



Cytotoxic T lymphocyte antigen-4 (CTLA-4) expression in chordoma and tumor-infiltrating lymphocytes (TILs) predicts prognosis of spinal chordoma

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Abstract

Purpose Chordoma is a rare tumor of the skeletal system that is characterized by a high recurrence rate and treatment resistance. Given the common finding of immune dysregulation in chordoma, immunotherapy has emerged as potential treatment option. As an important immune checkpoint regulator, we evaluated cytotoxic T-lymphocyte antigen-4 (CTLA-4) expression and its prognostic significance for patients with chordoma of the spine.

Methods CTLA-4 expression was analyzed immunohistochemically in 32 chordoma tissues and 14 nucleus pulposus tissues to examine the specificity of CTLA-4 expression in chordoma. Univariate log-rank analysis was used to evaluate the association of CTLA-4 expression in tumor cells and tumor-infiltrating lymphocytes (TILs) with survival. Cox multivariate analysis was used to identify independent factors of survival.

Results Positive CTLA-4 expression was observed in all of the TILs and tumor cell cytoplasm, and partial in the membrane or in both the membrane and nucleus, with a markedly higher positivity rate than that observed in normal nucleus tissues. Higher CTLA-4 expression in the tumor but not in TILs was significantly associated with shorter continuous disease-free survival (CDFS) and overall survival (OS). CTLA-4 expression in tumor cells and TILs were independent predictors for CDFS, whereas only tumor cell expression was a significant predictor of OS. Furthermore, the combination of CTLA-4 expression in the tumor and TILs had higher prognostic value.

Conclusions Targeting CTLA-4 may be a potential novel therapeutic strategy for chordoma patients.

Keywords CTLA-4 · Immunohistochemistry · Chordoma · TILs · Prognosis

Abbreviations

CDFS	Continuous disease-free survival	LSS	Lumbar spinal stenosis
CTLA-4	Cytotoxic T lymphocyte antigen 4	LDH	Lumbar disk herniation
DAB	Diaminobenzidine	OS	Overall survival
FCEP	Favorable CTLA-4 expression profile	PD-1	Programmed cell death protein 1
IHC	Immunohistochemistry	TILs	Tumor-infiltrating lymphocytes

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Introduction

Chordoma is an extremely rare malignant mesenchymal tumor of the skull base and axial skeleton that is considered to originate from notochordal remnants. Chordoma is typically characterized by slow growth and local invasion, accounting for 1–4% of primary malignant bone tumors and up to 20% of all spine tumors [1]. Current clinical treatments for chordoma include en-bloc resection combined with radiotherapy before and after surgery [2]. However, the predilection site of chordoma proximal to vital neurovascular structures and invasive growth into surrounding

soft tissue pose a challenge in achieving complete resection, ultimately leading to tumor recurrence [3, 4]. Moreover, in contrast to other tumors that are relatively sensitive to chemotherapy and radiotherapy, chordoma frequently shows drug resistance and radiotherapy insensitivity, hindering successful treatment [5]. Therefore, it is critical to obtain a better understanding of the biological properties of chordoma so as to develop novel therapeutic strategies that can improve treatment outcomes for patients.

Tumor-related immune dysregulation has recently been identified as a key feature of chordoma, leading to the consideration of immunotherapy as a promising therapeutic strategy [5]. Among available immunotherapy strategies, immune checkpoint blockades have shown good success in the treatment of various malignancies [6]. The mostly common and important immune checkpoint molecules include programmed cell death protein 1 (PD-1) and cytotoxic T lymphocyte antigen 4 (CTLA-4), and clinical trials of monoclonal antibodies targeting PD-1 and CTLA-4 have shown effectiveness for a variety of tumors [7–9]. Recent studies have also reported associations of the expression of PD-1 or its ligand PD-L1 with the metastatic potential of chordoma [10, 11]. However, to our knowledge, no study has explored the prognostic value of CTLA-4 expression in chordoma and its corresponding tumor-infiltrating lymphocytes (TILs) to date.

CTLA-4 is a well-known immune checkpoint protein that is commonly expressed on T cells, which is a CD28 homolog but shows much higher binding affinity for B7 ligands [12], and is mainly localized in the intracellular compartment of resting naive T cells. However, upon T cell activation, CTLA-4 expression is upregulated and the protein is translocated to the cell surface where it outcompetes with CD28 for B7 ligands, thereby antagonizing the effector T cell response by disturbing the cell cycle and cytokine release [13, 14]. Therefore, the relative binding of CTLA-4 and CD28 to B7 determines whether T cells will be activated or silenced, and results in regulating the immune environment.

