histopathology according to criteria described in a previous study [4]. In the current study, we collected retrospective clinicopathological data from the patients' medical records, including patient age and gender, tumor size, location, grade, stage and muscle invasion status, primary *vs*. recurrent chordoma, the type of surgical resection performed, tumor hemorrhage, necrosis and the extent of tumor-infiltrating lymphocytes (TILs; **Table 1**). None of the enrolled patients had received any form of tumor-specific therapy before surgical excision of the tumor lesion, including chemotherapy and radiotherapy. This study was approved by the hospital ethical committee of The Second Xiangya Hospital, Central South University, and consent was obtained from each patient before participation in this study.

The number of TILs was evaluated using hematoxylin and eosin-stained sections. Following methods established in a previous study, TILs were scored as absent, rare/few, moderate or prominent (**Figure 1**) [28]. Tumor resections were categorized as wide, marginal, intralesional or palliative by a pathologist during macroscopic and histological evaluation of tumor specimens. These specimens were categorized as Enneking appropriate or Enneking inappropriate according to the Enneking principles [29]. Tumor invasion of surrounding muscle tissue was confirmed using preoperative magnetic resonance images and pathologic examination [1]. Preoperative recurrence indicated that the patient had previously received surgical tumor excision and had confirmed relapse upon admission. Tumor grade and stage were evaluated according to the Enneking staging system for the surgical staging of malignant bone and soft tissue tumors [2, 30].

Follow-up of patients

Follow-up examinations of all patients were performed every three months during the first two years, every six months for the following three years and annually thereafter. Local relapse-free survival (LRFS) was defined as the time interval from tumor resection to the diagnosis of the first local recurrence. Recurrent tumors were diagnosed using clinical examinations and magnetic resonance imaging [3]. Similarly, overall survival (OS) was defined as the interval between the date of surgery and death related to any cause. Observations were censored when the patient was tumor-free (LRFS analysis) or alive (OS analysis) until September 2015.

Tissue specimens

Twenty nucleus pulposus tissue samples were also collected as control tissues from 20 patients who underwent surgical resection to treat disc herniation. These control patients (14 males and 6 females, 24 to 79 years of age, with a mean age of 48.30 ± 16.90 years) were randomly selected and the differences in sex and age distribution between the chordoma and control patient groups were not statistically significant ($\chi^2 = 0.175$, $p = 0.67$ and $t = 1.919$, $p = 0.059$, respectively). Tissue samples from both patient groups were immediately obtained after surgery, fixed in 10% buffered formalin and embedded in paraffin. The paraffin blocks from tumor and normal tissues were processed into 4 μM thick sections. To confirm chordoma diagnosis, tissue sections were stained with

hematoxylin and eosin and then examined by two pathologists who confirmed the diagnosis of chordoma of conventional subtype in all patients.

Immunohistochemistry and data scoring

Immunohistochemical staining was conducted using a peroxidase-labeled streptavidin-biotin technique [20]. In brief, tissue sections were deparaffinized with two xylene incubations, rehydrated in a series of ethanol washes and then subjected to antigen retrieval using a pressure cooker at 121°C for 15 min in 0.01 M citrate buffer, pH 6.0. Tissue sections were then incubated with 3% H_2O_2 in methanol for 15 min to quench any endogenous peroxidase activity. After being rinsed in phosphate buffer solution (PBS), sections were incubated in 10% normal goat serum for 30 min at room temperature to block any potential non-specific binding of the secondary antibody. Next, sections were incubated overnight at 4°C with anti-hTERT (ab183105, Abcam, Cambridge, MA, USA) at a dilution of 1:50 or anti-Ki-67 (ab16667, Abcam) at a dilution of 1:100. The next day, tissue sections were rinsed with PBS and then incubated with biotinylated goat anti-rabbit immunoglobulin at room temperature for 30 min, followed by a streptavidin-peroxidase conjugate (Auragene, Changsha, Hunan, China). Antibody binding was visualized by brief incubation with 3,3'-diaminobenzidine solution. Finally, sections were lightly counterstained using hematoxylin. Negative controls were generated by replacing the primary antibody with PBS or 10% normal serum, and positive controls were created using tissue sections generated from human tonsil tissue according to the manufacturer's suggestion.

