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ORIGINAL ARTICLE

Role of immunohistochemical cyclo-oxygenase-2 (COX-2) and osteocalcin in differentiating between osteoblastomas and osteosarcomas

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Abstract

Background/Aims: Differential diagnosis between aggressive osteoblastoma and low grade osteosarcoma may be very difficult or even impossible on a small biopsy. This study was designed to assess the usefulness of immunoexpression of COX-2 and osteocalcin in the differential diagnosis of the two tumour types. *Methods:* Immunostaining of COX 2 and osteocalcin were studied in 9 osteoblastomas and 30 osteosarcomas. *Results:* All osteoblastomas and 11/20 (55%) high-grade osteosarcomas showed COX-2 immunoreactivity. All low grade osteosarcomas were COX-2 negative. COX-2 was significantly higher ($p<0.002$) in osteoblastomas 9/9 (100%) than in osteosarcomas 13/30 (43%) and in aggressive osteoblastomas versus low grade osteosarcomas ($p<0.01$). Osteocalcin was found in tumour cells of all osteosarcomas and osteoblastomas and in the osteoid matrix of 84% of osteosarcomas and 78% of osteoblastomas. Strong osteocalcin was significantly higher ($p<0.02$) in osteoblastomas (78%) than in osteosarcomas (27%). *Conclusion:* COX-2 is a valuable marker in distinction between osteosarcoma and osteoblastoma. Negative COX-2 could confirm the diagnosis of low grade osteosarcoma versus aggressive osteoblastoma. Intensity and distribution of osteocalcin may indicate the degree of osteoblastic differentiation.

Keywords: COX-2, osteocalcin, osteosarcoma, osteoblastoma, immunohistochemistry

INTRODUCTION

Osteoblastoma and osteosarcoma are primary bone tumours of osteoblastic origin. Osteoblastoma is an uncommon neoplasm accounting for about 1% of all primary bone tumours.[1,2] It usually affects young patients in the age range of 10-30 years.[3] The most common sites for osteoblastoma are the vertebral column and long bones.[4] It has a distinctive predilection for the metaphysis and diaphysis. Epiphyseal location is rare.[5]

Microscopically, osteoblastoma is composed of proliferating osteoblasts along with anastomosing woven bone trabeculae and rich vascular fibrous stroma.[6] It is important to examine the edges of an osteoblastoma because the tumour does not infiltrate and isolate pre-existing lamellar bone structures as does osteosarcoma. In addition, no sheets of spindle cells are seen in osteoblastomas. The osteoblasts may show some mitoses but they are not atypical.[7]

Osteoblastomas may be misdiagnosed as an osteosarcoma if correlation of clinical history, radiology, and histology is not carefully considered or if the several variants of osteoblastoma are not recognized. These variants lie on a morphological spectrum between conventional osteoblastoma and osteosarcoma. Aggressive osteoblastoma is one such subtype. As the name implies, the histological features of aggressive osteoblastoma may appear malignant, and its biological behaviour may separate it from conventional osteoblastoma.[8] Aggressive osteoblastoma is a rare bone-forming neoplasm composed of large plump osteoblasts, with bizarre hyperchromatic nuclei and prominent nucleoli. It demonstrates locally invasive growth with a high rate of recurrence but no metastatic potential.[9, 10]

On the other hand, osteosarcoma is the commonest, non-hematopoietic, malignant primary tumour of bone.[11] Its incidence peaks in those aged 10-20 years. A second small peak is seen in those older than 60 years.[12] Osteosarcoma arising in bones distal to the wrists

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and ankles is extremely unusual.[13,14] Variants of osteosarcoma include conventional types (i.e. osteoblastic, chondroblastic, fibroblastic) and telangiectatic, multifocal, parosteal, and periosteal types. Classic, or conventional, osteosarcoma represents the most common variant, accounting for approximately 75% of all osteosarcomas.[15]

Conventional osteosarcoma is composed of pleomorphic tumor cells that may be spindle shaped, epithelioid, plasmacytoid, fusiform, ovoid, or rounded. Giant cells may also be present, and these may be mononucleated or multinucleated. Osteoid material is usually present, which appears as a lacelike, dense, pink, amorphous intercellular material.[16, 17]