CTLA-4 expression has also been found in various types of tumor tissues, including tumors of the hematological system [15] and solid tumors [16]. The differential expression of CTLA-4 in tumors and TILs compared to normal cells suggests that CTLA-4 could be a potential cancer marker; however, related results are controversial. Most studies indicated that high CTLA-4 expression in tumors and TILs was associated with a poorer patient prognosis, including for patients with breast cancer [17], nasopharyngeal carcinoma [18], and thymoma [19], suggesting that CTLA-4 overexpression may facilitate tumors in evading the host immune response. However, other studies showed that high CTLA-4 expression in tumors and TILs was related with a good clinical outcome and survival rate for patients with mesothelioma [20] and

non-small cell lung cancer [21], indicating that CTLA-4 overexpression may enhance anti-tumor immunity. These contradictory findings might indicate different roles of CTLA-4 in different tumor types, leading to tumor-specific effects on clinical outcome.

Considering this background, we sought to identify whether CTLA-4 expression can be a prognostic marker and potential therapeutic target for chordoma. Toward this end, we evaluated the CTLA-4 expression pattern in chordoma and normal nucleus pulposus tissues, and investigated the association between CTLA-4 expression and survival time as well as clinicopathological features of patients with chordoma of the spine.

Patients and methods

Patients and data collection

A total of 32 chordoma patients (23 men and 9 women) with a mean age of 50.53 years (range, 11–70 years) were enrolled in the study. All patients had undergone primary tumor resection at the Department of Orthopedics, Peking University Third Hospital from 2006 to 2019. 14 normal nucleus pulposus tissues as control samples were acquired from patients with lumbar spinal stenosis or lumbar disk herniation. Inclusion criteria were pathologically confirmed chordoma with availability of paraffin-embedded specimens of the primary tumor and relatively complete follow-up data. Patients were excluded if they had undergone any anti-cancer treatments prior to surgery, such as chemotherapy or radiotherapy, which may have an influence on the expression of proteins interest.

We retrospectively reviewed the clinicopathological characteristics, including age, gender, tumor size, tumor location, tumor stage, tumor type, surrounding muscle invasion, recurrence, extent of TILs, and Ki67 staining index. Resected tumor specimens were evaluated by anatomic pathologists and data were recorded based on the Enneking principles [22]. Surrounding muscle invasion of the tumor was verified by magnetic resonance images and histological analysis, and tumor recurrence was diagnosed according to clinical symptoms and imaging examination during follow-up [23]. Events were defined as the first evidence of local recurrence or distant metastasis for continuous disease-free survival (CDFS) and as death related to any cause for overall survival (OS). Patients were followed up every 3 months for the first 2 years, at 6-month intervals for the third year, and then annually until death. All patients were followed until May 2019 and observations were censored when a patient was disease-free or alive at the time of the last clinical follow-up (May 2019).

Immunohistochemistry

Paraffin-embedded tissue Sects. (5 µm thick) were dewaxed with xylene and subsequently hydrated with various concentrations of ethanol, followed by antigen heat repair with high-pressure Tris- ethylenediaminetetraacetic acid buffer. The sections were incubated with 3% H₂O₂ for 15 min to prevent activity of endogenous peroxidase and then blocked with 10% normal goat serum at room temperature for 30 min to avoid interference of non-specific antigens. The sections were incubated with primary anti-CTLA-4 antibody (Abcam, ab134090, 1:300 dilution) overnight at 4 °C, followed by incubation with the secondary antibody (EnVision K500711, Dako, Denmark) at 37 °C for 30 min. The antibody portion of the sections was visualized by 3,3'-diaminobenzidine staining for 40 s and then the nucleus was counterstained with hematoxylin.

Immunohistochemistry scoring

Two independent pathologists blinded to the clinical data evaluated the density of TILs along with CTLA-4 expression on tumor cells and TILs for each section. The immunohistochemistry staining result was scored based on the immunoreactive score (IRS) system, which takes both the intensity and percentage of stained cells into consideration. The intensity of stained cells was scored as 0 (none), 1 (weak), 2 (moderate), and 3 (strong), and the proportion of stained cells was scored as 0 (0–5%), 1 (5–25%), 2 (25–50%), and 3 (> 50%). The final scores (ranging from 0 to 12) were obtained by multiplying the intensity and percentage values [21]. The result for CTLA-4 expression was defined as negative (score 0–1), weak (score 2–4), moderate (score 5–8), and strong (score 9–12). Samples with negative and weak scores were classified in the low CTLA-4 expression group, whereas those with moderate and strong scores were classified in the high CTLA-4 expression group. The extent of TILs was assessed as absent, rare/few, moderate, and prominent. The

groups with absent and rare/few TILs were considered as low intensity, whereas those with moderate and prominent TILs were considered as high intensity.