Immunochemical results were independently scored by two experienced pathologists (SXL and JY) who were blinded to the patients' clinicopathological data and outcomes. The pathologists reviewed immunostained tissue sections under a light microscope and counted brown cytoplasmic or nuclear staining of tumor cells as positive for hTERT expression and nuclear staining for Ki-67 expression. Tissue sections were initially scanned at low power, then five high power fields were randomly selected and at least 1000 cells were counted in each randomly selected section. The pathologists then semiquantitatively scored hTERT expression in chordoma and control tissue sections according to the methods described in a previous study [20]. The staining intensity of cells was graded as 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). Staining percentage was scored as the ratio of positively immunostained cells to the total number of cells counted. For each case, the staining intensity (0-3) and staining percentage (0-100%) scores were multiplied to obtain the final staining score. For the subsequent survival analysis, the mean hTERT expression scores were used as the cut-off values for high and low level hTERT expression. Additionally, the Ki-67-staining index score was obtained from the percentage of cells with nuclear staining, and these data were divided into low (< 10% of cells with positive nuclear staining) or high (≥ 10% of cells with positive nuclear staining) Ki-67 expression according to the methods described in a previous study [1].

Statistical analysis

All statistical analyses were performed using SPSS 17.0 software from SPSS Inc. (Chicago, IL, USA). Associations between hTERT expression levels, the clinicopathological characteristics of spinal chordoma patients (e.g. patient age and tumor characteristics) and the Ki-67-staining index were analyzed using (as appropriate) Student's *t* tests and One-Way ANOVA tests. The receiver-operating characteristic curve (ROC) was used to analyze the sensitivity of hTERT expression [31]. LRFS and OS were calculated using Kaplan-Meier curves and analyzed using the log-rank test. hTERT was divided into low and high expression groups according to mean expression scores to determine the survival probability associated with different levels of hTERT expression. To account for potential confounding factors, multivariate analysis was performed using a Cox proportional hazard model to assess whether hTERT expression could independently predict patient outcome. This analysis included factors found to be significant by univariate analysis. All *p* values were two-sided, and a *p* value less than 0.05 was considered statistically significant.

Results

Patient characteristics

Tissue specimens were collected from 54 patients with spinal chordoma who were admit-

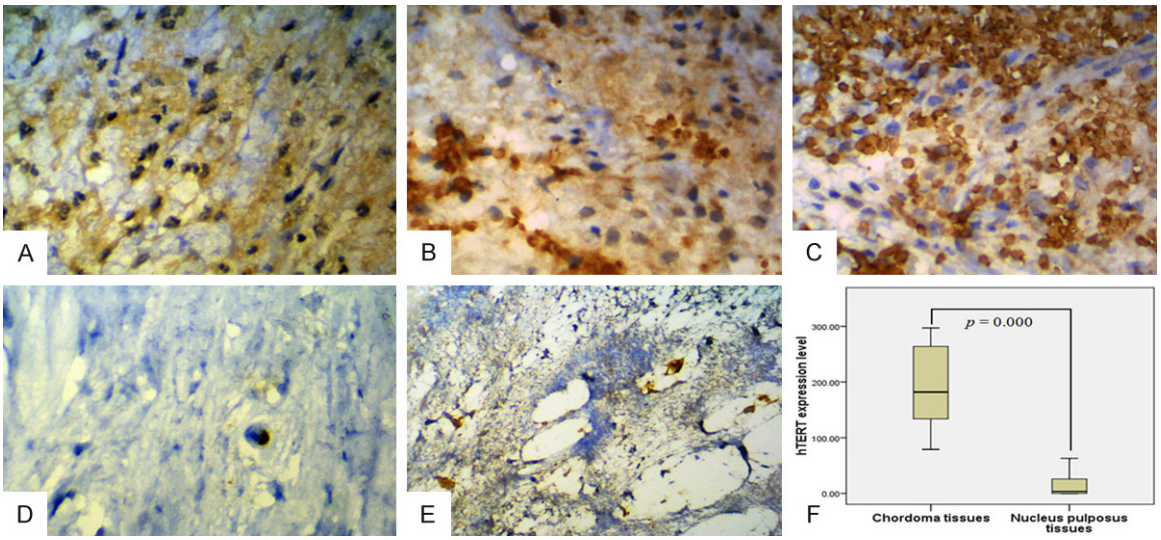

Figure 2. Immunohistochemistry analysis of hTERT expression levels. (A) Weak expression of hTERT in chordoma tissues; (B) Moderate expression of hTERT in chordoma tissues; (C) Strong expression of hTERT in chordoma tissues; (D) Negative hTERT expression in nucleus pulposus tissues; (E) Weak hTERT expression in nucleus pulposus tissues (×400). (F) Summary of hTERT expression data in chordoma and nucleus pulposus tissues.

ted to The Department of Spine Surgery, The Second Xiangya Hospital for surgical treatment. There were 35 males and 19 females, with an average age of 55.59 ± 13.56 years (range, 23 to 79 years). Forty-three of these patients had primary spinal chordoma and 11 had recurrent chordoma. All patients were followed until September 2015, with a mean follow-up period of 42.39 months (range, 5 to 158 months). All other patient characteristics are summarized in **Table 1**.

