

Evaluation of Indoleamine 2,3-Dioxygenase (IDO) Expression in Osteosarcoma: Insights From a 10-Year Retrospective Cohort

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Introduction: Osteosarcoma, a prevalent bone malignancy in children and adolescents, is currently treated through surgical resection and chemotherapy. Advancements in cancer research are targeting immune checkpoint molecules, such as indoleamine 2,3-dioxygenase, to advance the development of immunotherapy. However, the scarcity of research on IDO in osteosarcoma results in an absence of comprehensive data, highlighting the conflicting findings surrounding IDO's role in various cancers. Our study aims to explore IDO expression in primary tumors and metastatic lesions among osteosarcoma patients, investigating its association with clinicopathological characteristics and assessing its impact on survival outcomes.

Methods: 150 patients diagnosed with osteosarcoma were selected between 2009 and 2019 from the Shaukat Khanum Memorial Cancer Hospital and Research Centre, Lahore, Pakistan. FFPE tissue samples of primary tumors and metastatic lesions were retrieved to conduct immunohistochemical analysis. Moreover, the clinicopathological data of these patients were gathered from the hospital information system.

Results: Out of 150 patients, primary tumors were accessible for 134 individuals, while metastatic lesions were available for 49 patients. IDO expression was identified in 9 (6.71%) primary tumors and 2 (4.08%) metastatic lesions among osteosarcoma patients. Furthermore, 3 patients exhibited high expression (27.3%), while 8 displayed low IDO expression (72.7%).

Conclusion: Our comprehensive study findings indicate that most osteosarcoma patients do not exhibit expression of IDO. This absence of expression aligns with the characteristic “cold” tumor microenvironment observed in osteosarcoma. Further investigations are imperative to provide deeper insights into the intricacies of this immunomodulatory factor in the context of osteosarcoma.

Keywords: osteosarcoma, indoleamine 2, 3-dioxygenase, biomarkers, metastatic lesions, immunohistochemistry

Introduction

Osteosarcoma, the predominant bone malignancy, primarily affects children and adolescents, with another susceptible group being individuals aged 60 and above.¹⁻⁴ Recent data from the US National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER) program reported 3970 new cases of bone and joint cancers, resulting in 2170 deaths.⁵ Despite substantial focus on osteosarcoma's epidemiology, prevalence, and prognostic factors,⁶⁻¹³ a global lack of comprehensive data persists, particularly in regions like Pakistan.¹⁴⁻¹⁶ The current treatment involves surgical resection and chemotherapy, with advanced-stage patients undergoing radiation therapy and metastasectomy for resectable pulmonary disease.¹⁷⁻²⁰ Despite efforts elevating 5-year survival rates to 70% for localized osteosarcoma, progress remains limited for progressive and metastatic cases.^{1,21-26} Clinical trials consistently report lower-than-expected survival rates in metastatic osteosarcoma patients.^{25,26}

Osteosarcoma, characterized by significant heterogeneity, manifests a complex immune environment, often termed a “cold” tumor.²⁷ To enhance immunotherapy’s efficacy, decoding the regulatory mechanisms governing anti-cancer immunity in osteosarcoma is crucial.²⁸ Recent advancements target immune checkpoint molecules, including potential intervention in indoleamine 2,3-dioxygenase (IDO), a checkpoint protein pivotal in creating an immunosuppressive tumor microenvironment. IDO, an intracellular enzyme catalyzing tryptophan, not only modulates immune responses but also plays a pivotal role in tumorigenesis.²⁹ IDO catalyzes the breakdown of L-tryptophan into kynurenine and other metabolites, which reduces tryptophan availability and weakens T cell activity. Kynurenine can induce T cell apoptosis and promote immune tolerance by enhancing regulatory T cell (T-reg) differentiation.²⁹ IDO overexpression in tumors creates an immunosuppressive environment, aiding tumor survival and immune evasion.²⁹ Dysregulation of IDO expression thus supports tumor progression by contributing to immune suppression and tolerance. The role of IDO has been extensively studied in various cancers including breast cancer, esophageal cancer, endometrial cancer, hepatocellular carcinoma, and pancreatic cancer.^{30–34} IDO’s role in osteosarcoma was first reported by Urakawa et al, revealing its impact on clinical outcomes.³⁵ Recent studies show promise in combining chemotherapeutic agents and IDO inhibitors for osteosarcoma patients.^{36,37} However, limited research on IDO in osteosarcoma,^{38,39} results in a lack of comprehensive data. Conflicting findings regarding the role of IDO in different cancers and the cell types expressing this immunosuppressive mediator underscore context-dependent, and multi-dimensional mechanisms.⁴⁰ Our study aims to explore IDO expression in primary tumors and metastatic lesions among osteosarcoma patients, investigating its association with clinicopathological characteristics and assessing its impact on survival outcomes.

