Original article

Osteosarcoma: an immunophenotypic study for characterization and behavior

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Abstract:

Background: Osteosarcoma is the most common primary bone tumor occurring in second and third decades of life with a second peak later. Biopsy (needle or incision) is necessary for diagnosis along with imaging modalities (X-ray, CT scan etc) and serology. Due to diagnostic dilemma in certain cases and for prognosis of patients, immunohistochemistry is increasingly used. ***Aims:*** To assess the pathologic features and determinants of osteosarcoma in patients of the Indian subcontinent that would put an insight into its appearance and behavior. ***Methods and Material:*** Forty cases of biopsy proven osteosarcoma were selected over a period of three years. Histopathology was done for tumor typing, along with serology (pre and post-operative serum alkaline phosphatase). In all cases TNM staging and immunohistochemistry for antibodies to Osteonectin (ON) (diagnosis), S100 (differentiation), Ki 67 and Her2 (prognosis) was done. ***Results:*** Serum alkaline phosphatase was high in 37 (92%) cases initially and remained high in metastatic and recurrent lesions. Osteonectin was positive in 38 (95%) cases, S100 in 31 (77%), Ki 67 showed overlapping labeling indices between 4.8-18.8% and Her2 showed more positivity in higher stage tumors. ***Conclusions:*** Biopsy (along with imaging) is mandatory to diagnose osteosarcoma. Osteonectin is a good immunohistochemical marker to differentiate osteosarcoma from its mimics. For prognostication, serum alkaline phosphatase, post chemotherapy tumor necrosis (more than 90%), lack of Her2 expression are good parameters. S100 and Ki67 were found to have limited role in diagnosis and prognosis of osteosarcoma.

Keywords: alkaline phosphatase; immunohistochemistry; osteonectin; osteosarcoma

DOI: http://dx.doi.org/10.3329/bjms.v13i4.13573
Bangladesh Journal of Medical Science Vol. 13 No. 04 October '14. Page: 443-448

Introduction:

Osteosarcoma is the most common primary bone tumor occurring in second and third decades with another peak after 40 years[1,2]. Most osteosarcomas arise *de novo* in the metaphysis of long bones. It has large number of mimics like giant cell tumor, chondrosarcoma, fibrosarcoma and fracture callus. Hence correct diagnosis remains the cornerstone for effective management. Imaging techniques (X-ray, CT scan) and serology (alkaline phosphatase, lactate dehydrogenase) aid in the diagnosis. But biopsy (needle or incision) still remains the gold standard[3,4]. Diagnosis is being rendered more objective by the use of immunohistochemistry for accuracy

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and prognostication of their biological behavior.

Aims:

This study has been done to assess the pathologic features and determinants of osteosarcoma in patients of the Indian subcontinent that would put an insight into its appearance and behavior.

Material and methods:

The procedures followed were in accordance with the ethical standards of the institutional ethical committee on human experimentation. The study was done over a period of three years from April 2008 to March 2011. Forty cases of biopsy proven osteosarcoma were selected. TNM staging was done in all. Histopathological examination was done by routine method of formalin fixation, paraffin embedding and Hematoxylin and Eosin staining (Fig 1).

ferentiation of sarcoma), Ki67 (for detecting tumor proliferation) and HER2 (for prognosis) using monoclonal antibodies (except S100) and Novolink polymer detection system detection (RE7140-K) of Novacastra. Immunohistochemistry was done by polymer method following microwave antigen retrieval. Deep brown stain in varying cellular counterparts was considered positive for a particular stain.

Osteonectin (clone15G12) and S100 (RTU-S100p) (cytoplasmic) staining intensity was noted in tumor area (Fig 2).