Statistical analysis

Pearson's Chi-squared test was applied to analyze the association of CTLA-4 expression in tumor tissues and TILs with the clinicopathological characteristics of chordoma patients. Univariate analysis was performed based on the Kaplan–Meier curve (log-rank test) for associations with CDFS and OS. Multivariate analysis was performed with a Cox proportional hazard model to assess whether CTLA-4 expression independently predicted patient outcome (CDFS and OS) after controlling for the significant or highly clinically related factors based on univariate analysis. SPSS 20.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses, and a two-sided p value < 0.05 was considered to indicate a statistically significant effect.

Results

CTLA-4 expression on tumor and normal control tissues

The clinicopathological characteristics of the patients included in the study are summarized in Supplementary Table S1. TILs were detected in all the tumor samples with 7 (21.9%), rare/few in 6 samples (18.8%), moderate in 10 samples (31.3%), and prominent in 9 samples (28.1%) (Fig. 1), and CTLA-4 staining in TILs was low in 9 samples (28.1%), weak in 5 samples (15.6%), moderate in 7 samples (21.9%), and strong in 11 samples (34.4%) (Fig. 2a–d). CTLA-4 expression in the tumor was scored as low in 5 patients (15.6%), weak in 10 patients (31.3%), moderate in 2 patients (6.3%), and strong in 15 patients (46.9%) (Fig. 2e–h). In addition, among the 32 chordoma samples, CTLA-4 expression was observed either as focal dots or scattered patterns in chordoma cells with

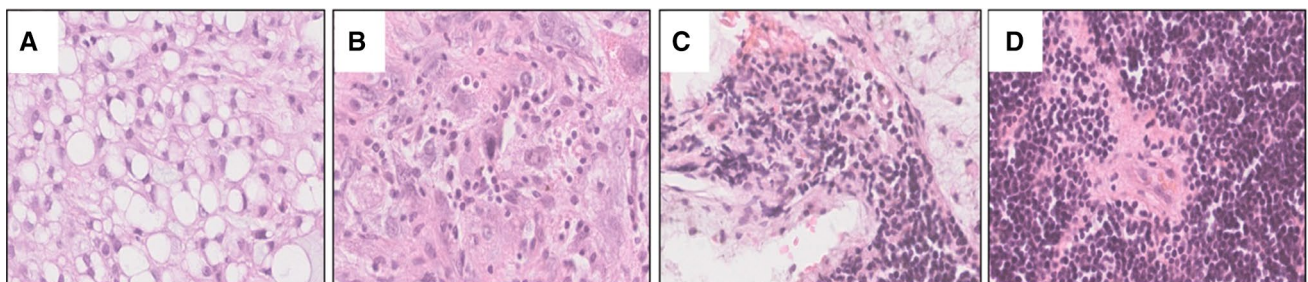


Fig. 1 Representative illustration of tumor-infiltrating lymphocytes (TILs) in chordoma tissues by HE staining. **a** Absent; **b** Rare/few; **c** Moderate; **d** Prominent. 400× magnification