Material and Methods

Patients and Data

We conducted a retrospective cross-sectional analysis involving osteosarcoma patients registered at Shaukat Khanum Memorial Cancer Hospital and Research Centre (SKMCH&RC) in Pakistan. The study included 150 patients diagnosed with osteosarcoma between 2009 and 2019. Formalin-fixed, paraffin-embedded (FFPE) blocks of primary tumors and metastatic lesions were sourced from the pathology department at SKMCH&RC. The primary tumors were derived from diagnostic specimens, ensuring that all specimens were treatment-naïve. Analysis was conducted using metastatic lesions from the same patients for whom primary tumors were accessible. Comprehensive patient data, encompassing demographics, pathological and radiological features, and treatment details, were extracted from the electronic medical records system of SKMCH&RC. For survival analysis, patient follow-up continued until January 2024. The study received approval from the institutional review board (IRB) of SKMCH&RC (EX-05-06-23-06), with the IRB granting a waiver of informed consent due to the minimal risk posed to patients’ rights, safety, and well-being, considering the data and FFPE samples were derived from archived records. Our study complies with the declaration of Helsinki.

IDO Expression Analysis by Immunohistochemistry

FFPE sections of primary tumors and metastatic lesions were cut at a thickness of 4 µm. The immunohistochemical analysis for IDO involved staining with an anti-IDO antibody (Cell Signaling Technology, Massachusetts, the United States; #86630). Heat-mediated epitope retrieval using a Tris-EDTA buffer was performed, and immunoreactivity was detected using the Dako EnVision kit (K5007). Normal reactive human lymph nodes served as a positive control. Slides were deparaffinized, and antigen retrieval was performed concurrently with the target retrieval solution (#GV805 Dako). The visualization of slides was conducted with an optical microscope (Provis AX-70, Olympus, Melville, NY). Two pathologists blindly numerically scored the slides through histopathologic evaluation as described earlier.³³

Statistical Analysis

Statistical analysis was carried out using SPSS software (version 26.0; SPSS, Chicago, IL, USA). Percentages (proportions) were used for categorical variables while mean and standard deviation was used for continuous variable. Bivariate analysis was done using chi-square or fisher exact test (when necessary). For continuous explanatory variable such as

age, one way ANOVA was performed. The Kaplan-Meier method was used to estimate overall survival and survival differences. Statistical significance was defined as a two-tailed p-value of less than 0.05.

Results

Clinicopathological Profiles of Osteosarcoma Patients

The average age at the time of osteosarcoma diagnosis was 14 years, with a notable variability of ± 17.66 years and a wide age range spanning from 6 to 54 years. The study population comprised 60.7% males and 39.3% females. Predominantly, patients diagnosed with osteosarcoma hailed from the Punjab region, followed by Khyber Pakhtunkhwa. Additionally, the study encompassed 22 patients from Afghanistan who were undergoing treatment at SKMCH&RC, highlighting the regional diversity of the patient cohort.

