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ORIGINAL ARTICLE

Evaluation of tumor-infiltrating lymphocytes in osteosarcomas of the jaws: a multicenter study

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Abstract

The aim of the present study was to investigate the profile of tumor-infiltrating lymphocytes (TIL) in osteosarcomas of the jaws (OSJ). A total of 21 OSJ samples were analyzed in a retrospective and cross-sectional multicenter study. Immunohistochemistry was performed to determine the recognition of TIL such as $CD4^+$, $CD8^+$, granzyme B^+ (GrB), programmed cell death $protein^+$ (PD-1), and cytotoxic T lymphocyte-associated antigen 4^+ (CTLA-4) in intratumoral and peripheral (stromal) regions. Positivity was determined based on the percentage and density of TIL^+ per square millimeter [1 = absent (< 25 cells/mm^2), 2 = low (25 to 130 cells/mm^2), and 3 = high (> 130 cells/mm^2)]. The association of TIL density with clinicopathologic data was determined by the Mann-Whitney test ($p < 0.05$). OSJ were positive for $CD8^+$ cells in 45% ($n = 9$) of cases, for $CD4^+$ cells in 30% ($n = 6$) of cases, and for CTLA-4^+ in 4.8% ($n = 1$) of cases, with a score of 2 (low TIL) in all cases. All cases were negative for GrB and PD-1 (score 1). No association was observed between immune infiltrate and clinicopathologic findings. OSJ showed a microenvironment with low TIL, including failure of effectiveness of the antitumor immune response (absence of GrB^+ cells), and few cells exhibited immunotherapeutic targets, such as CTLA-4 and PD-1.

Keywords Osteosarcoma · Tumor-infiltrating lymphocytes · Tumor microenvironment · Oral cancer · Immunotherapy

Introduction

Osteosarcomas of the jaws (OSJ) are the most frequent primary malignant bone tumors affecting adolescents and young adults [1, 2]. OSJ usually show an aggressive clinical course and represent a major therapeutic challenge [3–5]. These invasive and highly metastatic bone-forming tumors are characterized by the presence of an extracellular osteoid matrix produced by neoplastic cells surrounded by a microenvironment containing bone cells, blood vessels, stromal cells, and immune-inflammatory cells [6]. The immune-inflammatory cells present in osteosarcomas (OS) are mainly myeloid cells (monocytes, macrophages, and dendritic cells), T-lymphocytes, B-lymphocytes, and mast cells [6–8]. Tumor-infiltrating lymphocytes (TIL), mainly consisting of cytotoxic T-lymphocytes and subpopulations of T $CD4^+$ lymphocytes, represent the second most common population of cell-type infiltrates in OS of long bones [7–9].

Identification of TIL has led to the development of immunotherapeutic approaches since the selected population of T-lymphocytes exhibits high specific immunological reactivity against tumor cells despite the energy induced by the microenvironment and/or by neoplastic cells [10, 11]. TIL have the capacity to directly recognize and kill antigen-expressing cells with $CD8^+$ effector T cells and can manage diverse immune

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responses by $CD4^+$ helper T cells which integrate adaptive and innate effector mechanisms. Their action is regulated by a balance between co-stimulatory and inhibitory signals corresponding to the immune checkpoints [11]. Therefore, blockade of these immune checkpoints is a target of cancer immunotherapy. The most extensively investigated checkpoints are cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death 1 (PD-1) protein, which can be blocked separately or in association [12].

CTLA-4, also known as CD152, is a protein receptor that, functioning as an immune checkpoint, downregulates immune responses. CTLA-4 is expressed by activated CD4 and CD8 effector T cells, and was the first of this class of immunotherapeutic drugs to achieve approval by the US Food and Drug Administration [11, 12]. Human CTLA-4-blocking antibodies (ipilimumab and tremelimumab) have been used in clinical trials on patients with melanomas [13]. More recently, studies using immunotherapy drugs such as nivolumab and ipilimumab have been conducted on patients with OS [6, 14].