Ki67 (clone MM1)-labeling index (nuclear) = No. of cells X 100
1000 cells

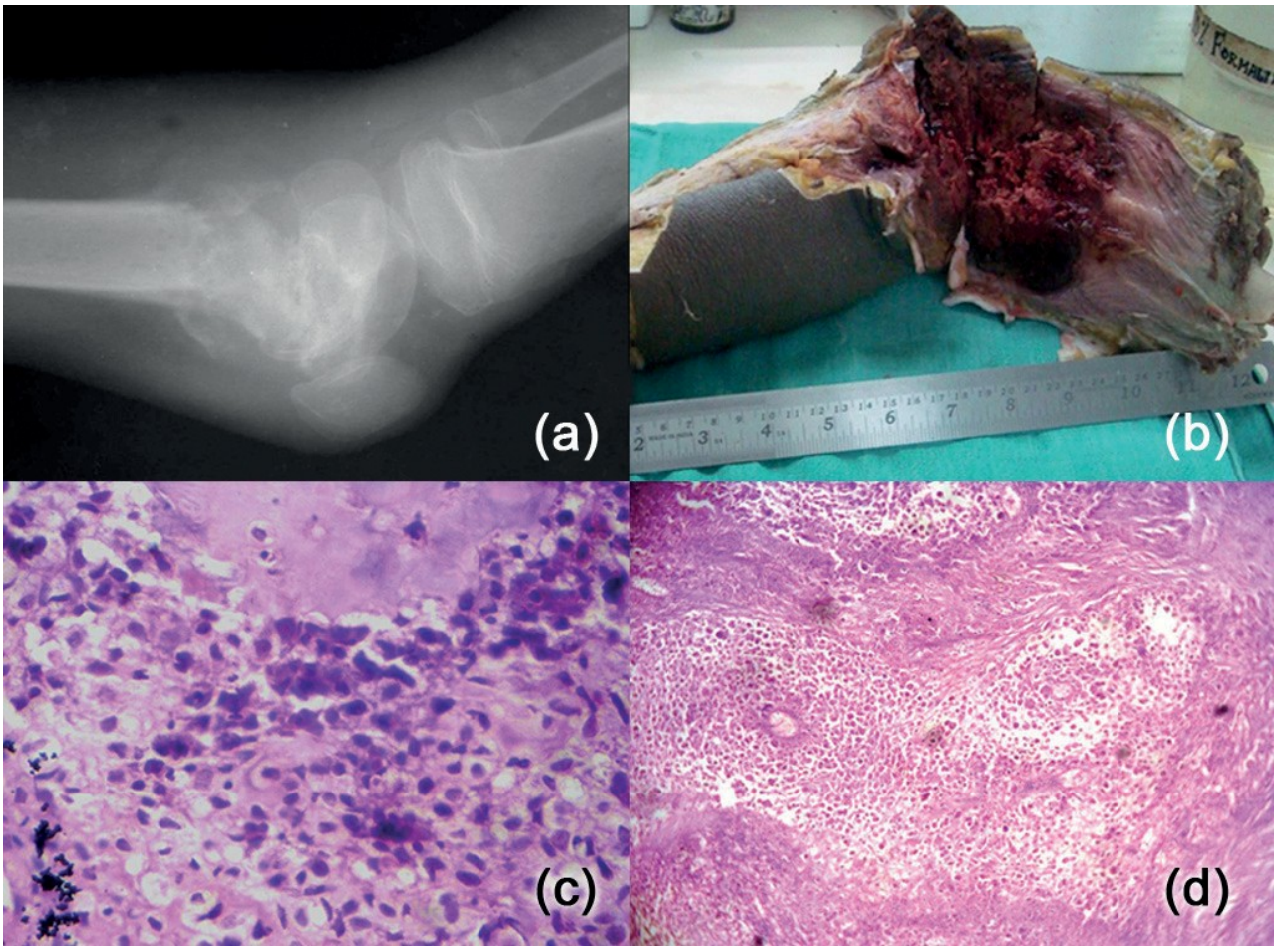

Fig.1: (a) Radiograph – osteosarcoma of lower end of femur (b) Gross – cut section of same tumor (c) Photomicrograph – osteoid production (H&E x400) (d) Post-chemotherapy necrosis (>90%) (H&E x400)

Post chemotherapy tumor necrosis along with pre and post operative serum alkaline phosphatase (SAP) was assessed in all. Immunohistochemistry (IHC) was done in these for antibodies to Osteonectin (ON) (for diagnosis and differentiation from other sarcomas), S100 (for chondroblastic dif-

HER 2/ c-erbB2 (clone CB11) – 0 (<10% cells stain, weak),
1+ (>10% cells stain, weak),
2+ (>10% cells stain, strong),
3+ (strong chicken wire appearance)