Within the cohort of 150 patients, osteosarcoma emerged as the predominant diagnosis, accounting for 130 cases. Further classification unveiled specific subtypes, including chondroblastic osteosarcoma (n=16), telangiectatic osteosarcoma (n=2), small cell osteosarcoma (n=1), and parosteal osteosarcoma (n=1) among the remaining 20 cases. A histological representation of osteosarcoma primary tumors and metastatic lesions is shown in [Figure 1](#). The radiological depiction of osteosarcoma tumors and metastatic lesions is illustrated in [Figure 2](#). The neutrophil-lymphocyte ratio (NLR) was assessed to evaluate immune responses in patients with osteosarcoma.⁴¹ In our cohort, most patients (n=97) had an NLR within the normal range of 0.7 to 3. Two patients had an NLR below 0.7, while 35 patients exhibited an NLR above 3. There was a significant association between NLR and T-stage of the osteosarcoma patients. However, in this study, we were unable to identify any significant association between NLR and factors such as tumor size, post-chemotherapy necrosis grade, IDO expression, or patient survival ([Supplementary Table 1](#)). In the context of treatment, among the 140 patients who underwent neo-adjuvant chemotherapy, responses exhibited variability. Specifically, 15 patients displayed a grade I response, 84 exhibited a grade II response, and 31 demonstrated a grade III

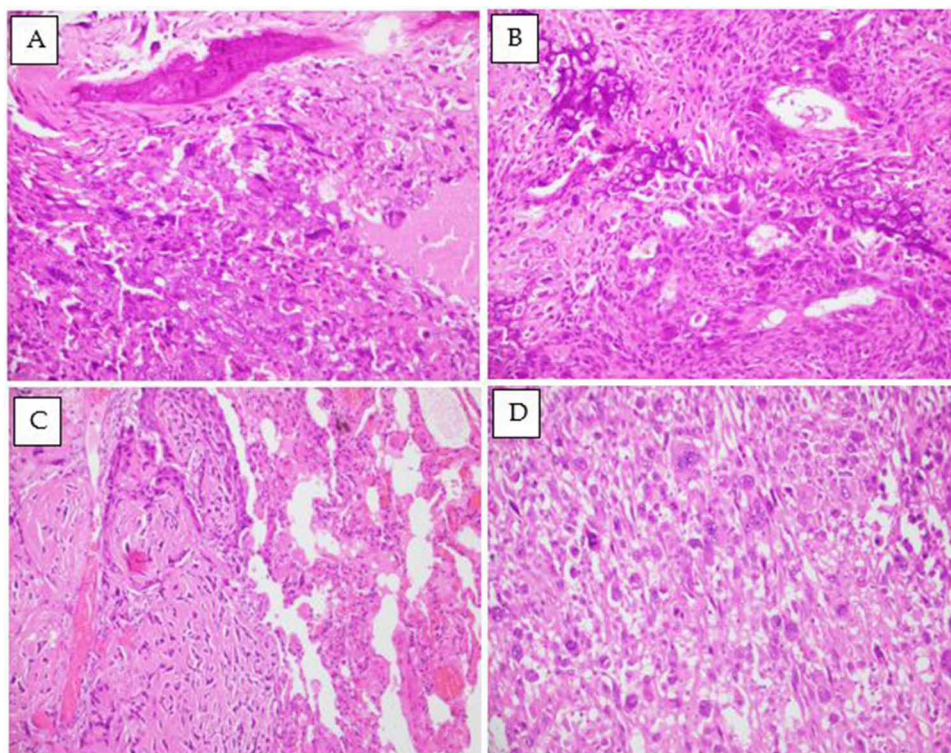


Figure 1 Histological representation of primary osteosarcoma tumors and metastatic lesions. **(A)** Fragments of bone with a malignant neoplasm composed of sheets of oval to spindle markedly pleomorphic cells with vesicular nuclei. Areas of necrosis are also noted. (H&E; 20X) **(B)** Sheets of oval to spindle pleomorphic cells with lace-like osteoid formation and tumor giant cells. (H&E; 20X) **(C+D)** Metastatic osteosarcoma in lung: **(C)** Lung alveoli with adjacent sheets of atypical oval cells. **(D)** Few high-grade areas with markedly pleomorphic cells and tumor giant cells. (H&E; 20X).