hTERT protein expression in chordoma tissue specimens

We analyzed hTERT expression in the surgical tissue specimens from the 54 spinal chordoma patients and 20 controls and found that hTERT protein was expressed in all tumor tissues. Immunostaining of chordoma cell nuclei was weak in 7 specimens (12.96%), moderate in 17 specimens (31.48%) and strong in 30 specimens (55.56%; **Figure 2A-C**). In contrast, hTERT expression was rarely observed in the nucleus pulposus control tissues (**Figure 2D**, **2E**). The mean hTERT-staining score was 190.20 ± 67.20% in chordoma tissues *vs*. 13.95 ± 19.23% in nucleus pulposus samples (t = 17.440, $p < 0.001$; **Figure 2F**). We conducted ROC analysis to determine the differential diagnosis value of hTERT expression in spinal chordoma *vs.* nucleus pulposus tissues. We found that hTERT levels could serve as a diagnostic marker for spinal chordoma (**Figure 3B**).

Association of hTERT levels with the Ki-67 proliferative index

To investigate the potential association of hTERT levels with tumor cell proliferation, we analyzed Ki-67 expression in chordoma tissues and found diverse intensities of Ki-67 immunostaining that were restricted to the cell nuclei. Our data indicated that the mean hTERT expression levels were significantly higher in chordoma tissues with a high Ki-67 proliferative index when compared with tissues with a low Ki-67 index (231.55 ± 56.04% *vs*. 142.24 ± 43.00%, respectively, t = -6.616, $p < 0.001$, **Table 1**). hTERT expression was closely associated with Ki-67 expression in chordoma tissues (**Figure 3A**; r = 0.716, $p < 0.001$; **Table 2**).

Association of hTERT expression with clinicopathological factors

We investigated the association of hTERT expression with other clinicopathological characteristics and found that mean hTERT expression levels were significantly associated with muscle tissue invasion by the tumor (215.17 ± 61.68% *vs*. 144.21 ± 51.65% in tumors with or without muscle tissue invasion, respectively, t = -4.496, $p < 0.001$). Increased muscle tissue