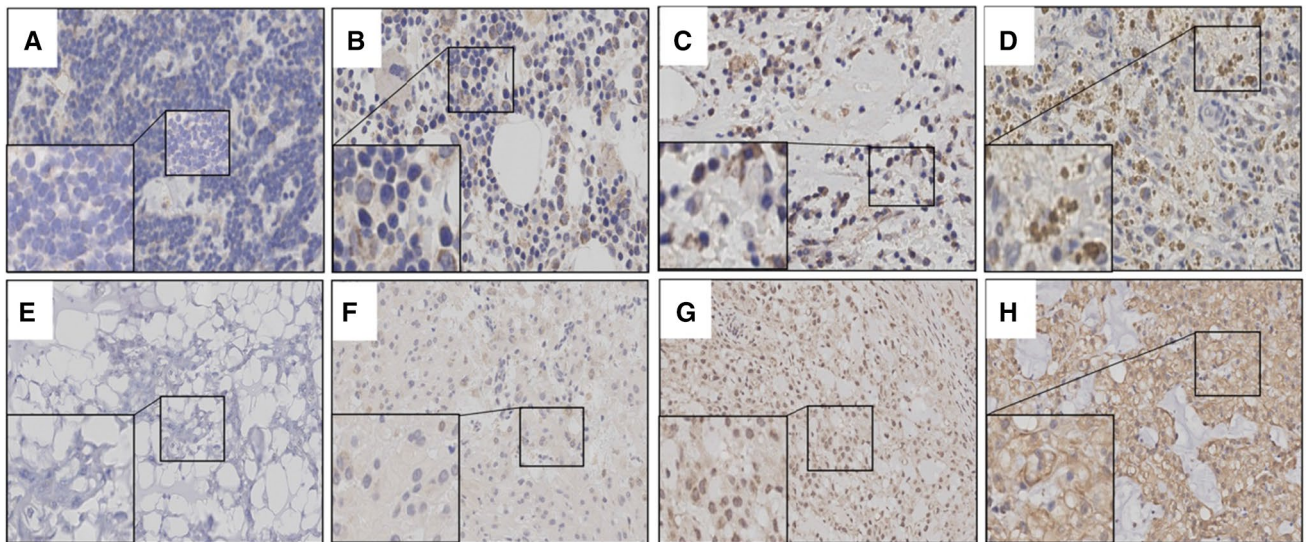


Fig. 2 CTLA-4 expression in TILs and chordoma tissues. TILs: **a** low CTLA-4 staining; **b** weak CTLA-4 staining; **c** moderate CTLA-4 staining; **d** strong CTLA-4 staining; Chordoma tissues: **e**

low CTLA-4 staining; **b** weak CTLA-4 staining; **c** moderate CTLA-4 staining; **d** strong CTLA-4 staining. 400 × magnification. Inset: a further magnification of the small black square

heterogeneous localization as follows: cytoplasm (15.6%), cytoplasm/membrane (25.0%), and cytoplasm/membrane/nucleus (59.4%) (Fig. 3). Furthermore, significantly higher CTLA-4 expression was observed on chordoma tissues than that on the nucleus pulposus tissues ($p = 0.014$; Fig. 4, Supplementary Table S4).

Association of CTLA-4 expression with clinicopathological characteristics

As shown in Table 1, Pearson’s Chi-square analysis revealed that high CTLA-4 expression in the tumor ($p = 0.005$) and TILs ($p = 0.016$) was significantly

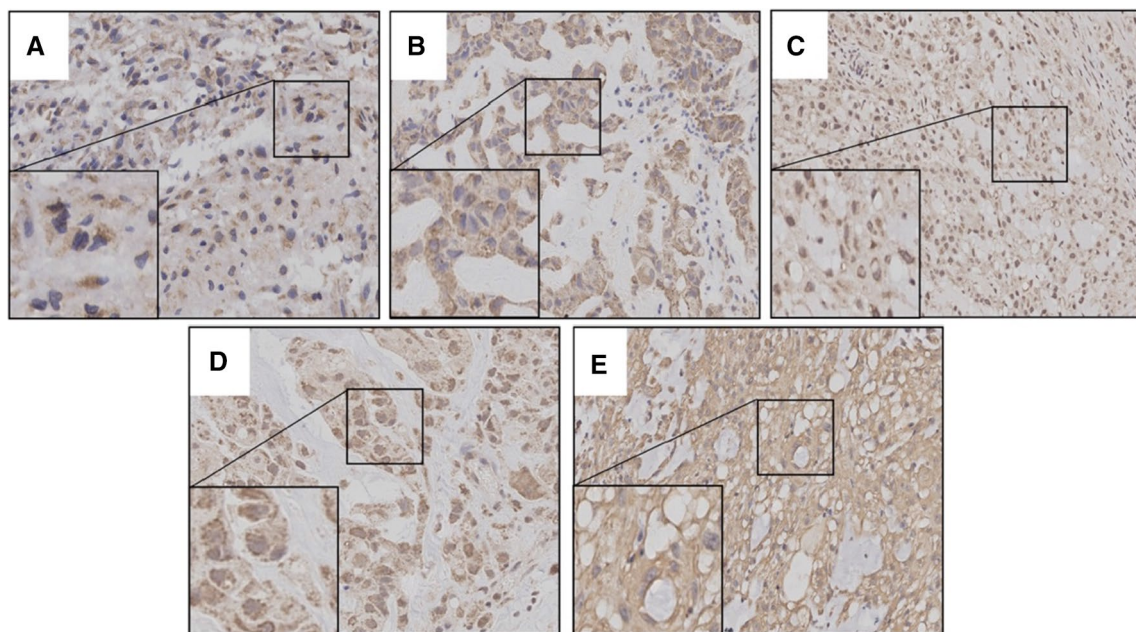
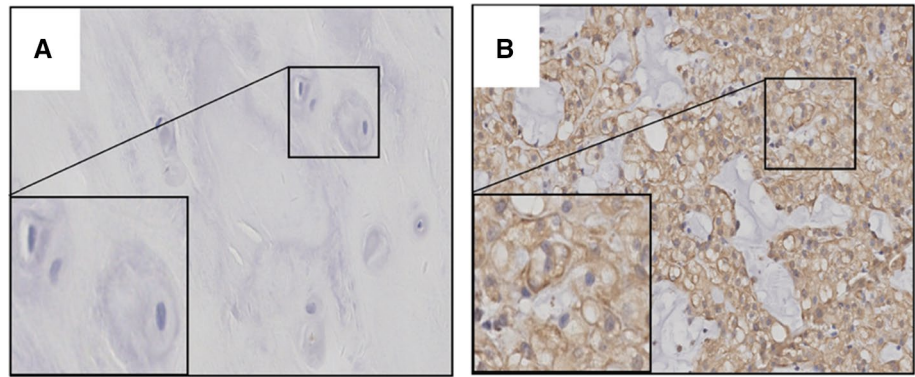