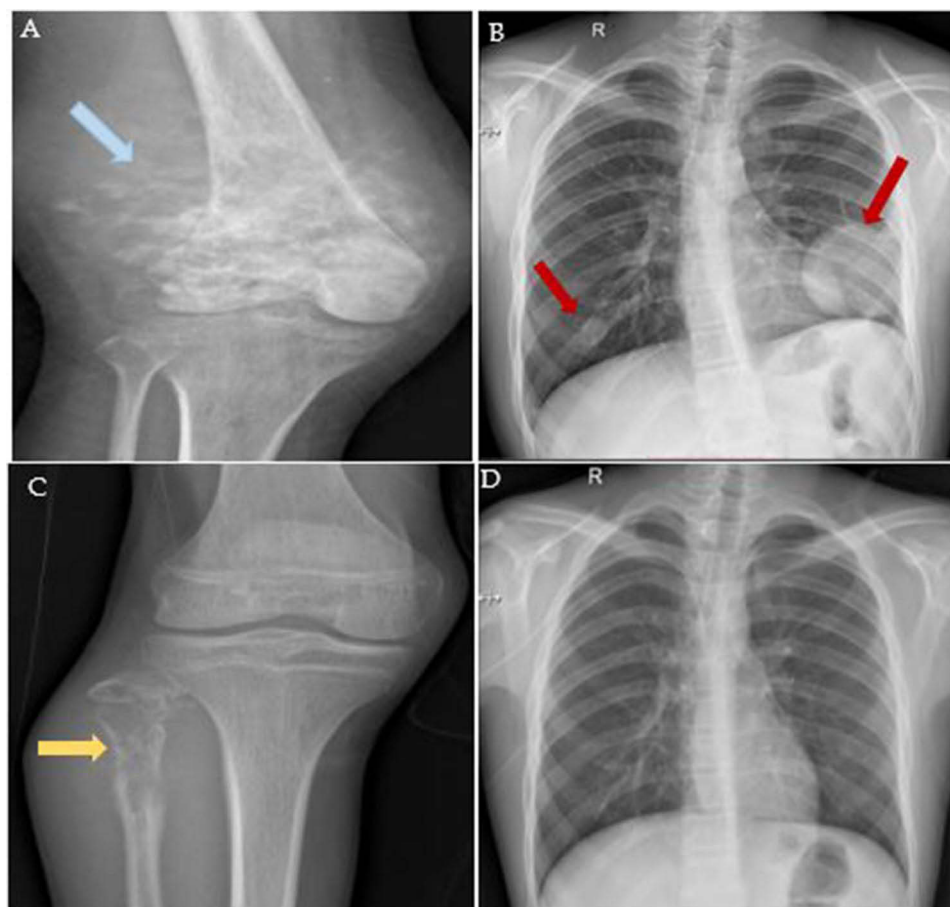


Figure 2 Radiological findings of osteosarcoma tumors. (A) a 22-year-old male patient exhibits an aggressive osseous lesion with a calcified soft tissue component at the distal end of the femur, confirmed by biopsy as osteosarcoma (indicated by the blue arrow). (B) the same patient shows pulmonary metastasis evident on the initial radiograph at the time of diagnosis (highlighted by red arrows). (C) displays an 8-year-old male patient with biopsy-confirmed osteosarcoma of the proximal fibula (depicted by the yellow arrow). (D) Remarkably, this patient did not develop pulmonary metastasis even after a 6-year follow-up chest radiograph.

response to chemotherapy. While only 1 patient exhibited a grade IV response, highlighting the range of treatment outcomes within the cohort (Table-1). The median survival duration was 69 months, with corresponding overall survival rates of 62% at three years and 54% at five years (as shown in Figure 3).

Immunohistochemical Analysis of IDO Expression

Within the cohort comprising 150 patients, FFPE blocks of primary tumors were accessible for 134 individuals, while blocks of metastatic lesions were obtainable for 49 patients. The immunohistochemical analysis revealed IDO expression in 9 (6.71%) patients' primary tumors and 2 (4.08%) patients' metastatic lesions. Out of the 9 patients who exhibited IDO expression in their primary tumors, metastatic lesions were accessible for two patients; however, both displayed no IDO expression in the metastatic sites. Likewise, among the two patients with IDO expression in their metastatic lesions, the primary tumor of one patient was accessible, but it did not demonstrate any IDO expression. Further categorization within the IDO-positive subgroup demonstrated high expression in 3 patients and low expression in the remaining 6 patients. Contrastingly, the majority of the cohort, totaling 125 patients, did not exhibit any discernible expression of IDO, as elucidated in detail in Table 1. We found no association of IDO expression with age, sex, histology or clinical outcome of osteosarcoma patients.