Osteoblastoma-like osteosarcoma is a low-grade osteosarcoma with characteristic histopathological features.[16,17] It has to be recognized by the pathologist to achieve the right treatment which is wide surgical excision Differential diagnosis may be very difficult or even impossible on a small biopsy.[18, 19]

To date, no single immunohistochemical stain can differentiate between aggressive osteoblastoma and osteosarcoma with certainty. However, one study showed that immunohistochemical detection of COX-2 in tumour cells supports the diagnosis of osteoblastoma and aids in distinguishing it from osteosarcoma.[20]

We aimed to study the immunohistochemical expression of COX-2 and osteocalcin in osteoblastoma and osteosarcoma and evaluating their utility in differentiation between aggressive osteoblastoma and osteosarcoma.

PATIENTS AND METHODS

This study included excisional and incisional biopsies of osteoblastoma and osteosarcoma sent to the Pathology Laboratories from the Orthopaedic Departments of Sohag and Assiut Universities Hospitals through the period from January 2003 to June 2006.

H&E staining

Five-micron tissue sections were prepared from the formalin-fixed, paraffin-embedded tissues of the tumour biopsies, stained with Hematoxylin and Eosin (H&E) and examined using light microscopy. Histological subtypes of osteosarcomas were categorised as osteoblastic, chondroblastic, or fibroblastic and histological subtypes of osteoblastomas as conventional and aggressive subtypes. Grading of osteosarcomas was done using a predetermined classification system, adapted from previously reported systems.[11, 21] Aggressive variant of osteoblastomas was separated using the criteria mentioned by Bonar *et al*.[17]

Immunohistochemistry

Immunostaining using peroxidase-labelled strepavidin-biotin technique to detect COX-2 and osteocalcin was done for all cases. The following primary antibodies were used: rabbit polyclonal antibody against human COX-2 (Catalogue # RB-9072-P0, 0.1ml, LabVision Corporation) and mouse monoclonal antibodies to Human/ Bovine osteocalcin (Clone BD1152, Catalogue # H95152M, 0.1ml, BioDesign Corporation).

Staining procedure

Five-micron tissue sections were mounted on Poly-Lysine coated slides, deparaffinized and rehydrated. Endogenous peroxidase activity was blocked using peroxidase blocking reagent (Catalogue # TP-012-HD, LabVision Corporation). The antigen sites were unmasked by immersing the slides in sufficient amounts of antigen retrieval solution (10 mmol sodium citrate buffer, pH 6.0). Sections were microwaved for 10-15 minutes (min), allowed to cool down for 20 min, washed in distilled water, then in phosphate buffered saline (PBS, pH 6.0). Tissue sections were incubated in normal goat serum (NGS) to block non-specific interactions.

Tissue sections were incubated for half hour at room temperature with 1/200 COX-2 and overnight at 4 C° in a humid chamber with 1/75 osteocalcin. The resulting immune-complex was detected by a universal staining kit (Catalogue # TP-012-HD, LabVision Corporation). Tissue sections were treated with biotinylated goat anti-polyvalent, then peroxidase-labelled streptavidin was applied for 10-15 min at room temperature, rinsed in PBS, incubated with 14-diaminobenzidine and 0.06% H2O2 for 5 min and counter-stained in Myer's Hematoxylin. Tissue sections were washed in tap water, dehydrated in alcohol, cleared in xylene, left to dry, then mounted with Canada balsam, and cover slipped.

Positive controls: Positive control slides were prepared from previously diagnosed colon carcinoma and normal bone tissue for detection of COX-2 and osteocalcin staining respectively. *Negative controls:* Negative control was achieved by omitting the primary antibody from the

staining procedure. The positive and negative controls were consistently immunoreactive and lacking reactivity respectively. These findings therefore confirmed the validity of our staining results.