Fig. 3 Characteristics of CTLA-4 expression on chordoma tissues. **a** CTLA-4 staining in cytoplasmic, **b** CTLA-4 staining in cytoplasmic combined with a membrane staining, **c** CTLA-4 staining in cytoplasmic combined with both membrane and nucleus staining; **d** CTLA-4

staining in chordoma tissues in a focal pattern; **e** CTLA-4 staining in chordoma tissues in a scattered pattern. 400× magnification. Inset: a further magnification of the small black square

Fig. 4 Representative CTLA-4 expression in normal control tissues and chordoma tissues groups. **a** Normal control tissues (nucleus pulposus tissues); **b** chordoma tissues.400× magnification. Inset: a further magnification of the small black square



associated with recurrence. Moreover, high CTLA-4 expression in TILs ($p=0.002$) but not in chordoma tissues ($p=0.513$) was significantly correlated with the extent of TILs. However, there was no significant correlations of CTLA-4 expression in tumor cells or TILs with other clinicopathological characteristics such as surrounding muscle invasion, tumor stage, or Ki67 staining index.

Association of prognostic factors and CTLA-4 expression with CDFS and OS

Kaplan–Meier log-rank analysis demonstrated that high CTLA-4 expression in the tumor was significantly associated with a poor CDFS ($\chi^2=8.548, p=0.003$; Table 2, Fig. 5a) and OS ($\chi^2=5.592, p=0.018$; Table 3, Fig. 5c), whereas CTLA-4 expression in TILs was not associated with