A comprehensive analysis of clinicopathological features in the 11 osteosarcoma patients expressing IDO is shown in Table 2. The majority of the tumors had histology of osteosarcoma while one patient had parosteal osteosarcoma. The absence of pulmonary metastasis at diagnosis is a consistent feature among the cases. The IDO expression levels

Table 1 Patients and Tumor Characteristics by IDO Low and High

Variables	Characteristics	IDO negative N = 125 (83.3%)	IDO low N = 6 (4.0%)	IDO high N = 3 (2.0%)	P-value
Age (years)					0.40
	Mean \pm SD*	17.1 \pm 7.3	13.8 \pm 2.7	13.8 \pm 4.9	
Gender					0.29
	Male	78 (62.4)	2 (33.3)	2 (66.7)	
	Female	47 (37.6)	4 (66.7)	1 (33.3)	
Diagnosis					0.12
	Parosteal osteosarcoma	-	-	1 (33.3)	
	Small cell osteosarcoma	1 (0.8)	-	-	
	Telangiectatic osteosarcoma	2 (1.6)	-	-	
	Chondroblastic osteosarcoma	15 (12.0)	-	-	
	Osteosarcoma	107 (85.6)	6 (100.0)	2 (66.7)	
Laterality					0.73
	Left	54 (43.2)	4 (66.7)	1 (33.3)	
	Right	64 (51.2)	2 (33.3)	2 (66.7)	
	Unknown	7 (5.6)	-	-	
Size (cm)					0.13
	Mean \pm SD*	11.5 \pm 5.9	6.1 \pm 4.5	11.8 \pm 3.5	
Necrosis grade (post-chemo)					0.29
	1	11 (8.8)	1 (16.7)	1 (33.3)	
	2	72 (57.6)	2 (33.3)	1 (33.3)	
	3	25 (20.0)	2 (33.3)	-	
	4	1 (0.8)	-	-	
	Unknown	16 (12.8)	1 (16.7)	1 (33.3)	
Lymph-vascular invasion					0.63
	Negative	63 (50.4)	2 (33.3)	2 (66.7)	
	Positive	2 (1.6)	-	-	
	Unknown	60 (48.0)	4 (66.7)	1 (33.3)	
pT stage					0.73
	pT0	2 (1.6)	-	-	
	pT1	33 (26.4)	3 (50.0)	1 (33.3)	
	pT2	83 (66.4)	3 (50.0)	2 (66.7)	
	Unknown	7 (5.6)	-	-	
Status					0.97

(Continued)

Table 1 (Continued).

Variables	Characteristics	IDO negative N = 125 (83.3%)	IDO low N = 6 (4.0%)	IDO high N = 3 (2.0%)	P-value
	Alive	42 (33.6)	2 (33.3)	1 (33.3)	
	Death	53 (42.4)	2 (33.3)	1 (33.3)	
	Unknown	30 (24.0)	2 (33.4)	1 (33.4)	

Notes: *Standard deviation. Percentages (proportions) were used for categorical variables while mean and standard deviation were used for continuous variables. Bivariate analysis was done using chi-square or fisher exact test (when necessary). For continuous explanatory variables such as age, one way ANOVA was performed. The Kaplan-Meier method was used to estimate overall survival and survival differences.

demonstrate variability, with cases exhibiting both low and high expressions, as shown in [Figures 4 and 5](#). Interestingly, patient diagnosed with parosteal osteosarcoma, showed high IDO expression while maintaining a status of being alive.