Results

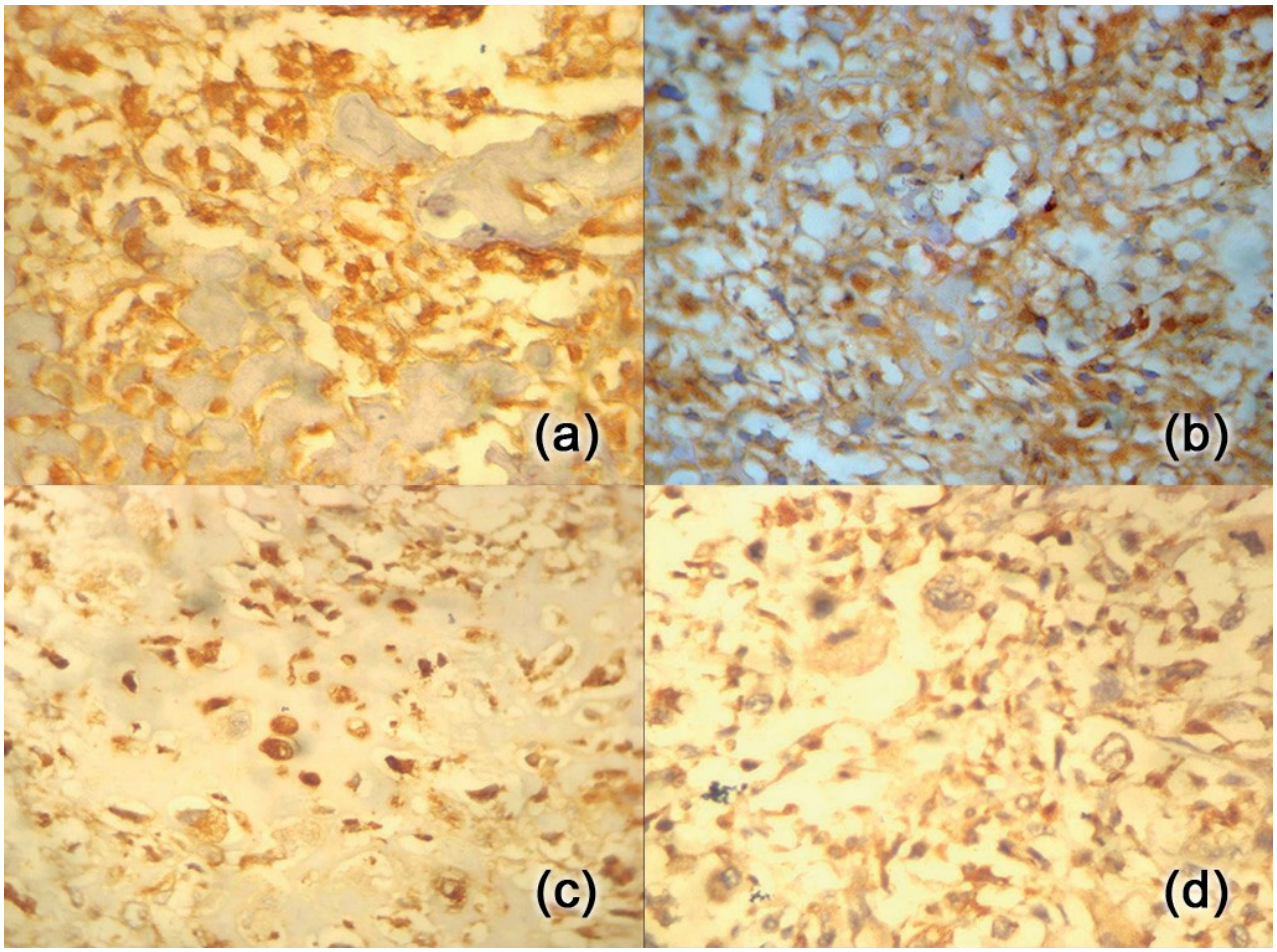

Fig.2: Immunohistochemistry (x400) (a) Osteonectin positivity (b) Staining for S100 protein (c) Ki-67 (++) positivity in chondroblastic variant (d) Her2 (3+) postivity

Out of 25,246 all new cases in our institution over a period of 3 years, 15,634 cases (61.9%) were proved to be malignant. Primary malignant bone tumor (PMBT) was detected in 296 cases (1.9% of all malignancies). Osteosarcoma was confirmed in 89 cases (30% of PMBTs). Forty (45%) cases of osteosarcoma could be followed up at least for 6 months and were selected; 59 cases (55%) either died prematurely or were lost on follow up. Of the 40 subjects male female distribution was 3:1.