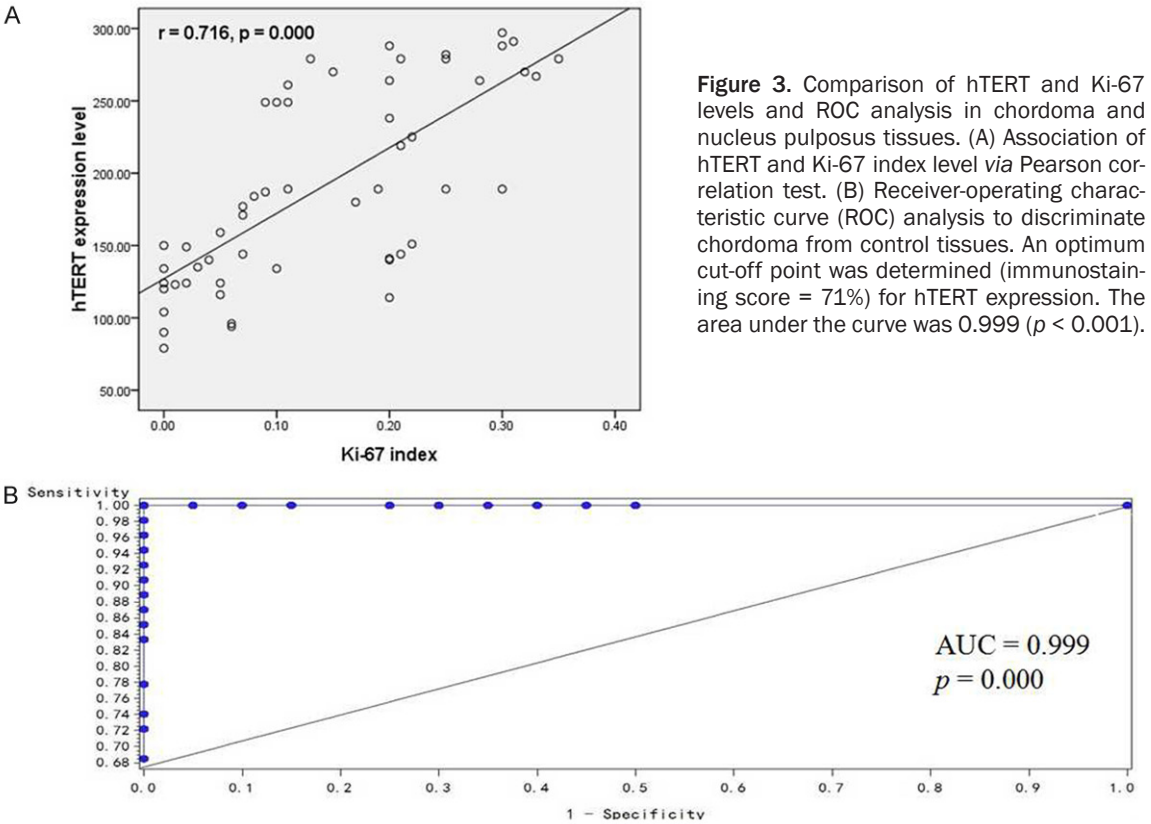

Figure 3. Comparison of hTERT and Ki-67 levels and ROC analysis in chordoma and nucleus pulposus tissues. (A) Association of hTERT and Ki-67 index level *via* Pearson correlation test. (B) Receiver-operating characteristic curve (ROC) analysis to discriminate chordoma from control tissues. An optimum cut-off point was determined (immunostaining score = 71%) for hTERT expression. The area under the curve was 0.999 ($p < 0.001$).

Table 2. Association between hTERT expression and Ki-67 proliferative index in spinal chordoma tissues

Ki-67 index	hTERT expression		*p*-value
	Low	High	
Low	23	2	0.000
High	10	19	

hTERT, human telomerase reverse transcriptase.

infiltration by TILs was also associated with high hTERT expression (F = 5.278, $p = 0.008$). Mean hTERT expression in tumor tissues with rare/few, moderate and severe TIL infiltration levels was 159.17 ± 63.44%, 204.00 ± 56.76% and 221.88 ± 65.67%, respectively. Subgroup analysis indicated that there was a significant difference in hTERT expression between tumor tissues with rare/few and moderate levels of TIL infiltration ($p = 0.035$, LSD test), and between rare/few and severe levels of TIL infiltration ($p = 0.003$). No statistically significant associations were seen between any of the other clinicopathological factors examined and hTERT expression (**Tables 1** and **2**).

hTERT expression and spine chordoma patient survival

Patient outcomes were followed-up until September 2015. Median LRFS and OS were 21.0 months (range, 3 to 45 months) and 48.0 months (range, 5 to 158 months), respectively. During the follow-up period, 41 patients (75.93%) had local recurrence of chordoma and 24 patients (44.44%) died. Kaplan-Meier curve analysis stratified according to high and low hTERT expression (mean immunostaining score, 190.20 ± 67.20%) indicated that patients with high hTERT expression exhibited significantly shorter LRFS when compared with patients with low hTERT expression (**Table 3** and **Figure 4A**). We also found that patients with a high Ki-67 proliferative index had significantly shorter LRFS when compared with patients that had a low Ki-67 index (**Table 3** and **Figure 4B**). High hTERT expression levels and a high Ki-67 index were associated with much poorer survival when compared with low hTERT expression and low Ki-67 index tumors (median, 26 *vs.* 12 months, $p < 0.001$ *via* the

Table 3. Univariate and multivariate Cox proportional hazard analyses of prognostic factors for local recurrence-free survival

Factors	Categories	Univariate analysis		Multivariate analysis	
		χ^2	*p*-value	*p*-value	HR (95% CI)
Sex	Male/Female	2.949	0.08		
Age	≤ 50/> 50	6.560	0.01	0.10	1.95 (0.86-4.40)
Tumor size	≤ 5 cm/> 5 cm	0.278	0.59		
Tumor location	Sacral vertebra/Cervical or thoracic or lumbar vertebra	0.003	0.95		
Preoperative recurrence	Yes/No	0.954	0.32		
Surrounding muscle invasion	Yes/No	24.585	0.000	0.006	4.66 (1.54-14.04)
Grade	High/Low	0.231	0.63		
Stage	IA/IB/IIA/IIB/III	0.960	0.91		
Type of resection	EI/EA	16.472	0.000	0.003	3.47 (1.54-7.80)
Tumor hemorrhage	Yes/No	4.031	0.04	0.30	1.81 (0.58-5.62)
Tumor necrosis	Absent/mild/moderate/severe	0.189	0.97		
Level of TILs	Absent/Rare or few/moderate/Prominent	6.559	0.038	0.27	0.77 (0.48-1.23)
hTERT expression	High/Low	19.075	0.000	0.016	1.01 (1.00-1.02)
Ki-67 index	High/Low	14.163	0.000	0.029	3.00 (1.12-8.04)
hTERT/Ki-67 coexpression	Low/Low	21.885	0.000[a]		
	High/Low and Low/High				
	High/High				