Discussion

Osteosarcoma is considered as a “cold tumor”, displaying insufficient local immune activation and inadequate recognition of cancer cells, even when immune cells are present within the tumor microenvironment.^{42–44} The immune microenvironment in osteosarcoma constitutes a complex system characterized by significant heterogeneity.⁴⁵ This complexity facilitates tumor immune escape and tumorigenesis, leading towards metastasis.⁴⁵ Understanding and addressing the immune markers is crucial for devising innovative therapeutic strategies to enhance immune responsiveness and counteract the challenges posed by osteosarcoma’s immune microenvironment. Several studies have demonstrated the roles of immune markers in cancer development and progression including osteosarcoma. Among these markers, IDO has been extensively investigated in breast cancer, colorectal cancer, hepatocellular carcinoma, ovarian cancer, head and neck cancers, pancreatic cancer and glioblastomas.^{30,33,34,46–48} In the context of cancer, IDO expression and activity have been observed not only in tumor cells but also within the tumor-associated stroma, which includes endothelial cells, immune cells, fibroblasts, and mesenchymal cells.⁴⁹ Furthermore, IDO expression has been detected in peripheral blood, highlighting its systemic involvement in immune regulation.⁴⁹ Increased IDO expression can be targeted through IDO inhibitors to regulate the immune responses in cancer. Several IDO inhibitors are part of clinical trials as co-therapy of various cancers. These include Epcadostat, Indoximod, KHK2455 and BMS-986205.⁵⁰

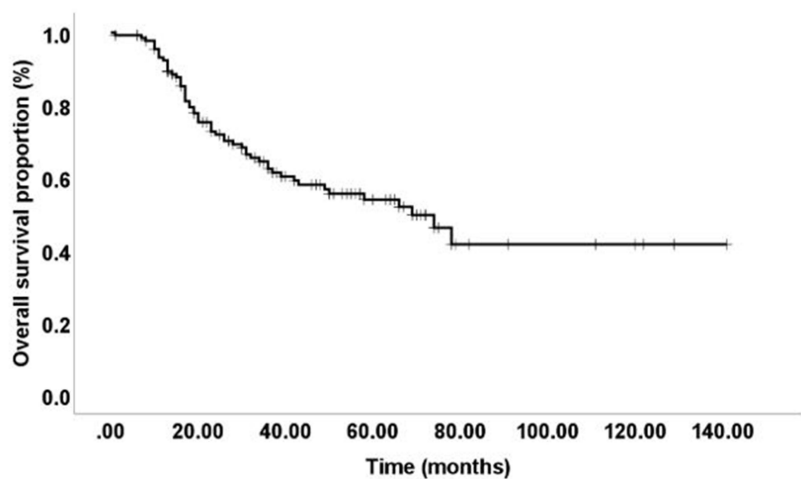


Figure 3 Survival analysis of osteosarcoma patients. The median survival duration of osteosarcoma patients was 69 months. Furthermore, the proportions of overall survival at three and five years were 62% and 54%, respectively.

Table 2 Clinicopathological and Radiological Features of Osteosarcoma Patients With IDO Expression

Case	Gender	Age (Y)	Tumor Site	Histology	Neutrophil Lymphocyte Ratio	Pulmonary Metastasis at Diagnosis	Necrosis Grade (post-chemo)	Status	IDO
01	F	11	Lower Limb	Osteosarcoma	1.81	-ve	2	UNK	Low
02	F	12	Lower Limb	Osteosarcoma	2.18	-ve	2	UNK	Low
03	M	16	Lower Limb	Osteosarcoma	1.70	-ve	1	Alive	Low
04	M	9	Upper Limb	Osteosarcoma	2.14	-ve	UNK	UNK	High
05	F	17	Lower Limb	Osteosarcoma	3.40	-ve	3	Alive	Low
06	F	15	Lower Limb	Osteosarcoma	1.78	-ve	1	Deceased	Low
07	M	14	Lower Limb	Osteosarcoma	2.94	-ve	2	Deceased	High
08	M	11	Upper Limb	Osteosarcoma	1.58	-ve	3	Deceased	Low
09	F	18	Lower Limb	Parosteal Osteosarcoma	2.89	-ve	1	Alive	High
10	M	41	Lower Limb	Osteosarcoma	2.59	-ve	UNK	UNK	Low
11	F	19	Lower Limb	Osteosarcoma	3.25	-ve	1	UNK	Low