PD-1, also known as CD279, is a 55-kD member of the immunoglobulin superfamily and is expressed by activated T, B, and natural killer (NK) cells [11]. Programmed cell death 1 ligand 1 (PD-L1), also known as B-7H1, is a ligand of PD-1 and a member of the B7 gene family. PD-L1 is a glycoprotein expressed on the cell surface of T and B lymphocytes, dendritic cells, macrophages, and tumor cells [11]. The expression of PD-1 has been investigated in several sarcomas (e.g., OS, Ewing sarcomas, alveolar rhabdomyosarcomas, and others) [14–17]. Studies have investigated the expression of PD-1 in various subtypes of primary sarcomas [15], and specifically in OS of long bones [14, 16, 17]. We have investigated the expression of human leukocyte antigen-G (HLA-G), human leukocyte antigen-E (HLA-E), and PD-L1 in a series of 13 cases of OSJ in a single-center study [18]. However, to our knowledge, no study has been conducted thus far regarding PD-1 and CTLA-4 in OSJ. Thus, the aim of the present multicenter study was to evaluate the immune profile ($CD4^+$, $CD8^+$, GrB^+, $PD\text{-}1^+$, and $CTLA\text{-}4^+$ cells) in the microenvironment of OSJ and to associate these cells with clinicopathologic data.

Materials and methods

Study design and ethical approval

This retrospective and cross-sectional study evaluated 21 paraffin-embedded tissue specimens of OSJ. The records were obtained from a consortium of four services of oral and maxillofacial pathology in four Brazilian regions: Midwest, North, Northeast, and Southeast (Table 1). Of the 21 cases of OSJ diagnosed at four Brazilian centers, eight were investigated in a previous study by Costa Arantes et al. [18]. Inclusion criteria were OSJ patients who had undergone an incisional biopsy for diagnosis. Only specimens with representative tissue and demonstrating a portion of tumor parenchyma and stroma were included. Exclusion criteria were records without demographic data. All patients included in this study were diagnosed at an oral and maxillofacial pathology service and were referred to head and neck services for oncologic treatment across Brazil. The study was approved by the Ethics Committee of Universidade Federal de Goiás (Approval No. 011.2011). Patient anonymity was guaranteed according to the Helsinki Declaration.

Clinical data and histopathologic analysis

Clinical data were collected from the records of the services where the diagnosis was made. Affected individuals were analyzed regarding gender, age, anatomical location, histopathologic diagnosis, and treatment and follow-up data when available.

Table 1 Sources of the cases reviewed for immunohistochemical analysis

Institution	State	Years	Lesions biopsied during the study period	Sarcomas of the jaws	OSJ	%[a]
UFG[b]	Goiás	1996–2017	11,076	26	8	0.07
UFPA[c]	Pará	2008–2017	5500	38	7	0.12
UFMG[d]	Minas Gerais	1953–2017	37,029	25	5	0.01
UEPB[e]	Paraíba	2008–2017	2968	3	1	0.03
Total	–	–	56,573	92	21	0.03

OSJ osteosarcomas of the jaws

[a] Percent of the OSJ sample at each center

[b] Department of Stomatology, School of Dentistry, Universidade Federal de Goiás (Midwest region)

[c] Service of Oral Pathology, School of Dentistry, Universidade Federal do Pará (North region)

[d] Department of Oral Surgery and Pathology, School of Dentistry, Universidade Federal de Minas Gerais (Southeastern region)

[e] Service of Oral Pathology, Department of Dentistry, School of Dentistry, Universidade Estadual da Paraiba (Northeastern region)

For the histopathologic analysis, 5-μm thick histologic sections were cut from the formalin-fixed paraffin-embedded tumors and stained with hematoxylin and eosin. OSJ were categorized according to the 2017 classification of the World Health Organization (WHO) and by histological subtype, i.e., osteoblastic, chondroblastic, fibroblastic, or small-cell type [19].

