Journal of Bone Oncology 25 (2020) 100332

Contents lists available at ScienceDirect

Journal of Bone Oncology

journal homepage: www.elsevier.com/locate/jbo

Adoptive transfer of TILs plus anti-PD1 therapy: An alternative combination therapy for treating metastatic osteosarcoma

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ARTICLE INFO

Keywords:
Metastatic osteosarcoma
TILs
Immunotherapy
Prognostic factor
PD1
Adverse effects
Phenotype
Retrospective

ABSTRACT

Aim: We sought to investigate the efficacy of adoptive transfer of TILs plus anti-PD1 therapy in metastatic osteosarcoma patients.

Materials and methods: A total of 30 patients received anti-PD1 therapy (Group 1) while 30 patients were subjected to TILs plus anti-PD1 therapy (Group 2). Progression-free survival time (PFS) and overall survival time (OS) were analyzed using Kaplan-Meier analysis. Potential prognostic factors were analyzed using univariate and multivariate analyses.

Results: The ORR in Group 2 is 33.3%, which is significantly higher than Group1 (6.67%). In addition, we found significantly prolonged mPFS (5.4 months) and mOS (15.2 months) in Group 2 compared to those in Group 1, which recorded mPFS and mOS of 3.8 and 6.6 months, respectively. Univariate and multivariate analyses indicate that patients with more infusions of TIL numbers and $CD8^+$ TILs or less infusions of $CD8^+$ $PD1^+$ TILs and $CD4^+FoxP3^+$ TILs show increased PFS and OS. Moreover, $PD1^{hi}$ is another good prognostic factor that predict PFS and OS.

Conclusion: Overall, these findings indicated that TILs plus anti-PD1 therapy has significant clinical outcomes in metastatic osteosarcoma patients. However, further studies are essential to validate and characterize the therapeutic activity of TILs plus anti-PD1.

1. Introduction

Osteosarcoma (OS) is the most prevalent primary malignant bone tumor occurring in children and adolescents, with a peak age of about 20 years old [1]. The disease manifests as highly aggressive and early systemic metastasis, with approximately 20% of osteosarcoma patients exhibiting these symptoms after first diagnosis [2]. The prognosis for patients diagnosed with osteosarcoma has significantly improved with the advent of multiagent chemotherapy regimens for neoadjuvant and adjuvant treatment. Consequently, these multi-modality treatment approaches guarantee a cure in three quarters of all osteosarcoma patients, with 90–95% of those diagnosed effectively treated with limb-sparing approaches rather than amputation [3]. Furthermore, survival rates have improved to approximately 60% in patients with localized osteosarcoma [1]. However, those with systemic metastasis, lung metastasis remains the most prominent cause of osteosarcoma-related deaths. In fact, previous studies have shown that only 11–30% of osteosarcoma patients with metastasis can survive after a combination of surgical resection and chemotherapy [2,4,5]. This indicates that osteosarcoma metastasis is an obstacle to successful treatment, necessitating development of novel therapeutic strategies for metastatic osteosarcoma for improved prognosis.

Immunotherapeutic approaches harness the immune system by attacking and destroying tumors [6]. In normal circumstances, the immune system regulates itself and maintains self-tolerance, ensuring that no unnecessary damage is done to the body following response to a foreign antigen. In cancer patients, some immune cells upregulate well-characterized cell surface molecules cytotoxic T-lymphocyte associated protein 4 (CTLA-4) and programmed cell death protein 1 pathway (PD-1/PD-L1), which serve as immune checkpoints for regulating activation and function of T cells [6]. Inhibition of T cells, by these molecules, is highjacked by cancer cells and used to evade recognition by the immune system. Consequently, cancer therapies employ the immune checkpoint blockade to reverse T-cell tolerance, by blocking inhibitory interactions between tumor and infiltrating T cells, thereby allowing antitumor immune responses [7]. Previous studies have reported excellent clinical efficacy using anti-PD-1/PD-L1 antibodies in trials targeting several cancer types, including osteosarcoma [8–12]. However, to date, objective response rate (ORR) has been achieved in only approximately 5% of non-selective osteosarcoma cases [9]. One of the

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https://doi.org/10.1016/j.jbo.2020.100332
Received 2 July 2020; Received in revised form 14 September 2020; Accepted 6 October 2020
Available online 16 October 2020

reasons for the low ORR in anti-PD-1/PD-L1 therapy is that tumor-reactive cytotoxic T lymphocytes (CTLs) are induced during development of metastatic osteosarcoma, but become exhausted in the tumor microenvironment [13–15]. Therefore, anti-PD1 therapy alone may not be an effective approach for treating metastatic osteosarcoma.

Previous studies have reported the use of adoptive cell therapy (ACT) in managing tumor-infiltrating lymphocytes (TILs). In fact, the approach has generated satisfactory efficacy in metastatic melanoma patients, with 40–70% ORR reported [16–19]. Despite the success of the approach in management of numerous malignancies, its use for treating osteosarcoma patients remains unknown [20–23]. A pre-clinical study found that TILs extracted from osteosarcoma could penetrate the tumor microenvironment and generate cytotoxic effects against allogeneic tumor cells, indicating the potential for this therapy in treatment of osteosarcoma [24]. Since anti-PD1 therapy depends on TILs in the microenvironment, we hypothesized that a combination of anti-PD1 therapy and TILs may generate potential antitumor effects in metastatic osteosarcoma patients. Therefore, we sought to evaluate the safety and activity of combined adoptive transfer of TILs and anti-PD1 therapy in metastatic osteosarcoma patients and identified potential prognostic biomarkers.