In the current study, we investigated IDO expression in primary tumors and pulmonary metastases of osteosarcoma patients using immunohistochemistry. Intriguingly, most patients did not exhibit IDO expression. Among those expressing IDO, 8 demonstrated low expression levels, while 3 exhibited high expression. Notably, within our dataset, a patient with the highest IDO expression (score =9) was observed to have survived. Toda et al noted comparable IDO expression patterns in their study, with 12 out of 56 osteosarcoma patients exhibiting IDO expression in primary tumors, while 11 patients displayed IDO expression in metastatic lesions.³⁸ In contrast, Urakawa et al observed a higher prevalence of IDO expression, with 45 out of 47 osteosarcoma patients demonstrating this expression in their cohort.³⁵ Ligon et al examined IDO expression in tumor-infiltrating lymphocytes (TILs) and discovered elevated levels of IDO expression at the interface of pulmonary metastases, surpassing the levels observed in primary tumors of osteosarcoma patients.³⁹

Wu et al observed the poor immune cell infiltration within the microenvironment of osteosarcoma. This deficiency, coupled with diminished T cell activity, a scarcity of immune-stimulating neoantigens, and the existence of various immune-suppressing pathways, collectively converge to dampen the efficacy of immunotherapy responses.⁵¹ They categorized three distinct immune subsets characterized by low (C1), intermediate (C2), and high (C3) levels of immune infiltrate.⁵¹ Tumors with lower immune infiltrate exhibited a scarcity of various immune cell types,⁵¹ indicating an inadequate cell-mediated anti-tumor immune response within the C1 subset of patients. Similarly, Ligon et al demonstrated that the primary osteosarcoma tumors were devoid of meaningful immune infiltrates and were unable to generate effective anti-tumor immune responses.³⁹

IDO is an immunoregulatory enzyme which is induced in the tumor microenvironment through spontaneous inflammation and T cell activation.⁵² Although IDO suppresses the immune system, the presence of IDO indicates that an immune response was mounted against the tumor. In our cohort, the absence of IDO expression in osteosarcoma patients suggests an impaired ability of the immune system to initiate an anti-tumor response. Conversely, the small subset of patients expressing IDO may signify a more effective anti-tumor immune response. This could be a contributing factor to the survival of one patient with the highest IDO expression in our cohort. In earlier investigations, we identified high IDO expression in patients with breast cancer and hepatocellular carcinoma from Pakistan.^{30,33} The variability in IDO expression across diverse solid tumors underscores its context-dependent and multi-dimensional mechanisms. These distinct patient subsets expressing immune markers warrant further investigation to establish personalized immune profiles, paving the way for patient-specific as well as tumor-specific immunotherapies.