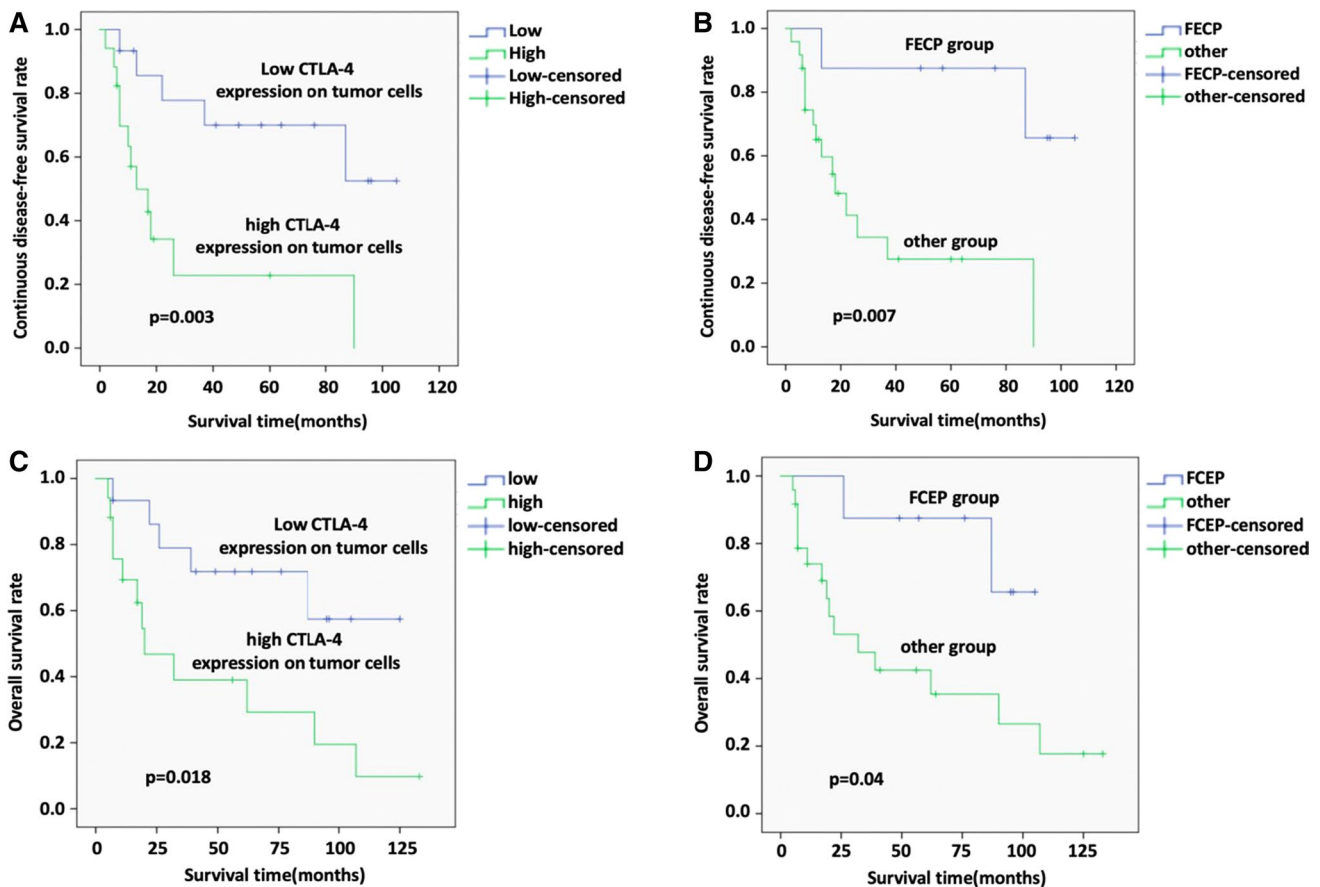


Fig. 5 Kaplan–Meier survival curves of the correlation between CTLA-4 expression and prognosis. (a, c) CDFS and OS of patients with high CTLA-4 expression were both shorter than that of patients

with low CTLA-4 expression in tumor cells; (b, d) CDFS and OS of patients in the FCEP group was longer than that of the other groups

Table 1 Association between CTLA-4 expression and clinicopathological features of 32 spinal chordoma patients

Clinicopathological factors	CTLA-4 expression on tumor		<i>p</i> -value	CTLA-4 expression on TILs		<i>p</i> -value
	Low (<i>n</i> = 15)	High (<i>n</i> = 17)		Low (<i>n</i> = 14)	High (<i>n</i> = 18)	
Age (years)						
≤ 50	7	8	0.982	7	8	0.755
> 50	8	9		7	10	
Sex						
Male	12	11	0.337	12	11	0.125
Female	3	6		2	7	
Tumor size						
≤ 5 cm	5	8	0.430	6	7	0.821
> 5 cm	10	9		8	11	
Tumor location						
Sacral vertebra/Cervical or thoracic or lumbar vertebra	4	7	0.388	3	8	0.174
Surrounding muscle invasion						
Yes	7	5	0.314	4	8	0.358
No	8	12		10	10	
Recurrence						
Yes	10	3	0.005	9	4	0.016
No	5	14		5	14	
Tumor stage						
IB	15	16	0.340	14	17	0.370
III	0	1		0	0	
Tumor type						
Typical	12	15	0.522	13	14	0.244
Dedifferentiated	3	2		1	4	
Extent of TILs						
Low	7	6	0.513	10	3	0.002
High	8	11		4	15	
Ki67 staining index						
Low	13	11	0.152	12	12	0.217
High	2	6		2	6	