2. Materials and methods

2.1. Patients

A retrospective study was performed to evaluate the clinical outcomes of the adoptive transfer of TILs plus anti-PD1 therapy for diagnosed patients with metastatic osteosarcoma. Summarily, a total of 60 metastatic osteosarcoma patients, were enrolled between 25th April 2017 and 1st June 2018. Subjects were recruited in the study if they: (1) were clinically diagnosed with metastatic osteosarcoma and had experienced disease progression after second line chemotherapy. (2) stopped any cancer therapy before enrollment; (3) were more than 12 years old; (4) had a life expectancy of greater than 3 months; (5) had an Eastern Cooperative Oncology Group (ECOG) performance status of 0–1; (6) showed adequate organ function; and (7) exhibited lesions that could be evaluated using the response evaluation criteria in solid tumors (RECIST 1.0 version 1.1) guidelines [25]. On the other hand, subjects were excluded from the study if: (1) had undergone previous treatment with anti-CTLA4 or anti-PD1/PDL1 therapy; (2) had any form of primary immunodeficiency or a history of autoimmune diseases; (3) had ongoing systemic infections and concurrent systemic steroid therapy; and (4) were recruited in other clinical trials.

2.2. Study design and follow-up

This single-center retrospective clinical study was approved by the Ethics Committee at the Affiliated Luoyang Central Hospital of Zhengzhou University. All methods and procedures associated with this study were conducted in accordance with the Good Clinical Practice guidelines and accorded ethically with the principles of the Declaration of Helsinki and local laws. All authors had access to the study data and reviewed and approved the final manuscript. The primary end point was objective response rate (ORR) and progression-free survival time (PFS). The secondary endpoints were overall survival time (OS) evaluated by Kaplan-Meier analysis. Potential prognostic factors were also analyzed by univariate and multivariate analyses based on adoptive transfer of TILs plus anti-PD1 therapy. The PFS was calculated from the date of immunotherapy to disease progression and patients with a stable state were censored at the time of last contact. The OS was calculated from the date of immunotherapy to the time of death, and patients who were alive at the time of last contact were censored. PFS and OS were calculated by the Kaplan-Meier method. A total of 70 patients were initially screened as follows; 40 of them agreed with adoptive transfer of TILs plus anti-PD-1 immunotherapy, whereas 30 agreed with anti-PD-1 immunotherapy. Ten patients were excluded owing to not meeting inclusion criteria or decline to participate or other reasons. Eventually, 60 patients met the aforementioned inclusion criteria and were equally assigned to Group 1 and Group 2, representing anti-PD-1 immunotherapy and adoptive transfer of TILs plus anti-PD-1 immunotherapy, respectively. Patients in both groups received a one-cycle infusion of anti-PD1 therapy, comprising 3 mg/kg Nivolumab, at our department for two weeks (if the total dosage of one cycle reach or exceed 240 mg, then use the maximum dose of 240 mg). All patients received at least 8 cycles of infusions. Otherwise, treatment continued until they experienced disease progression, exhibited unacceptable adverse effects (AEs) or withdrew from the study. Except this, patients in Group 2 also received one cycle of TILs transfusion during the first cycle of anti-PD-1 therapy. After immunotherapy, all patients were scheduled for follow-up evaluations at our hospital, from the date of initial treatment to 1st June 2020 (allocated follow-up deadline), or time of death. Clinical examinations, including complete blood examinations, chest and abdominal computed tomography (CT) scans or magnetic Resonance Imaging (MRI), were performed by our oncology specialists, after every 3 months. If follow-up evaluations showed progression, the patients were subjected to the best support care.

2.3. Measurement of outcomes

The primary aim of the study was to evaluate the ORR and PFS of infusions of TILs plus Nivolumab in the patients, whereas the secondary endpoint entailed assessing their OS. Safety evaluations primarily involved checking for clinical and laboratory abnormalities, and these were monitored throughout the study until two weeks after the last Nivolumab infusion. AEs were evaluated according to the guidelines described by the National Cancer Institute Common Toxicity Criteria version 4.0 [26]. In addition, treatment-associated AEs were assessed during the course of treatment and observation periods, and the highest observed grade recorded for each patient Lesions in each patient were evaluated using CT or MRI scans after every 3 months, whereas ORR were determined using RECIST version 1.1 [25].

2.4. Acquisition of tumor specimens and generation of TILs

Fresh tumor tissues from metastatic sites were obtained from each patient, using thick needle puncture, confirmed by two independent pathologists at our hospital, then TILs cultured according to our previously described protocol [27]. Briefly, tumor tissues were sliced into pieces (approximately 2–3 mm^3 in size), using a scalpel, followed by a 3-hour enzymatic digestion using collagenase type IV (Sigma-Aldrich, St. Louis, MO, USA, 1 mg/mL), DNase I (Sigma-Aldrich, St. Louis, MO, USA, 2U/mL), and hyaluronidase type V (Sigma-Aldrich, St. Louis, MO, USA, 0.5U/mL) at room temperature to obtain single-cell suspensions. The single-cell suspensions were then filtered, washed twice with phosphate-buffered saline (PBS), then seeded in a 12-well plate, at a concentration of 1.0×10^6 TILs/ml. The cells were thereafter cultured in X-VIVO medium (Muenchensteinerstrasse 38 CH-4002 Basel, Switzerland), supplemented with 7000 IU/ml recombinant human interleukin-2 (rhIL-2, Novartis, UK). This was considered Day 0. On Day 1, cell suspensions were removed and further purified via the Ficoll gradient. The purified bulk TIL culture was maintained, at a concentration of $1–2 \times 10^6$ cells/ml, in X-VIVO medium supplemented with 7000 IU/ml rhIL-2 until all other cells (including osteosarcoma cells) were eliminated and at least 5×10^7 TIL cells were achieved. This process took approximately 10 to 14 d. Finally, the cultured TIL cells were immediately assessed for large-scale expansion using anti-CD3 antibody (GE Healthcare Biosciences, Pittsburgh, PA, USA; 30 ng/ml) and 1000 IU/ml rhIL-2. Cultures showing expansion, to 5×10^9 TIL cells, were harvested. After detecting the immunophenotyping of TILs and confirming that the cell suspensions were free of bacterial and fungal contamination, negative for Mycoplasma, and contained < 5 Eu

endotoxin, TILs were infused back into patients.