EI, Enneking inappropriate; EA, Enneking appropriate; TILs, tumor-infiltrating lymphocytes; hTERT, human telomerase reverse transcriptase. [a]hTERT/Ki-67 coexpression was not used as a dependent variable in the multivariate analysis, because it depends on hTERT expression and Ki-67 index and seems to be confounded with them.

log-rank test; **Table 3** and **Figure 4C**). Old age (**Table 3** and **Figure 4D**), muscle invasion (**Table 3** and **Figure 4E**), tumor hemorrhage (**Table 3** and **Figure 4F**), and Enneking inappropriate resection (**Table 3** and **Figure 4H**) were significantly associated with poor LRFS. Additionally, TIL infiltration levels were significantly associated with LRFS (**Table 3** and **Figure 4G**); however, this difference was only observed between tumors with rare/few and moderate TILs (median, 24 *vs*. 16 months, $p = 0.004$ *via* the log-rank test).

Kaplan-Meier analysis indicated that muscle invasion (**Table 4** and **Figure 5A**), Enneking inappropriate resection (**Table 4** and **Figure 5B**) and tumor stage (**Table 4** and **Figure 5C**) were associated with poor OS. Multivariate Cox proportional hazards model analysis indicated that high hTERT expression (HR = 1.013, 95% CI: 1.002-1.024, $p = 0.016$; **Table 3**), a high Ki-67 index (HR = 3.002, 95% CI: 1.120-8.044, $p = 0.029$; **Table 3**), tumor muscle invasion (HR = 4.657, 95% CI: 1.545-14.040, $p = 0.006$; **Table 3**) and Enneking inappropriate resection (HR = 3.472, 95% CI: 1.545-7.805, $p = 0.003$; **Table 3**) were independent predictors of LRFS. However, this analysis did not indicate that these parameters were independent predictors of OS in spinal chordoma patients (**Table 4**).

Discussion

In this study, we assessed hTERT expression in spinal chordoma tissue samples and investigated the association of hTERT expression with the clinicopathological and survival data of the patients enrolled in the study. We found that hTERT protein expression was increased in chordoma tissues when compared with nucleus pulposus tissues. Upregulated hTERT protein expression was associated with tumor invasion of the surrounding muscle tissue, increased TIL infiltration, a high Ki-67 index and poor LRFS. hTERT expression was an independent predictor of LRFS in spinal chordoma patients; however, it was not associated with OS. This study indicates that measuring hTERT expression levels could be useful for predicting tumor progression and LRFS. Future studies are needed to evaluate whether hTERT could be an effective therapeutic target for spinal chordoma treatment.

Few studies have been conducted characterizing the molecular biology and molecular biomarkers of chordoma. Increased expression of various proteins (Survivin, SPHK1, PDGFR-α, EGFR, c-MET, VEGFR2 and iNOS) has been associated with the prognosis of spinal chordoma patients [32-35]. However, most of the