TNM staging of the selected cases showed: Stage I, 7 cases (17.5%), Stage II, 5 (12.5%), Stage III, 15 (37.5%) and Stage IV, 13 (32.5%). Pre- and post-chemotherapy serum alkaline phosphatase (SAP) levels for all the stages, showed initial greater values for higher stage tumors (Table 1). In all stage tumors, response to chemotherapy was associated with reduced levels apart from Stage II tumors which showed no significant change. One unresponsive Stage IV tumor showed higher post- chemotherapy SAP levels. Out of 40 cases, the histological types and their frequencies were as follows: Osteoblastic - 24; Chondroblastic - 11; Fibroblastic- 4; Small cell – 1. The sites involved were: Lower limb - 31; Upper limb - 8; Hand (metatarsal) – 1.

IHC with Osteonectin (ON) showed staining in 38/40 cases (95%). The two cases which turned out to be negative were higher stage tumors. S100 showed staining in only 31/40 cases (77.5%) (Table

TABLE 1: Serum alkaline phosphatase: pre and post chemotherapy levels

Alk phos level	Stage I		Stage II		Stage III		Stage IV	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
< 500 U/L	4	6	2	2	4	5	4	3
500 – 1000 U/L	3	1	2	2	5	8	3	6
> 1000 U/L	-	-	1	1	6	2	6	4

2).

Results of Ki67-labeling index showed unacceptable

TABLE 2: IHC for confirmation

Osteonectin* (Total 40 cases)						S100 (Total 40 cases)				
Staining intensity	**4+**	**3+**	**2+**	**1+**	**0**	**4+**	**3+**	**2+**	**1+**	**0**
Stage I	02	04	01	-	-	2	3	1	1	-
Stage II	02	02	01	-	-	1	2	1	-	1
Stage III	06	02	04	02	01	2	6	1	2	4
Stage IV	01	01	06	04	01	1	1	5	2	4

* Grading: 1 = focal (< 50%) weak staining; 2 = focal strong staining; 3 = diffuse (> or = 50%) weak staining; and 4 = diffuse strong staining.

overlap between Stage II, III and IV tumors. IHC with Her2/neu showed Stage I and II tumors (11/12 cases) to be negative and none of these were 3+. Stage IV tumors (8/13 cases) were recorded as 3+ and three were Her2 negative (Table 3).

Evaluation of post-chemotherapy tumor necrosis

TABLE 3: IHC for prognostication

	KI 67 LI	**HER 2/NEU***			
		0	**1+**	**2+**	**3+**
STAGE I (N=7)	0.6-4.8	5	1	1	-
STAGE II (N=5)	4.8-14.5	3	2	-	-
STAGE III (N=15)	4.5-18.8	2	4	5	4
STAGE IV (N=13)	5.2-28.8	0	3	2	8

* A test result of 0 to 1+ considered as negative and 2+ and 3+ as positive.

showed majority (7/12 cases) of Stage I and II tumors to undergo >90% necrosis. Only few (5/28 cases) of Stage III and IV tumors showed similar results (Table 4).

TABLE 4: Post-chemotherapy tumor necrosis

	> 90% necrosis	**<90% necrosis**
	N (%)	**N (%)**
STAGE I (N=7)	4 (57%)	3 (43%)
STAGE II (N=5)	3 (60%)	2 (40%)
STAGE III (N=15)	4 (27%)	11 (73%)
STAGE IV (N=13)	1 (8%)	12 (92%)

Discussion:

Osteosarcomas originate from osteoblasts whose function is the production of new bone. However, definite proof of this production is only possible in the case of highly differentiated osteosarcomas[5]. Even the classical type of osteoblastic osteosarcoma[6,7] may pose diagnostic difficulties. The interstitial matrix deposits, if not mineralized, are sometimes difficult to distinguish from the deposits of hyaline, which is a collagenous ground substance present in malignant soft tissue tumors such as synovial sarcoma, fibrosarcoma, malignant fibrous histiocytoma, or hemangiopericytoma. Immuno-cytochemical studies on a broad spectrum of bone and soft tissue tumors have showed that ON is demonstrable in bone-forming tumors only, but not in chondrosarcomas or soft-tissue sarcomas[8]. ON also has been found in tumor tissues that contain no, or only a slight amount of matrix, such as anaplastic osteosarcomas or fibroblastic osteosarcomas[5]. In a study by Fanburg-Smith JC *et al* ON was 93% sensitive for extraskeletal osteosarcoma cells (2 to 4+). ON was specific for osteoid matrix and nonreactive in both collagen and cartilage matrix. They concluded that ON will distinguish malignant bone from collagen and cartilage matrix, essential to the diagnosis of extraskeletal osteosarcoma[9].