2.5. Immunophenotyping of TILs

Examination of TILs immunophenotype was as described in our previous study [27]. Briefly, TIL immunophenotypes, after culture, were characterized by flow cytometry using anti-CD3 (Cat#: 555339, 1.5 μl/10^6 cells), anti-CD4 (Cat#: 557871, 2 μl/10^6 cells), anti-CD8 (Cat#: 563823, 2 μl/10^6 cells) , anti-CD56 (Cat#: 56275, 3 μl/10^6 cells), and anti-PD1 (Cat#: 561272, 5 μl/10^6 cells). The experiment was performed for 30 min on ice in darkness. The cells were washed once with, then resuspended in 400 μl PBS. Live and dead cells were distinguished using 7AAD, followed by running on a BD Fortessa (BD Bioscience), with Fluorescence minus one (FMO) included as a negative control. In addition, we performed FoxP3 staining, using intracellular staining protocol from BD bioscience [28]. Briefly, anti-CD3 and anti-CD4 were stained for 30 min on ice in dark. The TILs were then washed, fixed and permeabilized using the BD Fix Buffer I (Cat#: 557870, BD bioscience, USA) and Perm Buffer III (Cat#: 558050, BD bioscience, USA) according to the manufacturer's protocols. The specimens were thereafter washed three times with Perm Buffer III, then with anti-FoxP3 (Cat#: 560460, 5 μl/10^6 cells) for 30 min on ice in the dark. Finally, the cells were run on a Fortessa (BD Bioscience), with FMO also included as negative control. The PD1 expression by fresh $CD3^+CD8^+$TILs in tumors was also measured by flowcytometry. All flow cytometry data was analyzed using FlowJo software.

2.6. Statistical analysis

GraphPad Prism 7.0 and Spss17.0 software was used for statistical analysis. PFS and OS were calculated by Kaplan-Meier. PFS and OS were calculated from the start of immunotherapy. Univariable and multivariable Cox proportional hazards regression models were used to estimate hazard ratios along with associated confidence intervals and p-values. Other data used *t*-test or χ2-test. For all statistical analyses, significance is indicated as at least $p < 0.05$.

3. Results

3.1. Patient characteristics

A total of 60 patients met the aforementioned inclusion criteria and were therefore enrolled in this study. The patients were placed into 2 groups, with equal subjects per group. The average age of Group 1 and Group 2 were 21.2 years and 20.8 years, respectively. The most common sites of metastatic osteosarcoma in Group 1 and Group 2 seen in 80% vs. 73.33% and 20% vs. 26.67% were lung and other sites (liver and lymph node), respectively. The first line therapy of both group 1 and 2 is cisplatin (30 mg/m^2 d1-d3) epirubicin (45 mg/m^2 d1-d2) ifosfamide (2.0 g/m^2 d1-d5). The response rate of first line in group 1 and 2 is 39.8% vs 42.1%. there is no significant difference. The second line therapy of both group 1 and 2 is doxetaxel (75 mg/m^2 d1) and gemcitabine (1000 mg/m^2 d1, d8). There were no significant differences between the groups at presentation, with regards to demographic and clinical characteristics (Table 1).

3.2. TIL phenotypes

An average of 5.1×10^9 cells (range, 3.2–8.9 $\times$ 10^9) TILs were recorded at infusion time. These were primarily $CD3^+$ (93.78 ± 6.71%, N = 30), $CD8^+$ (68.77 ± 9.79%, N = 30), and $CD4^+$ T cells (26.81 ± 6.15%, N = 30), as well as NK (2.98 ± 2.76%, N = 30), and NKT cells (24.19 ± 8.97%, N = 30). PD-1 expression was found in 20.58 ± 7.66% of infused TILs, primarily on $CD8^+$ T cells (17.96% ± 4.57%, N = 30). Additionally, a subgroup of $Foxp3^+$ T regulatory cells (18.96% ± 7.79%) was also isolated from the $CD3^+CD4^+$ T cell population.

Table 1
The detailed baseline of the 60 patients.

Characteristics	Group1 (n = 30)	Group 2 (n = 30)	χ2	*P*-value
Age (years)				
< 20	24	23		
≥ 20	6	7	0.098	0.754
Gender				
Male	20	15		
Female	10	15	1.714	0.190
Site of primary tumor				
Femur and Tibia	23	22		
Others	7	8	0.089	0.766
Size of primary tumor (cm)				
≥ 5	14	20		
< 5	16	10	2.443	0.118
Histological classification				
Conventional	25	27		
Others	5	3	0.577	0.448
ECOG PS				
0	18	16		
1	12	14	0.271	0.602
Location of metastatic tumors				
Lung	24	22		
Others	6	8	1.000	0.542

3.3. Treatment-related toxicities

All patients exhibited an adverse event. Specifically, the most frequent grade 1 or 2 adverse events included fever, fatigue, anemia, anorexia, thrombocytopenia, rash, leukopenia and liver dysfunction. On the other hand, most frequent grade 3 or 4 adverse events were manifested as fever, anemia, and thrombocytopenia. Analysis of these adverse effects revealed no significant differences between the groups (Table 2), with none of these treatment-related adverse events found to be fatal. In fact, all of these events were controllable. For grade 1 or 2 adverse effects, they can resolve spontaneously within two days. When they had grade 3 or 4 adverse effects, support cares were given to patients. The patients with grade 3 and 4 fever were treated with non-steroidal anti-inflammatory drugs and resolved to a normal level within 48 h. The patients with grade 3 or 4 anemia or thrombocytopenia were treated with red blood cells or platelets transfusion and resolved to a normal level within 24 h. Overall, these findings indicated that TILs plus anti-PD1 therapy did not increase adverse effects compared to anti-PD1 therapy alone.