Immunohistochemical (IHC) staining

For the IHC study, 3-μm thick sections were obtained from paraffin-embedded tissue blocks and mounted on organosilane-coated slides (3-aminopropyltriethoxysilane; Sigma Chemical Co., St Louis, MO, USA). Deparaffinization, rehydration, and antigen retrieval steps were performed using Trilogy (Cell Marque, CA, USA) at a concentration of 1:100 in ultrapure water after immersing the slides in a digital water bath at 96 °C for 45 min (DeLeo, Porto Alegre, RS, Brazil). After washing with Tris-buffered saline (TBS, pH 7.4, Neon®, Suzano, São Paulo, Brazil), the slides were incubated with 3% hydrogen peroxide to block endogenous peroxidase and, after further washing with TBS, they were incubated with a protein block (Novolink™, Novocastra, Leica Biosystems Gmb, Wetzlar, Germany) for 5 min. The slides were then incubated with one of the following primary antibodies: anti-CD4 (clone 4B12, NovoCastra, Newcastle, UK, 1:200 dilution, overnight), anti-CD8 (clone C8/144B, Dako, Carpinteria, CA, USA, 1:600 dilution, overnight), anti-GrB (clone GrB-7, Dako, Carpinteria, CA, USA, 1:100 dilution), anti-CTLA-4 (clone F-8, Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:200 dilution, overnight), and anti-PD-1 (clone NAT105, Cell Marque, CA, USA, ready to use, overnight). Next, the sections were treated with the Novolink™ Max Polymer Detection System Kit (Novocastra, Leica Biosystems Gmb, Wetzlar, Hessen, Germany), and the reactions were developed with 3.3′-diaminobenzidine (DAB, Dako, Carpinteria, CA, USA). Finally, tissue sections were counterstained with hematoxylin from the NovoLink™ kit and coverslipped.

Samples of oral lichen planus or tonsils were used as positive controls for all proteins studied. Negative controls consisted of replacement of the primary antibody with 1% bovine serum albumin in the buffer solution.

IHC assessment

$CD4^+$, $CD8^+$, GrB^+, $PD\text{-}1^+$, and $CTLA\text{-}4^+$ cells were evaluated in peripheral (tumor stroma) and intratumoral (tumor parenchyma) regions. IHC analysis was assessed quantitatively ($cell^+$ density per mm^2). All sections were evaluated with an integration reticle (4740680000000-Netzmikrometer 12.5×, Carl Zeiss, Göttingen, Niedersachsen, Germany) connected to a light microscope (AxioScope; Carl Zeiss) in five alternating fields at × 40 magnification. At this magnification, the area of one field corresponds to 0.0961 mm^2. IHC analysis was performed by two oral and maxillofacial pathologists who were calibrated before data analysis (kappa = 0.90).

Immunostaining scores were attributed according to the method of van Erp et al. [15], i.e., 1 = absent (< 10 cells analyzed at × 20 magnification field, equivalent to < 25 cells per mm^2 at × 40 magnification field), 2 = low (10–50 cells, equivalent to 25 to 130 cells per mm^2) and 3 = high (≥ 50 cells, equivalent to > 130 cells per mm^2).

Data analysis

Descriptive and quantitative data analysis, with absolute and relative values, was performed using the IBM SPSS Statistics 22.0 software (IBM Corp., Armonk, NY, USA). The Kolmogorov-Smirnov test showed that the quantitative

Table 2 Clinicopathological features of cases of osteosarcomas of the jaws

Variable	*n* (%)
Gender	
Male	10 (47.6)
Female	11 (52.4)
Anatomical location	
Mandible	19 (90.5)
Maxilla	2 (9.5)
Histologic grading[a]	
Low	1 (4.8)
High	20 (95.2)
Histology/subtype	
Chondroblastic	12 (57.1)
Osteoblastic	6 (28.6)
Fibroblastic	2 (9.5)
Metastasis[b]	
Yes	
Lymph node	4 (57.1)
Lung	3 (42.9)
No	8 (53.3)
Recurrence[b]	
Yes	8 (53.3)
No	7 (46.7)
Treatment[b]	
Neoadjuvant chemotherapy + surgery	3 (20.0)
Surgery + chemotherapy	7 (46.7)
Surgery	4 (26.7)
Neoadjuvant chemotherapy + surgery + chemotherapy + radiotherapy	1 (6.6)
Death[b]	
Yes	10 (66.7)
No	5 (33.3)