Evaluation of immunostaining
Sections were histologically examined by bright field microscopy at low magnification (X40 and X100) to detect the sites of antibody positivity, then by higher power magnification (X200 and X400) to evaluate immunostaining.

COX-2 and osteocalcin positivity was expressed as the mean percentage (%) of positive cells and the staining intensity in at least three different fields. Cells positive for COX-2 were identified by the presence of both membranous and cytoplasmic brownish staining. Positive osteocalcin immunoreactivity was present as brownish staining in both the cytoplasm of bone cells and osteoid matrix. Semi-quantitation of COX-2 and osteocalcin immunoreactivities were calculated with a 12-point weighted score system as follows:

1. First, the percentage of positive cells (PP) in each area was scored with a 5-point scale: 0 for <5%, 1 for 5-25%, 2 for >25-50%, 3 for >50-75%, and 4 for over 75%.[22]
2. Second, the staining intensity (SI) of positive staining was scored with a 3-point scale: 0 for negative, 1 for weak, 2 for medium, and 3 for intense staining.[23-25]
3. Then, the average weighted score (AWS) for each area was calculated by multiplying PP by the SI. The results were scored as negative (0-1), weak (2-3), moderate (4-6) and strong (8-12).[22, 26]

Statistical analysis
Results were statistically analyzed using Statistical Package for Social Sciences (SPSS) for windows. Chi Square Test and ANOVA (Analysis of Variance) were used to assess the statistical significance of the relationships of COX-2 and osteocalcin expression in osteoblastomas and osteosarcomas.

RESULTS

Clinical features
The study group consisted of 39 patients, including 27/39 (69%) males and 12/39 (31%) females, ranging in age from 10 to 58 years. Patients presented with a swelling or mass and/or an osteolytic bone lesion. All patients were informed about the study and the study was approved by the local ethics committee.

H&E review
Cases included 9 osteoblastomas and 30 osteosarcomas, of which 27 were osteoblastic osteosarcomas (20 high grade and 7 low grade), two chondroblastic and one fibroblastic osteosarcoma. Figures 1A-C illustrate examples.

Immunohistochemical features

COX-2 expression
Tumour cells of all osteoblastomas showed diffuse cytoplasmic and membranous COX-2 expression with varying degrees of immunoreactivity. COX-2 was strong in 3/9 (33%), moderate in 4/9 (45%) and weak in 2/9 (22%) cases of osteoblastomas (Table 1 & Figures 2 A-B). All aggressive type of osteoblastomas (3 cases) were COX-2 immunoreactive (100%). COX-2 was also expressed in the osteoclasts of osteoblastomas (Table 2 & Figure 2 C).

All osteoblastic low grade osteosarcomas (7 cases) were COX-2 negative (Table 2). Positive COX-2 immunoreactivity was observed in 11/20 (55%) of high-grade osteosarcomas and in the two chondroblastic osteosarcomas, with most of these positive cases (8/13; 62%) showing weak immunoreactivity (Table 1 & Figures 2 D-E). COX-2 immunoexpression was significantly higher ($p<0.002$) in osteoblastomas (100%) than in osteosarcomas (43%).

The expression of COX-2 was significantly higher in aggressive osteoblastomas than in osteoblastic low grade osteosarcomas ($p<0.01$). The osteoid matrix in both osteoblastoms and osteosarcomas was COX-2 negative.

Osteocalcin expression
All cases of osteosarcoma showed positive osteocalcin in the cytoplasm of malignant osteoblasts, with varying degrees of immunoreactivity; strong in 27%, moderate in 43% and weak in 30% of cases (Table 3 & Figure 3 A). Most of the osteocalcin-positive cells are the relatively well-differentiated osteoblasts that are rimming and surrounding the areas of osteoid production in a case of osteosarcoma (Figure 3 B). Osteocalcin expression in the osteoid matrix was strong in 47%, mild to moderate in 37% and negative in 16% of cases of osteosarcomas (Figure 3 A).