3.4. Treatment outcomes

In this study, 30 patients were treated with nivolumab while 30 others were subjected to TILs plus nivolumab. The deadline for the exercise was 1st June 2020. All patients in Group 1 died by the last follow-up evaluation, whereas 10 out of 30 survived in Group 2. ORR was observed in 2 (6.67%) patients under nivolumab therapy in Group 1, and 10 (33.3%) subjects treated with TILs plus nivolumab therapy (Group 2). Interestingly, we found 2 patients with complete response (CR) and 8 others with partial response (PR) Group 2. On the other hand, 2 and 1 patient exhibited ORR and PR, respectively in Group 1. Additionally, one patient in Group 2 exhibited vitiligo after TILs plus nivolumab therapy (Fig. 1A), albeit with CR (Fig. 1B). This patient was still alive by the cutoff date, recording a PFS and OS of 15.0 and 30.1 months, respectively. Furthermore, mPFS for Groups 1 and 2 were 3.8 and 5.4 months ($P = 0.0018$), respectively, whereas mOS were 6.6 and 15.2 months ($P < 0.0001$), respectively (Fig. 2A and B). Conclusively, these results indicated that TILs plus nivolumab therapy significantly increased the treatment efficacy for metastatic

Table 2
Distribution of adverse events.

	Group 1				Group 2			
Side effects	Grade 1	Grade 2	Grade 3	Grade 4	Grade 1	Grade 2	Grade 3	Grade 4
Fever	6	7	1	1	7	8	2	1
Fatigue	8	8	0	0	7	10	0	0
Anemia	3	5	1	1	4	5	0	1
Anorexia	5	2	0	0	6	2	0	0
Thrombocytopenia	3	3	1	0	3	4	1	0
Rash	3	2	0	0	2	2	0	0
Leukopenia	2	3	0	0	3	4	0	0
Liver dysfunction	2	2	0	0	3	3	0	0
Arthralgia	1	1	0	0	1	2	0	0
Nausea	1	0	0	0	1	0	0	0
Vitiligo	0	0	0	0	1	0	0	0

osteosarcoma patients.

3.5. Prognostic factors of combined TILs and anti-PD1 therapy

We examined potential prognostic factors in Group 2, owing to the high mPFS and mOS in subjects herein. Specifically, we analyzed gender, ages, ECOG PS, site and size of the primary tumor, histologic classification, location of metastatic tumors and the number of TILs infused, infusion of $CD8^+$TIL percentage, infusion of $CD8^+PD1^+$ TIL percentage and infusion of $CD4^+FoxP3^+$ TIL percentage. Univariate analysis revealed no significant differences in mPFS and mOS based on gender, age, ECOG PS, site and size of the primary tumor, histological classification, and location of metastatic tumors (Table 3). Conversely, more TILs and $CD8^+$TILs infused, as well as less $CD8^+PD1^+$ TILs and $CD4^+FoxP3^+$ TILs infused were significantly associated with increased mPFS (6.7 months vs. 3.8 months, $P < 0.0001$; 6.75 months vs. 4.3 months, $P = 0.0009$; 6.5 months vs. 3.85 months, $P = 0.0001$ and 6.3 months vs. 3.8 months, $P = 0.0082$) (Fig. 3A–D) and mOS (17.8 months vs. 8.5 months, $P = 0.0010$; 20.0 months vs. 8.75 months, $P = 0.0002$; 16.7 months vs. 8.25 months, $P < 0.0001$ and 18.7 months vs. 8.2 months, $P < 0.0001$) (Fig. 4A–D). Multivariate Cox proportional hazard model revealed that these differences were significant ($P < 0.0001$) for mPFS (Table 4) and mOS (Table 5). Overall, these findings suggested that more TILs and $CD8^+$TILs infused as well as less $CD8^+PD1^+$ TILs and $CD4^+FoxP3^+$ TILs infused may be potential prognostic factors for predicting clinical response to combined TILs and anti-PD1 therapy.

3.6. PD1 expression by fresh $CD3^+CD8^+$TILs in biopsy samples showed better prognostic effects in both Groups 1 and 2

We then measured PD1 expression by $CD3^+CD8^+$TILs from fresh tumor specimens and analyzed the correlation between $CD3^+CD8^+PD1^+$TILs with prognosis of osteosarcoma in both Groups 1 and 2. The percentages of $CD3^+CD8^+PD1^+$TILs in Groups 1 and 2 were 38.24% ± 2.98% (N = 30) and 37.86% ± 3.21% (N = 30), respectively. There were no significant differences between Groups 1 and 2 of PD1 expression on $CD3^+CD8^+$TILs. Based on the expression of PD1

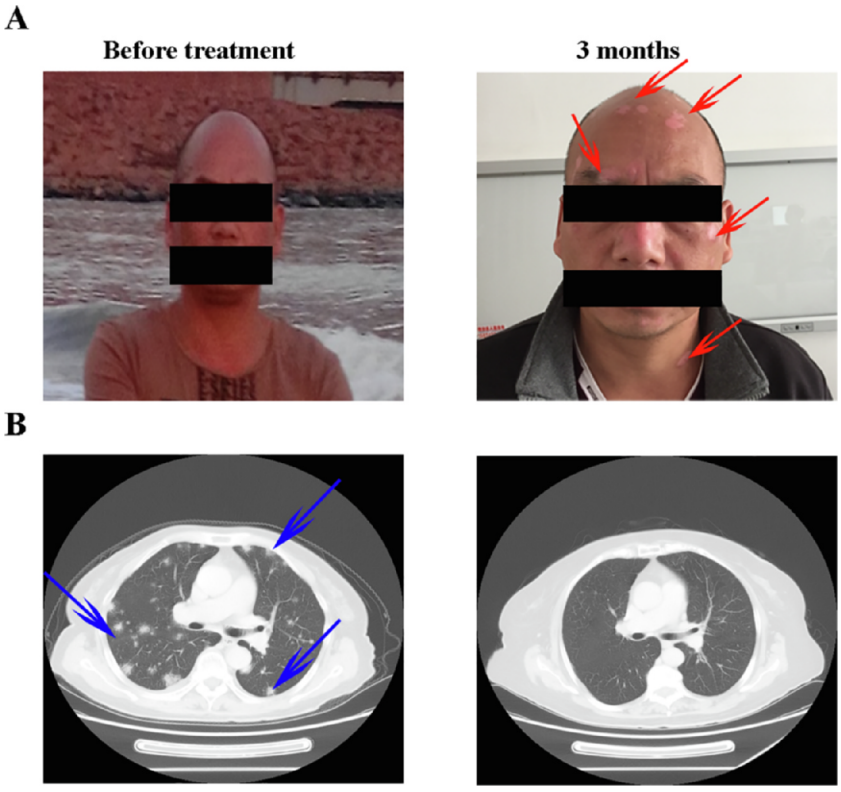

Fig. 1. Patients exhibiting a CR of multiple lung metastases and vitiligo after TILs plus anti-PD1 therapy. (A) Patient exhibited multiple vitiligo (the red arrows) in the face and neck after 12 weeks of therapy. (B) The patient achieved a CR of multiple lung metastases (the blue arrows) after 12 weeks of therapy. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