Out of eighteen osteosarcoma cases, Hasegawa T *et al* detected S-100 protein-positive tumor cells not only in all four tumors of the chondroblastic type, but also in three of the osteoblastic type, one of the low-grade central type, and in the solitary giant cell-rich type[10]. Gao FX found positive reactions to S-100 protein, collagen type IV, UEA-1 and factor XIII in 26, 20, 36 and 13 of the total 48 cases respective-

ly. Their results confirmed that osteosarcoma possesses the unique multidirectional differentiation potential toward osteoblastic, chondroblastic, myofibroblastic, fibrohistiocytic and epithelial cells, and also indicate that immunophenotypic analysis is useful for the diagnosis, differential diagnosis and classification of the tumor cells of osteosarcoma[11]. In our study, S-100 protein was found to be positive in 31 cases (77.5%), of which 9 showed chondroblastic, 21 showed osteoblastic and 1 showed small cell type of differentiation. Hence our study shows, S-100 is sensitive (82%) but not specific (24%) for demonstration of chondroblastic differentiation of osteosarcoma. Over expression of the HER-2/neu oncogene has prognostic significance in breast cancer. Kilpatric SE *et al* observed at least focal cytoplasmic positivity (1-3+) in 98% of osteosarcomas. However, intensity of the cytoplasmic staining did not correlate with histologic subtype/grade, response to chemotherapy, metastasis or survival in their study[12]. In their study, Onda M *et al* found that 42% of osteosarcomas expressed Her-2. Expression strongly correlated with early pulmonary metastasis and poor survival rate. Their data suggested that Her-2 plays a significant role in aggressive tumor growth and in the promotion of metastatic potential in osteosarcomas[13]. Gorlick R *et al* observed higher frequencies of expression in samples from patients with metastatic disease at presentation and at the time of relapse. Expression of Her2/erbB-2 correlated with a significantly worse histologic response. In patients presenting with non-metastatic disease, expression of HER2/erbB-2 at the time of initial biopsy was associated with a significantly decreased event-free survival[14]. In our study, strong intensity of Her/2µ staining was noted in 10 out of 13 cases of stage IV (77%) and 9 out of 15 cases of stage III (60%), whereas negative staining is noted in 86% and 100% of cases of stage I and stage II disease respectively. The specificity of Her/2µ staining is around 92% for detection of advanced disease. Hence in this study, Her/2µ staining indicates poor prognosis.

Levine AM *et al* found that tissue alkaline phosphatase levels of primary osteosarcomas is strongly correlated with prognosis with respect to development of pulmonary metastases.[15] Thorpe WP *et al* showed that out of 17 osteosarcoma patients with elevated preoperative SAP levels, 12 recurred. Of 13 patients with normal preoperative levels, only 4 recurred. Thus, preoperatively elevated alkaline phosphatase levels were correlated with poor prognosis[16]. Bacci G *et al* found the percentage of patients with increased SAP levels to be significantly higher in the metastatic group than in the group of patients with localized disease (91.5% versus 61.3%). In the latter group, treated with adjuvant and neoadjuvant chemotherapy, the relapse rate was significantly higher in patients with elevated pretreatment SAP levels than in those with normal levels (55.1% versus 26.4%)[17]. In the present study, SAP level remained markedly elevated (?500U/L) even after treatment with chemotherapy in 67% and 77% of patients of stage III and stage IV disease respectively.

In a multivariate analysis, Davis AM *et al* found that the most important prognostic variable for patients with osteosarcoma of the extremity was tumor necrosis evident following preoperative chemotherapy. However, there is no consensus as to any prognostic variable that might be used to stratify patients before the onset of therapy[18]. The strongest association with local recurrence in patients undergoing limb-sparing resection is chemotherapy response, followed closely by surgical margins. Picci P *et al* concluded chemotherapy-induced tumor necrosis is one of the most important prognostic factors for local control of patients with osteosarcoma[19]. Post chemotherapy tumor necrosis correlated well with the stage of the tumor in our study. Most of the patients with lower stage disease developed >90% necrosis of tumor following chemotherapy (stage I - 57% and stage II – 60%) whereas in minority of cases of higher stage disease developed the same (stage III – 27% and stage IV – 8%).

In conclusion, biopsy (along with imaging) is mandatory to diagnose osteosarcoma. ON is a good immunohistochemical marker to differentiate osteosarcoma from its mimics. For prognostication, serum alkaline phosphatase, post chemotherapy tumor necrosis (more than 90%), lack of Her2 expression are good parameters. S100 and Ki67 were found to have limited role in diagnosis and prognosis of osteosarcoma.

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