[a] According to the 2017 WHO classification

[b] Some data were not available

variables ($CD4^+$, $CD8^+$, GrB^+, CTLA-4^+, and PD-1^+) exhibited non-normal distribution. Therefore, the Mann-Whitney test was used to determine possible differences in the median number of TIL according to the clinicopathologic data of OSJ. Statistical significance was set at $p < 0.05$.

Results

Clinicopathological data

A total of 56,573 histopathologic records of oral and maxillofacial biopsies were diagnosed at four referral services; 92 cases were sarcomas of the jaws (0.16%) and 21 of them (0.03% of all cases) were OSJ. Table 1 presents the distribution of OSJ by center.

The sample consisted of 10 men (47.6%) and 11 women (52.4%) with a mean age of 32.6 years (range 5 to 70 ± 14.3 years); the mean age of the men and women was 28.9 and 36.0 years, respectively. Regarding anatomical location, 90.5% ($n = 19$) of cases affected the posterior mandible and 9.5% ($n = 2$) the maxilla (Table 2).

Histological grading revealed that 95.2% ($n = 20$) of OSJ were high-grade tumors. The histological subtype of the tumors was chondroblastic in 57.1% ($n = 12$) of cases, osteoblastic in 28.6% ($n = 6$), and fibroblastic in 9.5% ($n = 2$) (Table 2).

Follow-up information (metastasis, recurrence, survival, and death) was available for 15 individuals. Metastasis was present in seven patients (46.7%), including lymph node ($n = 4$) and lung ($n = 3$), recurrence occurred in eight subjects (53.3%), and 10 patients (66.7%) died. Mean overall survival was 37.6 months (range 2 to 156 months) (Table 2).

Immunoexpression of $CD4^+$, $CD8^+$, GrB^+, PD-1^+, and CTLA-4^+ cells

IHC staining demonstrated that CD4, CD8, PD-1, and CTLA-4 exhibited a brown staining pattern in the membrane and GrB in the cytoplasm. Immune-inflammatory cells were predominantly distributed diffusely over a peripheral area and few cells were seen in the intratumoral area (Fig. 1).

All cases exhibited absent or low densities of CD4-, CD8-, and GrB-positive cells. Of $CD8^+$ cells, 57.1% ($n = 11$) and 42.8% ($n = 9$) showed 25 to 130 cells per square millimeter (score 2) in peripheral and intratumoral regions, respectively. Regarding $CD4^+$ cells, 52.4% of the samples ($n = 11$) and 23.8% ($n = 5$) also exhibited a score of 2 in peripheral and intratumoral areas. GrB^+ cells were absent in 14 cases (66.6%) and were present in numbers ranging from 1.4 to 23.4 (score 1) in the other seven (Table 3).

Most cases were negative for CTLA-4 ($n = 19$) and PD-1 ($n = 14$). One case (4.8%) showed positivity for CTLA-4, with a density of 28.1 for peripheral regions and of 29.1 for intratumoral regions (score 2). For PD-1, seven cases (33.3%) exhibited a density of less than 25 cells per square millimeter (score 1) (Table 3). No significant association was observed between immune cells and demographic data or histologic subtypes.