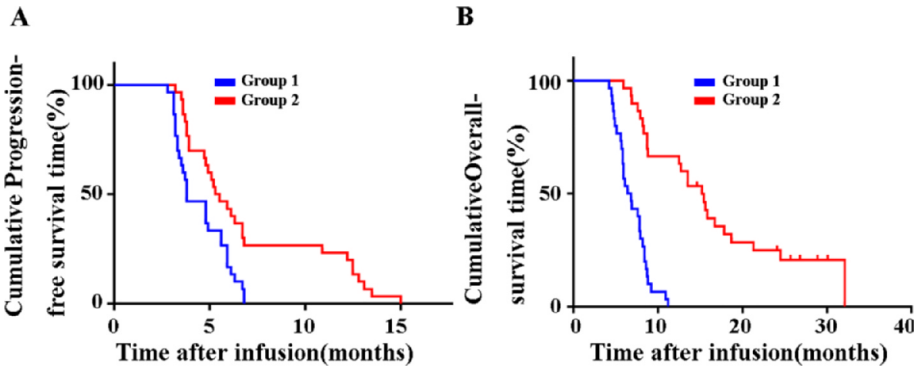

Fig. 2. Kaplan–Meier curves for PFS and OS of metastatic osteosarcoma patients in Group 1 (anti-PD1 chemotherapy) and Group 2 (TILs plus anti-PD1 therapy). (A) Patients' PFS curve. (B) Patients' OS curve.

Table 3
Univariate analysis of factors related to mPFS and mOS of patients in this study (N = 30).

Characteristics	mPFS (months)	*P*-value	mOS (months)	*P*-value
Gender				
Male	4.8		13.5	
Female	5.9	0.377	15.8	0.185
Age (years)				
≥20	6.7		15.4	
<20	5.1	0.154	15.2	0.951
Site of primary tumor				
Femur and Tibia	5.6		15.5	
Other	5.2	0.526	14.4	0.575
Size of primary tumor(cm)				
≥5	5.4		14.0	
<5	5.6	0.925	15.2	0.655
Histological classification				
Conventional	5.5		16.7	
Others	5.3	0.929	15.2	0.496
ECOG PS				
0	6.2		15.7	0.915
1	5.0	0.292	13.5	
Location of metastatic tumors				
Lung	6.5		15.2	
Others	5.0	0.167	14.5	0.995

on $CD3^+CD8^+$TILs, we divided patients into $PD1^{hi}$ (≥20%) and $PD1^{low}$ (<20%) in Groups 1 and 2 as previously described [27]. Interestingly, the ORR patients in Group 1 and Group 2 are both in $PD1^{hi}$ Group. The efficacy of anti-PD1 immunology relay on the expression of PD1 on immune cells and the expression of PDL1 on tumor cells. Consistent with previous studies, $PD1^{hi}$ (N = 16) had increased mPFS (5.9 months vs. 3.3 months, $P < 0.0001$) and mOS (8.4 months vs. 5.65 months, $P < 0.0001$) compared with $PD1^{low}$ (N = 14)in Group 1 (Fig. 5A and B). In addition, $PD1^{hi}$ (N = 19) also had increased mPFS (12.5 months vs. 4.7 months, $P < 0.0001$) and mOS (32.1 months vs. 8.8 months, $P = 0.0002$) compared with $PD1^{low}$ (N = 11) in Group 2 (Fig. 5C and D). Interestingly, multivariate analyses suggested that $PD1^{hi}$ was an independent prognostic element in osteosarcoma patients for mPFS and mOS (HR = 4.668, 95% CI 1.897 to 11.48, $P < 0.0001$ and HR = 4.908, 95% CI 1.967 to 12.24, $P < 0.0001$, receptively) in Group 1. Moreover, $PD1^{hi}$ was an independent prognostic element in osteosarcoma patients for mPFS and mOS (HR = 3.749, 95% CI 1.736 to 8.098, $P < 0.0001$ and HR = 5.032, 95% CI 2.243 to 11.29, $P = 0.0006$, receptively) in Group 2. Taken together, our results demonstrate that $PD1^{hi}$ in fresh TILs is a good prognostic factor to predict the treatment efficacy of anti-PD1 therapy or TILs and anti-PD1 therapy.

4. Discussion

Chemotherapy is the first line of treatment for metastatic osteosarcoma patients, with its 2-year EFS and OS rates reported to be 21% and 55%, respectively [29]. However, the efficacy of patients with disease progression, following this therapy, is limited using chemotherapy, sorafenib, and everolimus among others [30–33]. Recently, development of immunotherapy and its application in the field of oncology has generated progress in management of malignancies. Generally, this success is largely attributed to of the effect of immune-checkpoint inhibitors. However, previous studies have shown that durability and efficacy of anti-PD1 therapy varies across different malignancies [8,9,34–36]. For example, Pembrolizumab monotherapy was used in SARC028, the first multi-center, open-label, phase 2 study of