TABLE 1: COX-2 expression in osteosarcoma and osteoblastoma

	Osteosarcoma n = 30	**Osteoblastoma n = 9**
Staining intensity:		
0 (Negative)	17/30 (57%)	-
1 (Mild)	6/30 (20%)	2/9 (22%)
2 (Moderate)	5/30 (17%)	4/9 (44%)
3 (Strong)	2/30 (6%)	3/9 (33%)
% of positive cells:		
0 (0-5%)	17/30 (57%)	-
1 (5-25%)	5/30 (17%)	1/9 (11%)
2 (25-50%)	3/30 (10%)	3/9 (33%)
3 (50-75%)	3/30 (10%)	1/9 (11%)
4 (>75%)	2/30 (6%)	4/9 (44%)
Weighted score:		
0-1 (negative)	17/30 (57%)	-
2-3 (weak)	8/30 (27%)	2/9 (22%)
4-6 (moderate)	4/30 (13%)	4/9 (44%)
8-12 (strong)	1/30 (3%)	3/9 (33%)

All osteoblastomas showed positive osteocalcin expression in the cytoplasm of neoplastic osteoblasts with varying degrees of immunoreactivity; strong in 78%, moderate in a single case (11%) and weak in another single case (11%) as shown in Table 3 and Figure 3 C . Osteocalcin staining in the osteoid was also variable; strong in 33%, mild to moderate in 45%, and negative in 22% of cases of osteoblastomas Figure 3D.

TABLE 2: COX-2 expression in low grade osteoblastic osteosarcomas and aggressive osteoblastomas

	Low grade osteoblastic osteosarcoma n = 7	**Aggressive osteoblastoma n = 3**
Staining intensity:		
0 (Negative)	7/7 (100%)	-
1 (Mild)	-	1/3 (33%)
2 (Moderate)	-	1/3 (33%)
3 (Strong)	-	1/3 (33%)
Percentage of positive cells:		
0 (0-5%)	7/7 (100%)	-
1 (5-25%)	-	1/3 (33%)
2 (25-50%)	-	-
3 (50-75%)	-	-
4 (>75%)	-	2/3 (67%)
Weighted score:		
0-1 (negative)	7/7 (100%)	-
2-3 (weak)	-	1/3 (33%)
4-6 (moderate)	-	1/3 (33%)
8-12 (strong)	-	1/3 (33%)

TABLE 3: Osteocalcin expression in osteosarcoma and osteoblastoma

	Osteosarcoma n = 30	Osteoblastoma n = 9
Staining intensity:		
0 (Negative)	-	-
1 (Mild)	10/30 (33%)	1/9 (11%)
2 (Moderate)	14/30 (47%)	3/9 (33%)
3 (Strong)	6/30 (20%)	5/9 (56%)
% of positive cells:		
0 (0-5%)	-	-
1 (5-25%)	1/30 (3%)	-
2 (25-50%)	6/30 (20%)	1/9 (11%)
3 (50-75%)	15/30 (50%)	2/9 (22%)
4 (>75%)	8/30 (27%)	6/9 (67%)
Weighted score:		
0-1 (negative)	-	-
2-3 (weak)	9/30 (30%)	1/9 (11%)
4-6 (moderate)	13/30 (43%)	1/9 (11%)
8-12 (strong)	8/30 (27%)	7/9 (78%)

n: number.