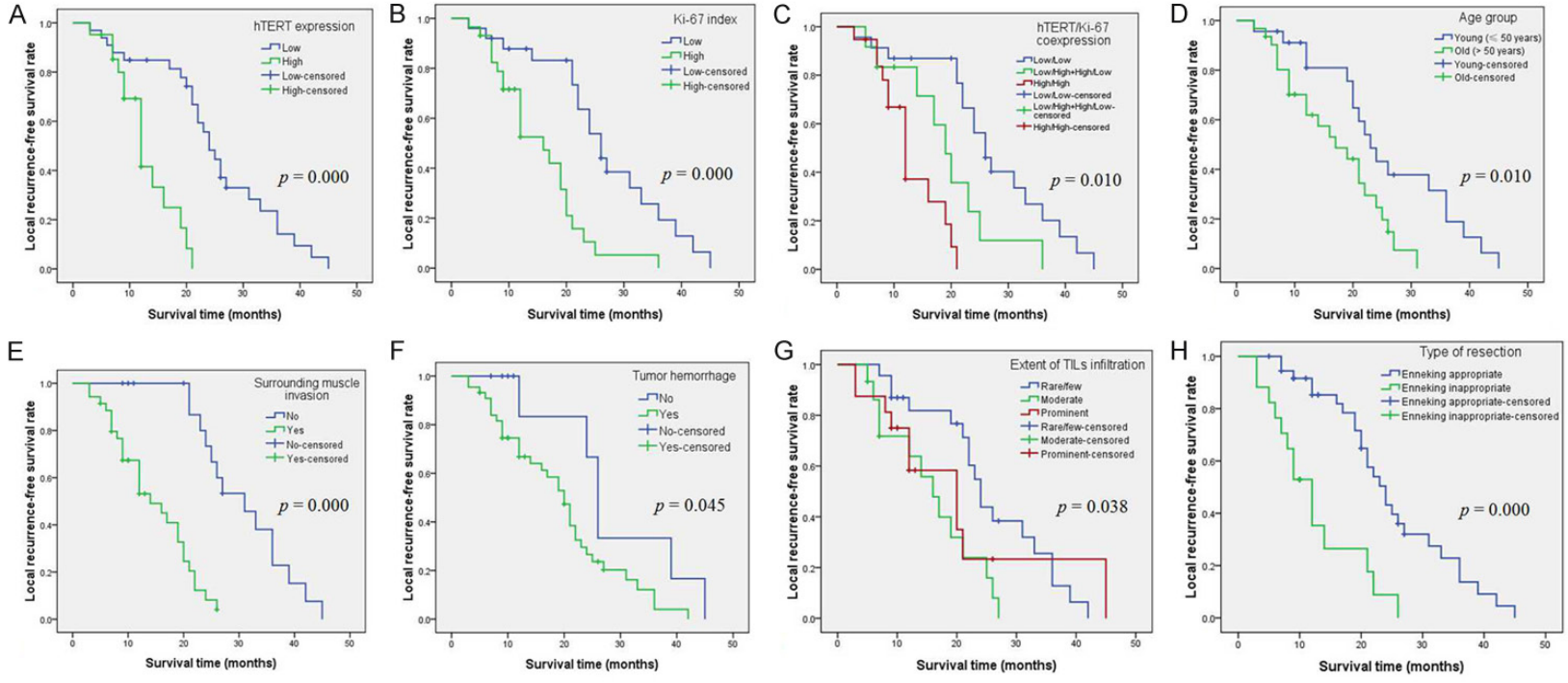

Figure 4. Kaplan-Meier curves of the local recurrence-free survival (LRFS) of patients. (A) Stratified by hTERT expression ($p < 0.001$ *via* log-rank test); (B) Stratified by Ki-67 expression ($p < 0.001$); (C) Stratified by hTERT/Ki-67 coexpression ($p = 0.010$); (D) Stratified by age ($p = 0.010$); (E) Stratified by tumor invasion into the surrounding muscle ($p < 0.001$); (F) Stratified by tumor hemorrhage ($p = 0.045$); (G) Stratified by tumor-infiltrating lymphocyte level ($p = 0.038$); and (H) Stratified by type of surgical resection ($p < 0.001$).

Table 4. Univariate and multivariate Cox proportional hazard analyses of prognostic factors for overall survival

Factors	Categories	Univariate analysis		Multivariate analysis	
		χ^2	*p*-value	*p*-value	HR (95% CI)
Sex	Male/Female	1.54	0.21		
Age	≤ 50/> 50	1.45	0.22		
Tumor size	≤ 5 cm/> 5 cm	0.12	0.72		
Tumor location	Sacral vertebra/Cervical or thoracic or lumbar vertebra	0.13	0.71		
Preoperative recurrence	Yes/No	0.54	0.46		
Tumor invasion into the Surrounding muscle	Yes/No	6.79	0.009	0.10	2.44 (0.82-7.22)
Tumor grade	High/Low	0.64	0.42		
Tumor stage	IA/IB/IIA/IIB/III	22.30	0.000	0.31	1.20 (0.84-1.72)
Resection type	EI/EA	5.89	0.015	0.19	1.82 (0.74-4.46)
Tumor hemorrhage	Yes/No	0.31	0.57		
Tumor necrosis	Absent/mild/moderate/severe	4.03	0.25		
Level of TILs	Absent/Rare or few/moderate/Prominent	0.03	0.98		
hTERT level	High/Low	1.78	0.18		
Ki-67 index	Ki-67 index, High/Low	2.04	0.15		
hTERT/Ki-67 coexpression	Low/Low	3.13	0.20		
	High/Low and Low/High				
	High/High				

EI, Enneking inappropriate; EA, Enneking appropriate; TILs, tumor-infiltrating lymphocytes; hTERT, human telomerase reverse transcriptase.