Discussion

Malignant neoplasms exhibit many strategies for evasion of cytotoxic T-lymphocytes, including expression of immune inhibitory molecules such as PD-L1 [11]. Based on this context, four categories of tumor microenvironment have been proposed according to patterns of PD-L1 expression and TIL, i.e., type I: PD-L1 expression in the presence of TIL (adaptive resistance);

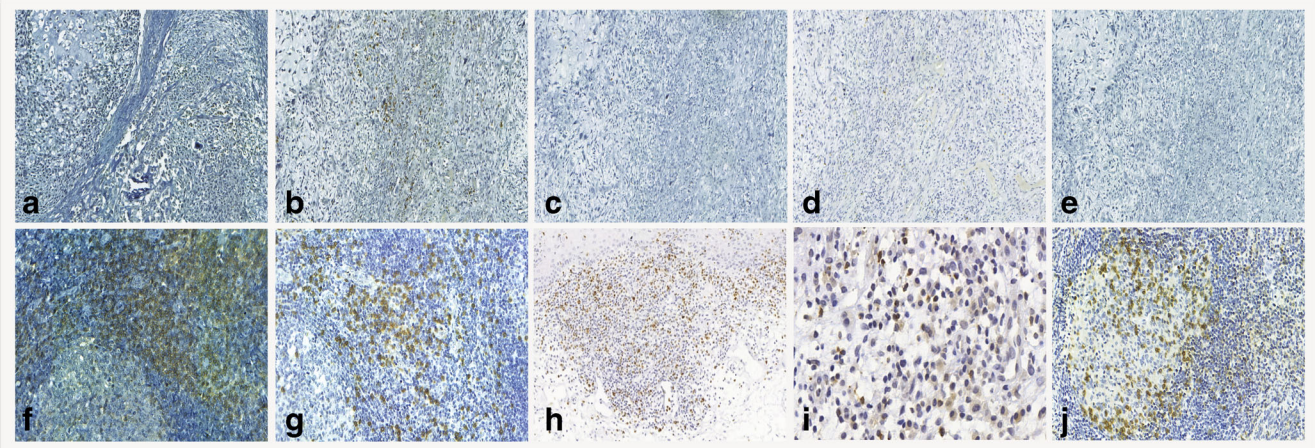

Fig. 1 Photomicrograph exhibiting tumor infiltrating T-lymphocytes in osteosarcomas of the jaws. **a**, **b** Low density of $CD4^+$ and $CD8^+$ TIL (100×). **c** Absence of GrB^+ cells (100×). **d**, **e** Presence of scarce positive cells for CTLA-4 and PD-1 (100×). **f**, **g**, **j** Samples of tonsils as positive controls for CD4, CD8, and PD-1 (100×). **h**, **i** Samples of oral lichen planus as positive controls for GrB (100×) and CTLA-4 (200×)

Table 3 Median, minimum, and maximum densities per square millimeter of CD4, CD8, granzyme B (GrB), CTLA-4, and PD-1 in peripheral and intratumoral regions of osteosarcomas of the jaws

Histologic grading (subtype)	Peripheral region					Intratumoral region				
	CD4 Median (min-max)	CD8 Median (min-max)	GrB Median (min-max)	CTLA-4 Median (min-max)	PD-1 Median (min-max)	CD4 Median (min-max)	CD8 Median (min-max)	GrB Median (min-max)	CTLA-4 Median (min-max)	PD-1 Median (min-max)
High grade ($n = 20$)										
Chondroblastic ($n = 12$)	33.6 (0–92)	39.9 (0–99)	0 (0–23.4)	0 (0–28.1)	0 (0–17.7)	13.5 (0–68.2)	28.4 (0–112)	0 (0–1.0)	0 (0–29.2)	0 (0–17.2)
Osteoblastic ($n = 6$)	38.6 (0–124.2)	72.4 (0–122)	0 (0–9)	0 (0–0)	0 (0–6.3)	25 (0–44.3)	17 (0–26.7)	0 (0–0)	0 (0–3.7)	0 (0–4.2)
Fibroblastic ($n = 2$)	2.3 (0–4.7)	45.1 (0–90.1)	4 (3.1–4.7)	0 (0–0)	1.3 (0.5–2.1)	7.8 (0–15.6)	54.1 (0–108.3)	1.8 (1.6–2.1)	0 (0–0)	3.3 (1.0–5.7)
Low grade ($n = 1$)	12.5	58.3	14.0	10.4	0	0	4.2	0	0	0

type II: lack of both PD-L1 expression and TIL (immunological ignorance); type III: PD-L1 expression in the absence of TIL (intrinsic induction); and type IV: lack of PD-L1 expression but presence of TIL (immunological tolerance) [20, 21].