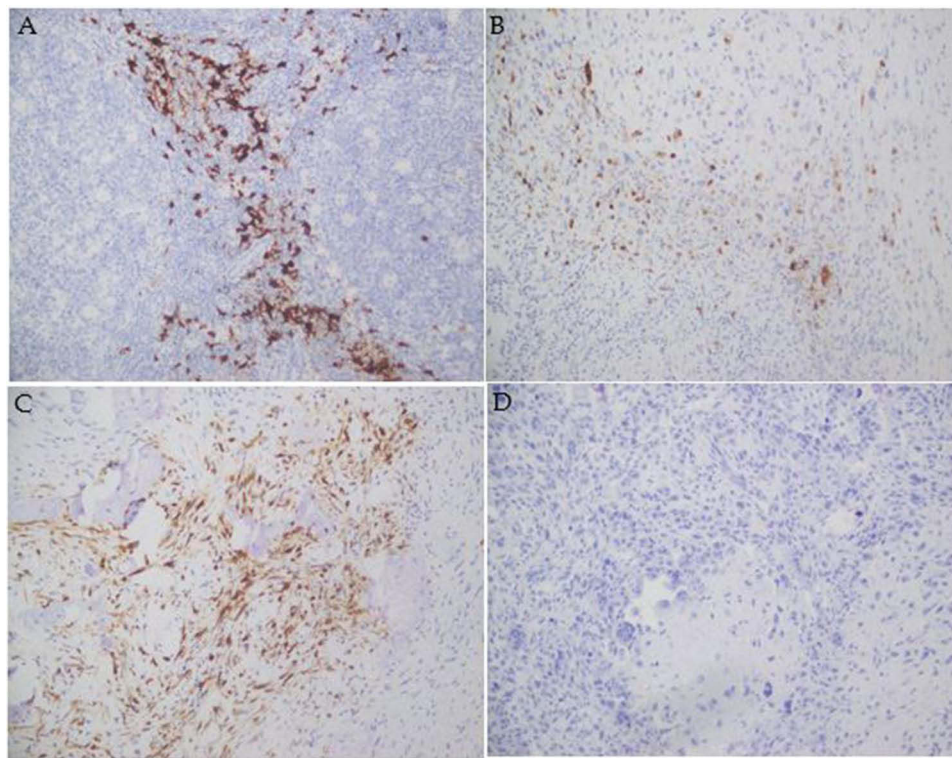


Figure 4 IDO Expression in primary osteosarcoma tumors detected by immunohistochemical staining. Representative images of immunohistochemical staining of IDO in primary tumors of osteosarcoma cases. **(A)** Positive control (normal human reactive lymph node). **(B)** Low IDO expression. **(C)** High cytoplasmic IDO expression. **(D)** Negative expression of IDO. All images were captured at 20X magnification.

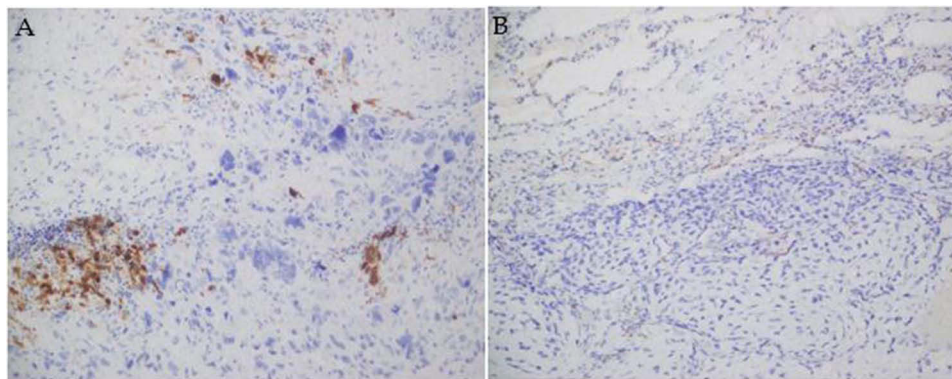


Figure 5 IDO Expression in metastatic lesion of osteosarcoma patients detected by immunohistochemical staining. Representative images of immunohistochemical staining of IDO in metastatic lesions of osteosarcoma cases. **(A)** Low IDO expression. **(B)** Negative expression of IDO. All images were captured at 20X magnification.

Our study has several limitations, primarily being a retrospective cohort study. Resource constraints have restricted our analysis to the expression of a single immune marker through IHC. Nevertheless, it is noteworthy that our study represents the largest cohort to date investigating IDO expression in osteosarcoma patients. Despite the limitations, our findings offer valuable insights into the potential utility of immune markers for identifying patient subsets that may derive benefits from immunotherapies. To advance the field, future studies with larger cohorts and comprehensive panels of immune markers are essential for developing impactful therapeutic strategies tailored to the unique challenges posed by osteosarcoma.

The aim of our study was to explore IDO expression in primary tumors and metastatic lesions among osteosarcoma patients, investigating its association with clinicopathological characteristics and assessing its impact on survival

outcomes. Our findings reveal that IDO is expressed in a limited subset of osteosarcoma patients. To comprehensively understand the immune landscape, it is imperative to explore the expression of a diverse array of immune markers in osteosarcoma patients, laying the foundation for the development of companion diagnostics. This approach holds promise in elucidating unique immune profiles associated with osteosarcoma, ultimately contributing to improved outcomes in the realm of immunotherapies.

Data Sharing Statement

All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

Statement of Ethics

The institutional review board (IRB) of SKMCH&RC approved the current retrospective study (EX-05-06-23-06). The IRB granted waiver for informed consent for this study. The patient data accessed complied with relevant data protection and privacy regulations.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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