studies investigating the expression of these proteins in chordoma did not perform multivariate adjustment for their statistical analysis [36], which may have introduced bias to the data [37]. In the current study, we assessed hTERT expression and performed a multivariate analysis to adjust for the influence of other clinicopathological data (such as age, gender and tumor stage) on hTERT expression and patient survival. We found that hTERT was predominantly expressed in spinal chordoma tissues, but was rarely expressed in nucleus pulposus tissues. This result is consistent with previous studies that reported hTERT detection in most human cancers, including chordoma, but not in benign or normal tissues [3, 38, 39]. Difference in hTERT protein expression between normal and malignant cells suggests that different cell types may have different proliferation or turnover rates [20]. Based on this speculation, the higher levels of hTERT expression in chordoma lesions when compared with nucleus pulposus tissues indicates that chordoma cells may need higher hTERT expression to maintain chromosomal stability and cell proliferation. Supporting this hypothesis, previous studies have demonstrated that hTERT is a telomerase activity marker and reported that a large tumor lesion expressed more hTERT due to a higher proliferation potential and the need for more telomerase activity to maintain tumor cell growth [20, 40]. This finding may at least partially account for the upregulated hTERT expression observed in a large spinal chordoma, due to its indolent and low-grade nature [1].

This study associated hTERT expression with the Ki-67 labeling index. This result is supported by a previous study showing an association between hTERT expression and the Ki-67 labeling index in colorectal cancer with hepatic metastases [40]. Ki-67 is an antigen that reflects cell proliferation, and it is reasonable to suggest that tumor cells with high Ki-67 expression would need increased telomerase activity to maintain cell growth. Furthermore, previous studies reported mutations of the hTERT promoter [41] and increased TERT transcription to induce hTERT expression in human cancers [42, 43]. The current study does not address whether or not the increased hTERT expression observed in chordoma is related to mutations in the TERT promoter.

The current study demonstrates a statistically significant association between elevated hTERT expression and muscle tissue invasion by chordoma tumors. Previous studies have demonstrated significant associations between hTERT