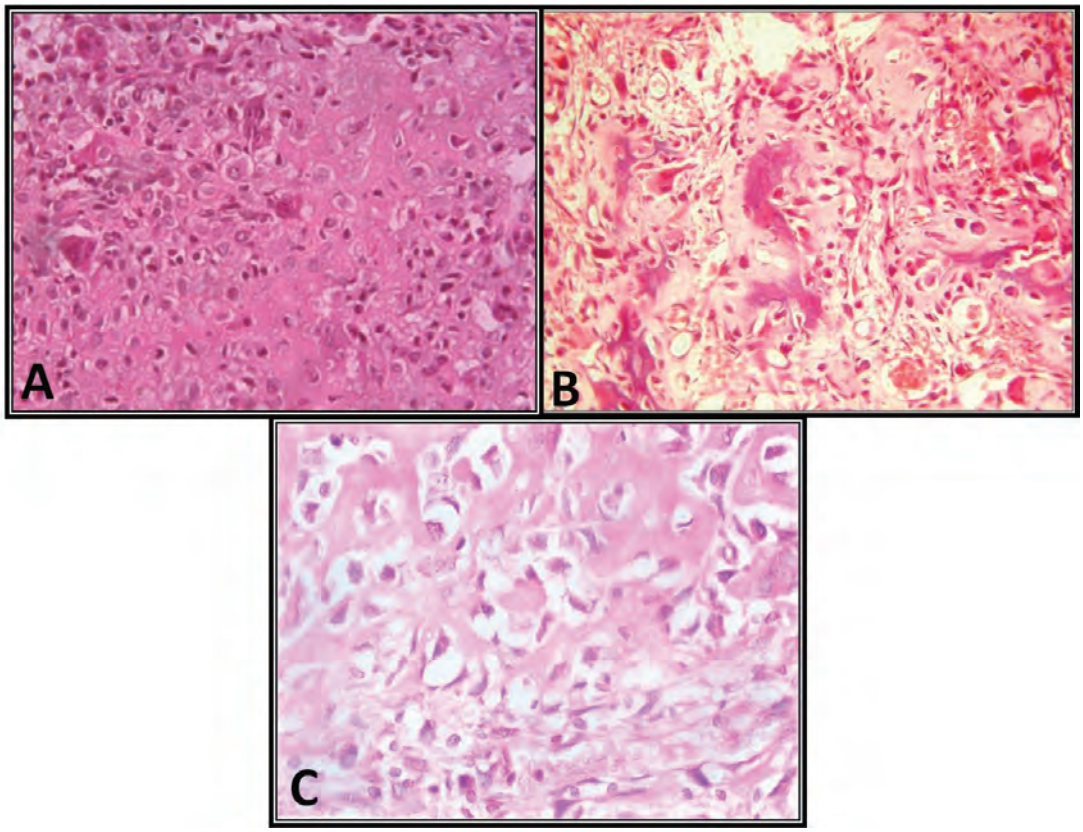

FIG. 1: H&E stain **(A)** Osteoblastoma showing proliferating benign-appearing osteoblasts with osteoid production (X200), **(B)** Aggressive osteoblastoma showing large plump osteoblasts, with bizarre hyperchromatic nuclei (X400), **(C)** Osteosarcoma showing highly pleomorphic neoplastic cells with areas of osteoid production (X400).