CDFS ($\chi^2 = 1.773$, $p = 0.183$; Table 2) or OS ($\chi^2 = 0.664$, $p = 0.415$; Table 3). Among the other clinicopathological factors, only recurrence was significantly related to CDFS ($\chi^2 = 4.872$; $p = 0.027$; Table 2). Multivariate analysis showed that CTLA-4 expression in tumor cells remained an independent predictor for CDFS ($p = 0.007$, hazard ratio [HR] = 4.393; Table 2) and OS ($p = 0.026$, HR = 3.319; Table 3). Moreover, after controlling for other variables, CTLA-4 expression in TILs showed a strong association with CDFS ($p = 0.038$, HR = 3.409; Table 2) but was still not associated with OS ($p = 0.183$, HR = 1.773; Table 3).

Association of combined CTLA-4 expression in tumor cells and TILs with patient survival

Previous studies have highlighted the importance of combined CTLA-4 expression in tumor cells and TILs for

predicting the prognosis of tumor patients [17, 24]. Therefore, we further divided the patients into four subgroups according to various combinations of CTLA-4 expression in tumor cells and TILs: Group 1 (CTLA-4^{high} tumor cells CTLA-4^{high} TILs), Group 2 (CTLA-4^{high} tumor cells CTLA-4^{low} TILs), Group 3 (CTLA-4^{low} tumor cells CTLA-4^{high} TILs), and Group 4 (CTLA-4^{low} tumor cells CTLA-4^{low} TILs). CDFS and OS were significantly longer in Group 4 (89.563 and 91.188 months, respectively) than those in Group 1 (36.727 and 59.623 months), Group 2 (14.444 and 31.833 months), and Group 3 (41.071 and 75.743 months) (Supplementary Table S2), indicating that high CTLA-4 expression in both tumor cells and TILs is strongly associated with a poor prognosis.

Table 2 Univariate and multivariate Cox proportional hazard analyses of prognostic factors for continuous disease-free survival time and overall survival time of spinal chordoma patients

Factors	CDFS			
	Univariate analysis		Multivariate analysis	
	χ^2	<i>p</i> -value	HR	<i>p</i> -value
CTLA-4 expression on tumor	8.548	0.003	4.393	0.007
CTLA-4 expression on TILs	1.773	0.183	3.409	0.038
FCEP group	7.221	0.007	6.561	0.017
Age	0.667	0.414		
Sex	0.007	0.953		
Tumor size	0.482	0.487		
Tumor location	0.678	0.407		
Surrounding muscle invasion	1.604	0.205		
Recurrence	4.872	0.027		
Tumor stage	0.035	0.853		
Tumor type	0.207	0.649		
Extent of TILS	0.254	0.110		
Ki67 staining index	0.003	0.955		

Table 3 Univariate and multivariate Cox proportional hazard analyses of prognostic factors for overall survival time of spinal chordoma patients

Factors	OS			
	Univariate analysis		Multivariate analysis	
	χ^2	<i>p</i> -value	HR	<i>p</i> -value
CTLA-4 expression on tumor	5.592	0.018	3.319	0.026
CTLA-4 expression on TILs	0.664	0.415	1.773	0.183
FCEP group	4.213	0.040	4.185	0.060
Age	1.149	0.284		
Sex	0.303	0.582		
Tumor size	0.177	0.732		
Tumor location	0.172	0.679		
Surrounding muscle invasion	0.162	0.687		
Recurrence	1.898	0.168		
Tumor stage	0.005	0.945		
Tumor type	0.001	0.980		
Extent of TILS	0.476	0.490		
Ki67 staining index	0.026	0.871		

Association of combined CTLA-4 expression in tumor cells and TILs with clinicopathological characteristics and prognosis

Based on the results described above, we defined Group 4 as the favorable CTLA-4 expression profile (FCEP) group, and Groups 1, 2, and 3 were combined as the Other

group. Pearson's Chi-squared analysis showed no significant differences in clinical characteristics between these two groups except for recurrence ($p=0.002$) and extent of TILs ($p=0.022$) (Supplementary Table S3). Univariate log-rank analysis showed that the CDFS ($\chi^2=7.221$, $p=0.007$; Table 2; Fig. 5b) and OS ($\chi^2=4.213$, $p=0.04$; Table 3, Fig. 5d) of the FCEP group were longer than those of the Other group. Multivariate analysis further demonstrated that the FCEP group was an independent predictor for improved CDFS (HR=6.561, $p=0.017$; Table 2) and OS (HR=4.185, $p=0.06$; Table 3).