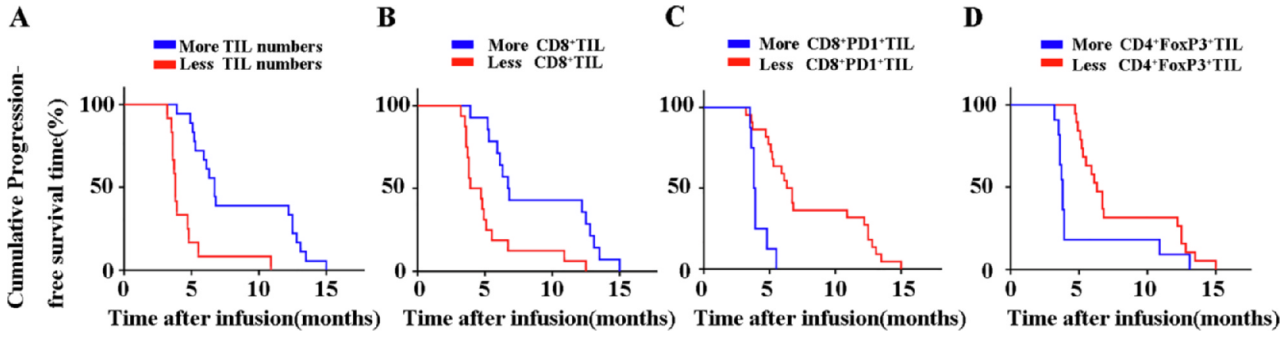

Fig. 3. Univariate analyses of more infusion of (TIL numbers and $CD8^+$TIL percentage) and less Infusion of ($CD8^+PD1^+$ TIL percentage and $CD4^+FoxP3^+$TIL percentage) based on PFS. PFS curve for; (A) Patients with more TIL numbers (≥5 × 10^9, blue line) and less TIL numbers (<5 × 10^9, red line); (B) Patients with more $CD8^+$TIL (≥60%, blue line) and less $CD8^+$TIL (<60%, red line); (C) Patients with more $CD8^+PD1^+$TIL (≥10%, blue line) and less $CD8^+PD1^+$TIL (<10%, red line); and (D) Patients with more $CD4^+FoxP3^+$TIL (≥20%, blue line) and less $CD4^+FoxP3^+$TIL (<20%, red line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

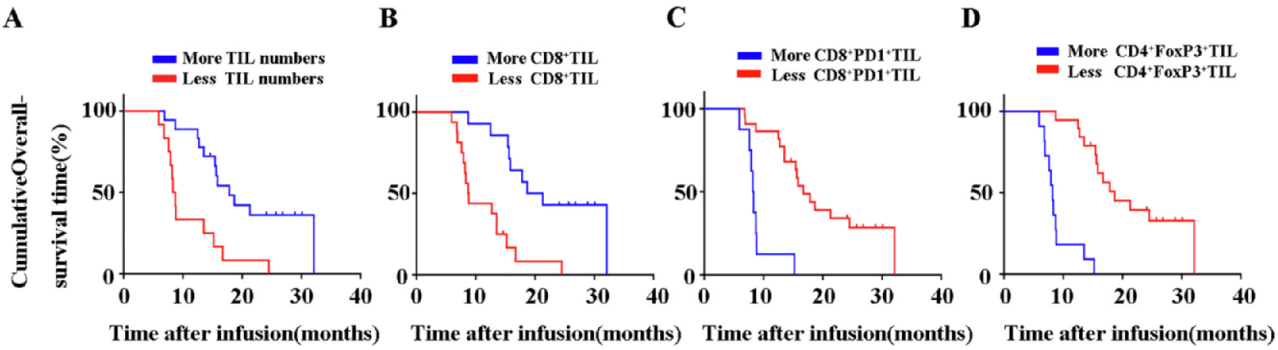

Fig. 4. Univariate analyses of more infusion of (TIL numbers and $CD8^+$TIL percentage) and less Infusion of ($CD8^+PD1^+$ TIL percentage and $CD4^+FoxP3^+$TIL percentage) based on OS. OS curve for; (A) Patients with more TIL numbers ($\geq 5 \times 10^9$, blue line) and less TIL numbers ($< 5 \times 10^9$, red line); (B) Patients with more $CD8^+$TIL ($\geq$60%, blue line) and less $CD8^+$TIL ($<$60%, red line); (C) Patients with more $CD8^+PD1^+$TIL ($\geq$10%, blue line) and less $CD8^+PD1^+$TIL ($<$10%, red line); and (D) Patients with more $CD4^+FoxP3^+$TIL ($\geq$20%, blue line) and less $CD4^+FoxP3^+$TIL ($<$20%, red line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Multivariate analysis (mPFS).

Parameters	Hazard ratio	95% confidence interval	*P*-value
Infusion of $CD8^+$ TIL numbers ($\geq 5 \times 10^9$ VS $< 5 \times 10^9$)	2.89	(2.21, 7.18)	$<$0.0001
Infusion of $CD8^+$ TIL percentage ($\geq$60% VS $<$60%)	2.66	(2.13, 6.53)	0.0003
Infusion of $CD8^+$ $PD1^+$TIL percentage ($\geq$10% VS $<$10%)	1.98	(1.45, 4.76)	0.0013
Infusion of $CD4^+$ $FoxP3^+$TIL percentage ($\geq$20% VS $<$20%)	2.23	(2.08, 6.09)	0.0008

Table 5
Multivariate analysis (mOS).

Parameters	Hazard ratio	95% confidence interval	*P*-value
Infusion of $CD8^+$ TIL numbers ($\geq 5 \times 10^9$ VS $< 5 \times 10^9$)	3.30	(2.58, 6.45)	0.0004
Infusion of $CD8^+$ TIL percentage ($\geq$60% VS $<$60%)	2.88	(2.24, 5.94)	0.0003
Infusion of $CD8^+$ $PD1^+$TIL percentage ($\geq$10% VS $<$10%)	5.89	(2.69, 12.38)	$<$0.0001
Infusion of $CD4^+$ $FoxP3^+$TIL percentage ($\geq$20% VS $<$20%)	4.74	(2.37, 9.87)	$<$0.0001