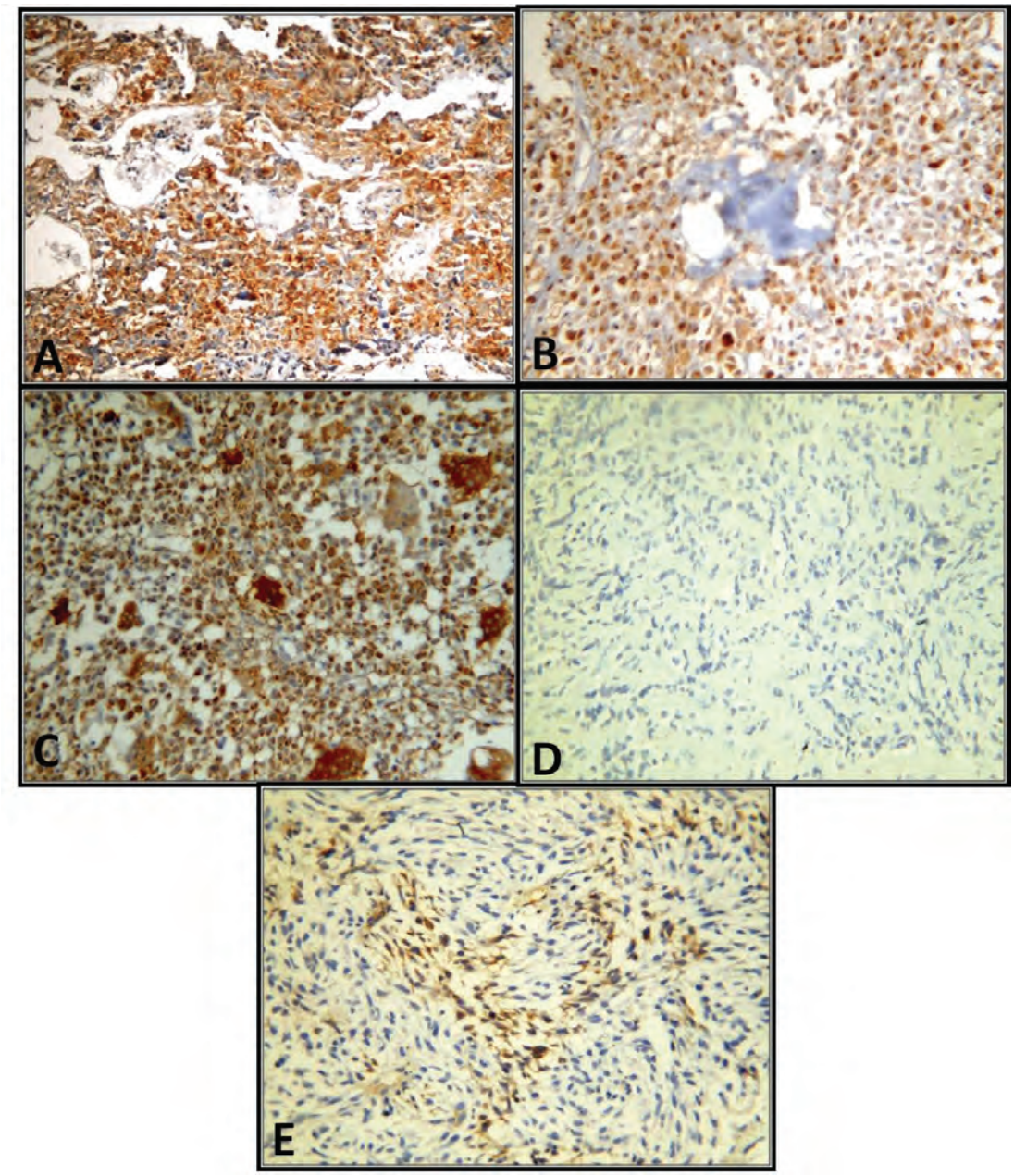

FIG. 2: COX-2 stain**. (A)** Osteoblastoma revealed strong COX-2 expression in the neoplastic osteoblasts (X200), **(B)** Aggressive osteoblastoma showing strong COX-2 immunoreactivity in the neoplastic osteoblasts (X400), **(C)** Osteoblastoma showing osteoclast-like giant cell positive for COX-2 (X400), **(D)** Negative COX-2 immunostaining in the neoplastic osteoblasts of osteosarcoma (X400), **(E)** Osteosarcoma showing focal COX-2 immunoreactivity in the neoplastic osteoblasts (X400).

In addition, the percentage of cases having strong immunoexpression for osteocalcin was significantly higher (p<0.02) in osteoblastoma (78% of cases) than in osteosarcoma (27% of cases).

DISCUSSION

Osteoblastoma is a benign tumor that is composed of proliferating osteoblasts along with small trabeculae of woven bone and rich vascular fibrous stroma.[6,27] These neoplastic osteoblasts may occasionally have atypical features, with