Discussion

Chordoma is a rare bone tumor characterized by local invasion and poor prognosis [25]. Currently, maximal tumor resection combined with adjuvant radiotherapy is considered the standard treatment for chordoma patients. However, frequent recurrence remains a major limitation to effective chordoma treatment despite wide resection and regular radiotherapy [26]. Therefore, searching for molecular biomarkers that can specifically target chordoma is an important research direction to improve the prediction of prognosis and development of molecular targeted drugs for chordoma patients.

In recent years, an increasing number of molecular biomarkers have been evaluated with respect to potential associations with prognosis of patients with spinal chordoma [27]. However, most of these prognostic biomarkers showed no significant associations after controlling for other potentially confounding factors in multivariate analysis. Thus, we sought to identify new biological markers that can independently predict the prognosis of chordoma patients. We found heterogeneous localization patterns of CTLA-4 expression in chordoma cells in accordance with previous studies [17, 28]. Our finding is consistent with the commonly accepted view that CTLA-4 is mainly localized in the sub-membrane vesicles (cytoplasm) similar to the Golgi apparatus, and will cycle from intracellular stores to a specific position upon activation. However, the relationship between the heterogeneous localization patterns of CTLA-4 expression and the prognosis of chordoma patients requires further investigation to better understand its role in cancer development and progression.

Although previous studies have demonstrated CTLA-4 overexpression in the tumor microenvironment, the relationship of CTLA-4 expression in tumor cells and TILs with patient prognosis remains controversial. The majority of related studies indicated that high CTLA-4 expression was associated with a poor prognosis of tumor patients, and antibodies for targeting CTLA-4 or in combination with other therapies could enhance the tumor-killing effect and

improve the survival rate of patients [29–31]. However, other studies conversely reported that CTLA-4 overexpression was related with an improved prognosis [32, 33]. Such discrepancy among studies suggests that CTLA-4 expression might have distinct clinical outcomes in different tumor types. In our study, both univariate and multivariate analyses demonstrated that high CTLA-4 expression in chordoma cells was associated with a poor CDFS and OS, suggesting that CTLA-4 overexpression in the tumor and TILs might result in an immunosuppressive tumor microenvironment. Two recent studies in breast cancer and esophageal cancer demonstrated that CTLA-4 expression in the tumor combined with that in TILs was a stronger predictor for patient prognosis [17, 24]. Similarly, in the present study, although tumor CTLA-4 expression did not differ from that in TILs, the HR for CDFS and OS of the FECF group (characterized by low CTLA-4 expression in both the tumor and TILs) was much higher than that of other groups, suggesting that the combination of high CTLA-4 expression in chordoma and TILs is also an even stronger poor predictor for chordoma patient prognosis.

Considering these results, we speculate that CTLA-4 expression in chordoma tumor cells or TILs may synergistically damage the function of activated tumor-specific T cells. In addition, CTLA-4 expression was much more frequent and stronger in chordoma than that in normal nucleus pulposus tissues, indicating that CTLA-4 overexpression might contribute to tumorigenesis. Nevertheless, the specific function of CTLA-4 and the underlying regulatory mechanism in chordoma require further experimental clarification.

Conclusion

To our knowledge, this is the first study to demonstrate an association of CTLA-4 expression in chordoma tissues and TILs with patient prognosis. Our results revealed that high CTLA-4 expression in tumor tissues or TILs was related with poor survival of chordoma patients and their combination was an even stronger poor prognostic predictor (see Schematic 1). These findings suggest that CTLA-4 blockade might be a promising immunotherapeutic strategy for treatment of chordoma patients. Nevertheless, as this was a single-center study with a relatively small sample and lack of functional assays, further *in vitro* and *in vivo* studies are needed to verify the role of CTLA-4 in chordoma. In particular, multicenter studies with larger patient cohorts will help to obtain a more comprehensive understanding of the biological function and underlying mechanism of abnormal CTLA-4 expression in chordoma and its clinical relevance.

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Author contributions Conceived and designed the experiments: GH, XL. Performed the experiments: GH, XP, XL, YM. Analyzed the data: GH, XP, XL, XL. Wrote the paper: GH, XL.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The study was approved by the ethics committee of Peking University Third Hospital (No. IRB00006761-2016048).

Consent to participate Informed consent for use of their specimens and data in this study was obtained from all adult patients or written parental consent was provided for minors before the study was conducted.

Availability of data and materials The data and materials are available from the corresponding author on reasonable request.

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