immune checkpoint blockade in patients with advanced soft-tissue or bone sarcoma. The findings therein indicated that the therapy was associated with clinically meaningful and sustained ORR in 18% of patients with soft-tissue sarcoma and 5% of those with advanced osteosarcoma [9]. To date, several studies have analyzed the use of anti-PD1 against osteosarcoma, although a 10% ORR has been found in non-selective patients, significantly lowering the effectiveness of anti-PD1 therapy to osteosarcoma [9,37]. The findings from these studies suggest that single-agent anti-PD1 therapy may not be an effective treatment strategy in these patients. Reports have also demonstrated that absence of TILs in the tumor microenvironment is one of the potential causes of tumor resistance to this type of immune checkpoint therapy [38]. Notably, TILs therapy has achieved successful clinical efficacy in treating melanoma since its first report by Rosenberg and colleagues more than 20 years ago [16]. The resulting success from this therapy has encouraged scientists globally to analyze the prospect of using it for management of other solid tumors, such as renal cell carcinoma, cervical and other epithelial cancers [20–23]. However, clinical response of TILs therapy to these tumors has generally been found to be lower than in melanoma. Notably, only a handful of studies, such as a pre-clinical trial [24], have reported the use of TILs in treating osteosarcoma. The current study, therefore, sought to expand knowledge previously generated by the aforementioned trial, by confirming and further characterizing the clinical activity of combined TILs and anti-PD1 therapy. Interestingly, we found promising antitumor effects and a satisfactory objective response, with clinical tumor regression observed in 10 out of the 30 patients (33.3%). However, anti-PD1 therapy achieved an ORR in 2 out of the 30 patients (6.67%), which was consistent with previous studies [9]. These findings indicated that combined TILs and anti-PD1 therapy may be a potential strategy for improving treatment of metastatic osteosarcoma.

Furthermore, effective treatment methods for patients with disease progression after first-line therapy are unavailable, necessitating the exploration of additional novel approaches. In the current study, we found that combined TILs and anti-PD1 therapy significantly increased ORR, PFS and OS of patients that exhibited disease progression after first-line therapy relative to anti-PD1 therapy alone. Mullinax et al. [39] used a combination of ipilimumab and tumor-infiltrating lymphocytes (TILs) in patients with metastatic melanoma and found the regimen to be feasible and well tolerated. In a previous study, our research group found that combined adjuvant chemotherapy and TILs therapy improved mDFS and mOS of osteosarcoma patients with poor response to neoadjuvant chemotherapy [27]. To date, however, it remains unclear whether combined TILs and anti-PD1 therapy has higher efficacy than anti-PD1 therapy alone. Despite being a single-center retrospective clinical study, our results suggest that combined TILs and anti-PD1 therapy may be an improved treatment method for metastatic osteosarcoma patients, with potentially high clinical response rates expected.

Previous studies have reported vitiligo in melanoma patients, with its development associated with excellent response to chemoimmunotherapy [40–42]. For example, several research groups have shown that many melanoma antigens are normal non-mutated genes recognized by TILs, which explains the increase in vitiligo incidence [43–48]. In the current study, we found one patient who exhibited vitiligo after TILs plus anti-PD1 therapy. Interestingly, this patient showed a CR with longer PFS and OS. Although the actual mechanisms through which osteosarcoma development causes breaking of immunologic tolerance in normal antigens are unknown, it is possible that it may involve overexpression of these antigens in cancer cells or unique inflammatory reactions and cytokines present at sites of tumor growth. Therefore, exploring the mechanisms between vitiligo and osteosarcoma regression should form the basis for future studies.

Numerous tumors continue to grow despite TILs infiltration into the tumor stroma. PD1 expression by TILs is thought to be one of weakened

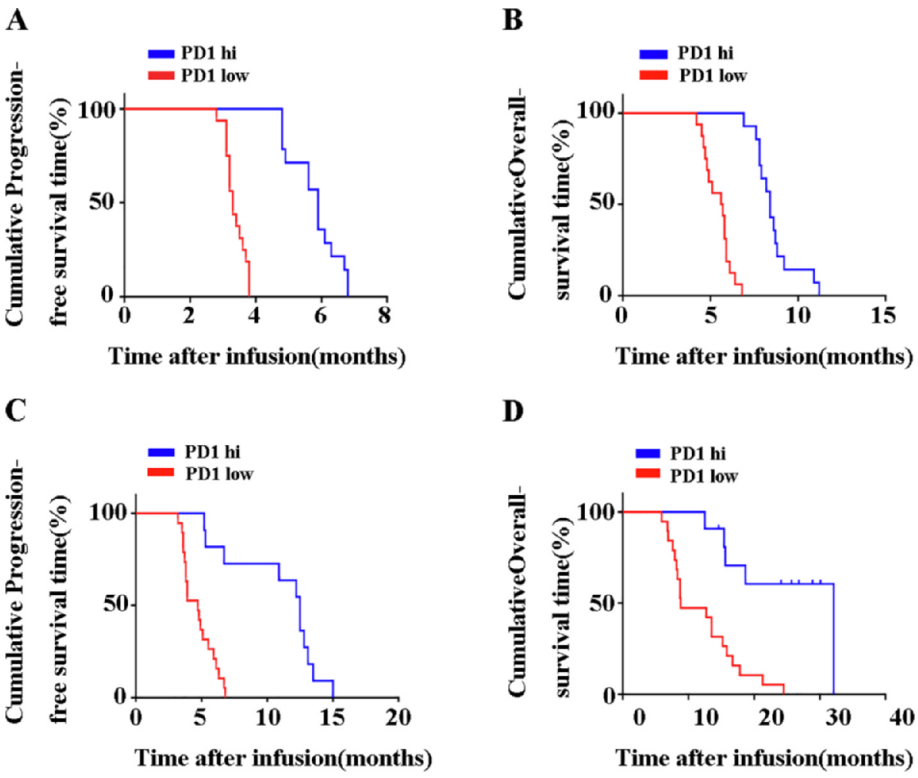

Fig. 5. Kaplan–Meier curves for PFS and OS of osteosarcoma patients according to $PD1^{hi}$ and $PD1^{low}$ in Group 1 and Group 2. (A) The PFS curves based on $PD1^{hi}$ and $PD1^{low}$ in Group 1. (B) The OS curves based on $PD1^{hi}$ and $PD1^{low}$ in Group 1. (C) The PFS curves based on $PD1^{hi}$ and $PD1^{low}$ in Group 2. (D) The OS curves based on $PD1^{hi}$ and $PD1^{low}$ in Group 2.