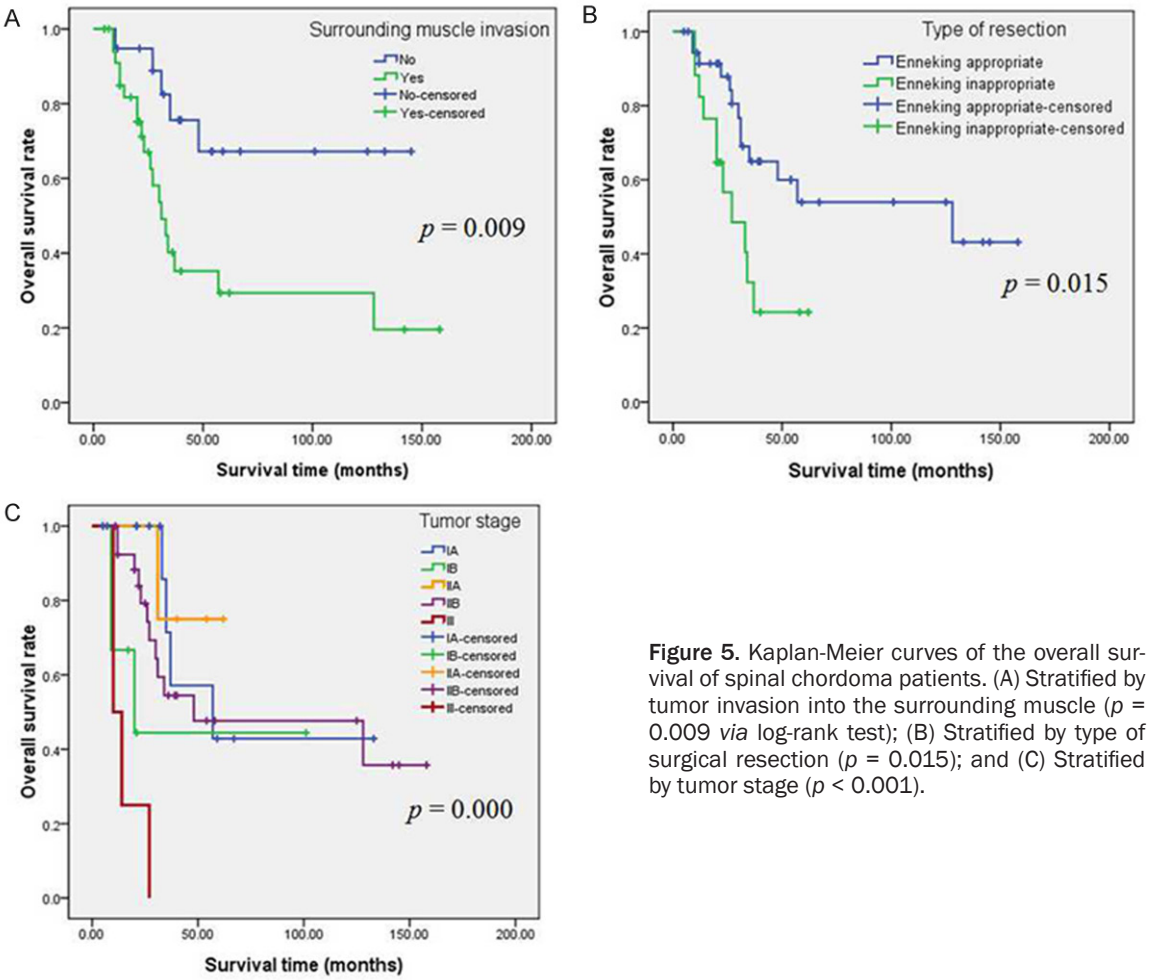

Figure 5. Kaplan-Meier curves of the overall survival of spinal chordoma patients. (A) Stratified by tumor invasion into the surrounding muscle (p = 0.009 *via* log-rank test); (B) Stratified by type of surgical resection (p = 0.015); and (C) Stratified by tumor stage (p < 0.001).

expression and tumor histological types, clinical staging and histological grading in various human cancer types [21, 44-46]. hTERT has also been shown to contribute to the invasion and metastasis of melanoma [47] and esophageal squamous cell cancer [48]. An *in vitro* study reported that exogenous hTERT expression also promoted invasiveness and metastasis of hepatoma HepG2 cells [49]. These data may support the non-reverse transcriptase activity of hTERT in promoting epithelial mesenchymal transition [50], which has been postulated to be a requirement for tumor invasion and metastasis [51]. The current study provides the first evidence that hTERT expression is associated with the level of TIL invasion into chordoma tissues. Previous studies have shown that TILs express programmed cell death 1 (PD-1) and are associated with the elevated expression of programmed cell death-1 ligand 1 (PD-L1) in various cancers [28, 52, 53]. The interaction of PD-1 with PD-L1 is a mechanism that may allow tumors to escape from the host's immune response [54]. Intratumoral infiltration of PD1-positive lymphocytes has been associated with the progression of human cancers [28, 53]. This suggests that hTERT expression may not only be a marker of cell proliferation, but also could be necessary for chordoma progression and play a role in tumor evasion from host immunity. However, although previous studies have demonstrated an analogous association [46], our current study failed to show any association between hTERT expression and the histological grade of chordoma. This inconsistency may be due to our small sample size and correspondingly low statistical power.

Previous studies have associated hTERT mRNA levels and telomerase activity with the prognosis of many cancers [20, 26, 38, 40]. In our cur-

rent study, log-rank analysis revealed that the LRFS of spinal chordoma patients was significantly shorter when hTERT expression was high. This indicates that hTERT is an oncoprotein, or that hTERT has oncogenic functions in spinal chordoma cells. We chose the mean value of hTERT expression in chordoma tissues as a cut-off point for high *vs.* low hTERT expression. Although this hTERT expression value represents an arbitrary choice, we were able to use this cut-off point to predict LRFS or OS of patients. Based on our current data and the results from previously published studies, hTERT should be further assessed as a potential therapeutic target for spinal chordoma. hTERT has recently emerged as a promising target for a therapeutic cancer vaccine [55-59]. In one example, Vx-001, an HLAA*0201-associated optimized cryptic peptide derived from TERT, induced tumor immunity but not autoimmunity in HLA-A*0201 transgenic mice [55]. *In vitro,* Vx-001 stimulated cytotoxic T-lymphocytes to kill hTERT-expressing tumor cells but not normal cells from healthy donors and prostate cancer patients [56]. In a phase I clinical trial, vaccination therapy with Vx-001 was administered to 19 patients with advanced cancers. Approximately 90% of the patients in the trial mounted a TERT-specific immune response, and the trial reported a favorable safety profile with no evidence of serious adverse events [57]. Additionally, several phase II clinical trials reported that patients exposed to Vx-001 achieved significantly improved overall survival when compared to non-responders [58, 59].

The current study has several limitations that must be considered. First, the study is a correlation study and lacks cell-function experiments. Thus, further study is needed to determine how hTERT expression promotes tumor progression and influences the clinical outcome of spinal chordoma patients. Second, the study was retrospective, conducted in a single institution and included only a small number of patients; therefore, additional well-designed prospective studies involving large sample sizes and long-term follow-up are needed to validate our current findings. Finally, a panel of biomarkers, including surrogate biomarkers of angiogenesis, tissue invasion, self-sufficiency in growth signals and insensitivity to antigrowth signals [60] should be evaluated along with hTERT to enable better prediction of the overall biologic behavior of spinal chordoma.

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Disclosure of conflict of interest

None.

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