Recently, we demonstrated high expression of PD-L1, HLA-G, and HLA-E by malignant osteoblastic cells in almost 70% of OSJ cases [18]. However, despite these findings, in the present study, we observed a microenvironment with low $CD4^+$ and $CD8^+$ TIL including the absence of GrB, as well as a significantly low number of CTLA-4^+ and PD-1^+ cells. In general, OSJ might be characterized as type III, suggesting an immunosuppressive microenvironment that could contribute to the aggressiveness of this neoplasm. Furthermore, Heymann et al. [6] demonstrated that OS cells could control TIL responses via PD-1/PD-L1, establishing a local immune tolerance environment favorable to tumor growth, drug resistance, and the occurrence of local and/or distant metastasis.

On the other hand, in OS of long bones, many studies have revealed a high density of $CD8^+$ TIL [9, 14–16, 22, 23], but low levels of PD-1 and PD-L1 [15, 23]. Interestingly, Gomez-Brouchet et al. [16] showed an association between lower numbers of $CD8^+$ cells and metastatic OS of the limb/axial regions, as well as a significant correlation between the presence of these cells and improved survival in zoledronate-treated patients. Moreover, these authors demonstrated that PD-1/PD-L1 markers were negative in more than 80% of OS cases and were not associated with outcome. In the present study, no association was observed between clinicopathologic features and TIL or immunotherapeutic targets (i.e., CTLA-4 and PD-1).

Some studies on cell lines and animal models have demonstrated that antineoplastic therapy for OS such as radiotherapy and adjuvant chemotherapy can enhance the anti-tumor immune response [24, 25]. In a study of OS cell lines, Ratti et al. [24] observed that trabectedin, a chemotherapy drug, is able to increase T cell recruitment (CD3, CD4, and CD8), adaptive immune response activation, overexpression of PD-1/PD-L1, and upregulation of CTLA-4, consequently also increasing tumor control. In addition, immunotherapy has yielded promising results for the treatment of mouse model metastatic OS [26, 27]. Also, the combination of immunotherapy modalities using PD-L1 and CTLA-4 antibodies in a mouse model of metastatic OS was found to be even more effective in controlling tumors [26]. In our previous investigation [18], OSJ showed high PD-L1 levels; however, herein, most cases were CTLA-4 negative. While immunotherapy has induced some responses in soft tissue and bone sarcomas, further research will help clarify optimal patient selection for future clinical trials and new combinations of immunotherapeutic strategies [28]. Thus, the immune characterization of OSJ in incisional biopsies can contribute to individualized therapy for affected patients.

Extrapolation of our results should be taken with caution. This multicenter study analyzed a series of 21 cases of OSJ

from biopsy records obtained at several referral centers in Brazil. Nevertheless, this was a small sample, although this is justifiable in view of the rarity of this lesion. The second limitation is due to missing data because some patients did not attend a follow-up appointment, a fact inherent to the retrospective nature of the study.

In summary, our findings suggest that OSJ show a microenvironment with low $CD4^+$ and $CD8^+$ TIL, including failure of effectiveness of the antitumor immune response due to the absence of GrB^+ cells, and few immunotherapeutic targets such as CTLA-4 and PD-1.

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Authors' contributions PMA, DACA, LLS, SFSC, and JAAA conducted a literature review, organized the data of the clinical cases, and conducted the immunohistochemical reactions, and morphological and immunohistochemical analysis of samples. HARP, RAM, CFWN, and EFM contributed cases from their services, and reviewed and classified, morphologically, all cases. ACB, PMA, and JAAA contributed to the design of the work. FPF was responsible for data interpretation. ACB, PMA, RAM, and HARP contributed to the conception of the work. All authors gave final approval for publication.

Compliance with ethical standards

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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

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