antitumor immune response [49]. In addition, immune checkpoint inhibitors, used as cancer therapies, reverse T cell tolerance and mediate a proliferative response of TILs by blocking inhibitory interactions between tumor cells and infiltrating T cells, thus allowing for an antitumor immune response. However, the origin of this response has not yet been established because chronic activation promotes terminal differentiation or exhaustion of TILs [50,51]. Immunotherapies aim at boosting anti-tumor immune responses and induce durable tumor control. The current immunotherapeutic regimens mainly include the use of adoptive cell therapy (immune accelerator) and checkpoint inhibitors (immune brake), which have yielded unprecedented clinical benefits in several types of tumor. Moreover, inhibiting the PD-1/PD-L1 pathway has been shown to release the brake in T lymphocytes, thereby restoring antitumor immune response and eliminate the tumor [52]. In the current study, we observed a subpopulation of $PD\text{-}1^{+}$T lymphocytes in the cultured TILs, suggesting that a PD-1 blockade may significantly increase cytotoxicity of TILs. Similarly, recent studies have reported that blocking the PD-1 pathway significantly increased antitumor effects of adoptive T lymphocyte immunotherapy performed with chimeric antigen receptor (CAR) T cells [53]. In addition, Mifarmutide, a chemotherapeutic agent was shown to increase immune-cell infiltration into osteosarcoma metastases, a crucial step for improving efficacy of anti-PD1 therapy [54]. Another report indicated that pembrolizumab-activated autologous DC-CIK cells exerted encouraging antitumor activity in advanced solid tumors [55]. These studies suggest that a combined TILs and anti-PD1 therapy may generate a synergistic and reciprocal increase in efficacy.

In the current study, univariate and multivariate analyses indicated that patients with more TILs and $CD8^{+}$TILs infused exhibited better PFS and OS. Conversely, more $CD8^{+}PD1^{+}$ TILs infused resulted in poor PFS and OS. These findings suggest that combined TILs and anti-PD1 therapy potentially increase survival times in metastatic osteosarcoma patients. Traditionally, $PD1^{hi}$ is a poor prognostic factor for tumor patients, while it is a good prognostic factor, when patients received anti-PD1 therapy [27]. Consistent with this, in this study, we observed that metastatic osteosarcoma patients with higher PD1 expression by fresh $CD3^{+}CD8^{+}$TILs in the tumor is a good prognostic factor when they received anti-PD1 therapy. In addition, higher PD1 expression by fresh $CD3^{+}CD8^{+}$TILs in the tumor is also a good prognostic factor when patients received TILs and anti-PD1 therapy. Although, we roughly divided patients into $PD1^{hi}$ and $PD1^{low}$ based on the percentage of PD1 on $CD3^{+}CD8^{+}$ TILs, our results still suggest that higher PD1 expression represent a good prognostic factor. We speculate that $CD8^{+}PD1^{+}$TILs in the tumor microenvironment may be exactly the population that is being chronically exposed to relevant tumor antigens but become exhausted. This finding is consistent with fresh $CD8^{+}PD1^{+}$TILs cannot make IFN-γ by stimulation with PMA-ionomycin in vitro [56]. However, clinical confirm that transfusion of cultured TILs have tumor reactivity after in vitro expansion mediates the regression of metastatic melanoma [16,17,56]. In the future, selectively cultured $PD1^{+}$ TILs may be optimal cells therapy in clinical applications and can yield higher anti-tumor reactivity.

Regulatory T cells (Tregs) have been shown to suppress T cell-mediated host immune response against self- and nonself-antigens [57–59]. In fact, studies have described a negative relationship between peripheral $CD4^{+}FoxP3^{+}$ regulatory T cell levels and clinical response to adoptive immunotherapy of human cancer [28], indicating that Tregs may play an inhibitory role in adoptive immunotherapy of human tumors. In a murine model, lymphodepletion by chemotherapy or chemoradiation seemed to enhance the antitumor effects of transferred T cells *in vivo* via several mechanisms. These mechanisms include elimination of suppressive T-regulatory lymphocytes, and cellular “sinks” for homeostatic cytokines, such as IL-7 and IL-15, as well as engagement of toll-like receptors on antigen-presenting cells following damage to the integrity of the gut epithelial lining [28]. In our study, we found that more $CD4^{+}FoxP3^{+}$ TILs infused resulted in poor PFS and OS, which was consistent with these studies.

One limitation of our study was that it employed a single-center, and retrospective clinical design, but not a randomized clinical trial. Although, we cannot exclude potential bias that might exist when patients are allowed to choose between anti-PD1 therapy or TILs and anti-PD1 therapy. Our success in combination of TILs and anti-PD1 therapies

for metastatic osteosarcoma patients still suggested that this combination may be a better treatment for these patients. However, whether TILs should be administered in combination with anti-PD-1 or as a single treatment option is still unclear for metastatic osteosarcoma. Further studies should be conducted to clarify this. Future studies are also needed to validate the characteristics of cultured TILs as biomarkers for predicting response to this combined immunotherapy.

5. Conclusion

Summarily, our results indicated that a combination of TILs and anti-PD1 therapy provides significant clinical implications to osteosarcoma patients. Taken together, these findings lay a foundation for future use of combined immunotherapies in metastatic osteosarcoma. Further studies are needed to validate these findings and characterize activity of TILs plus anti-PD1 therapy.

Author statement

Chao Wang did the experiment, analyzed the data and wrote the manuscript. Ming Li and Rong Wei collected and recorded clinical data. Junlong Wu designed experiment and edited the manuscript.

Acknowledgements

We thank all the enrolled patients and their cooperation. We thank all the staff of GMP lab for the preparation of TILs.

Competing interests

The authors declare that they have no competing interests.

Ethical approval

All procedures performed 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.

Informed consent

Institutional review board approval and data sharing agreements were obtained from all participating institutions. All data were anonymized.

Funding statement

There are no funding associated with this manuscript.

Data availability statement

All data generated in the study are included in the present article.

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