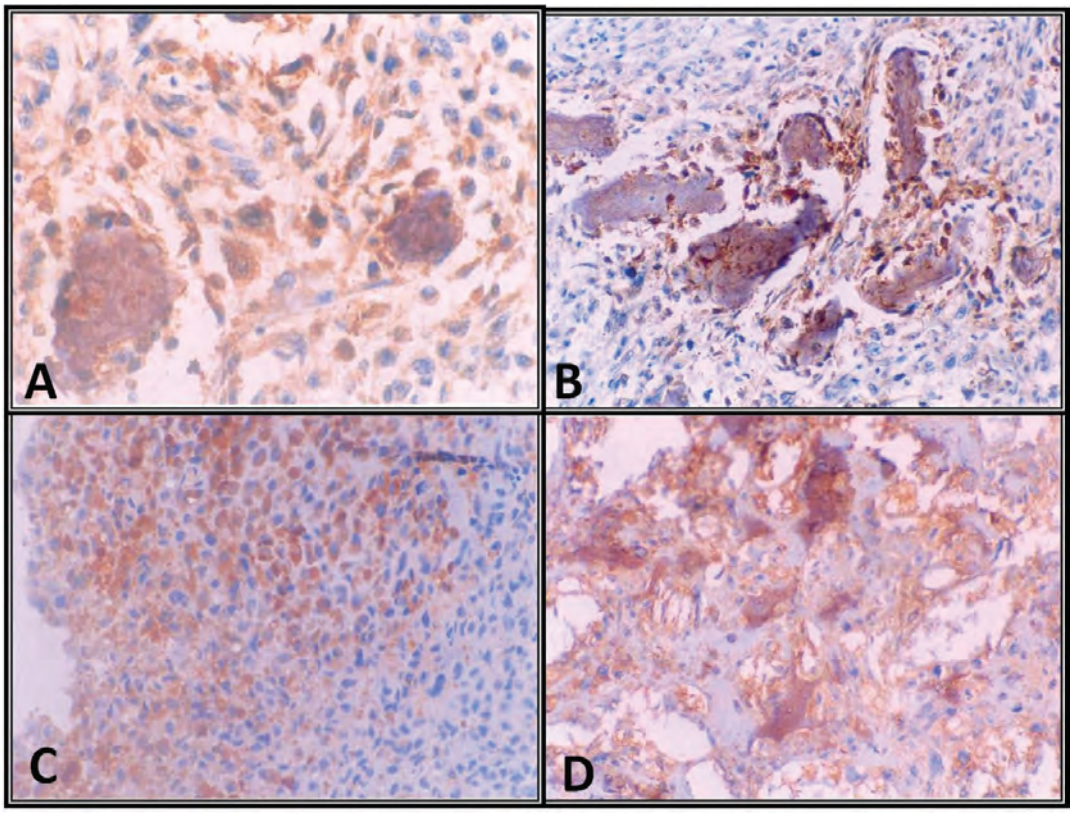

FIG. 3: Osteocalcin stain. **(A)** Osteosarcoma showing strong positive osteocalcin in both cytoplasm of the osteoblasts and the osteoid tissue (X400), **(B)** Lower power view showing osteocalcin-positive cells rimming and surrounding the areas of osteoid production in a case of osteosarcoma (X200), **(C)** Osteoblastoma revealed strong osteocalcin immunostaining in the cytoplasm of neoplastic osteoblasts (X200), **(D)** Osteoblastoma showing positive osteocalcin expression in areas of osteoid production (X200).

high cellularity and epithelioid appearances,[28] making its distinction from osteosarcoma and other bone tumours difficult. [7, 27]

In this study, it was hypothesized that the evaluation of COX-2 and osteocalcin expressions in osteoblastomas and osteosarcomas may be of practical value in their accurate diagnosis, and in the differentiation between them. To test this hypothesis, a total of 39 cases representing different types of bone forming tumours were examined for detection of COX-2 and osteocalcin expression.

Consistent with other studies, COX-2 was positive in all cases of osteoblastomas, with strong to moderate staining in 77% of cases. [20, 29] In contrast to osteoblastomas, the majority (57%) of osteosarcomas were COX-2 negative and most of those positive cases showed weak COX-2 immunoreactivity (62%). COX-2 immunoreactivity was restricted to high grade osteosarcomas, with negative reactivity in all osteoblastic low grade cases (seven cases) in agreement with Masi *et al*. [30] Chondroblastic osteosarcomas (two cases) expressed COX-2, consistent with Hosono *et al*.[20] These results suggest that COX-2 may be of considerable value in the differentiation between osteoblastomas and osteosarcomas especially in cases with small sample size.

To the best of our knowledge, this is the first study correlating COX-2 expression in low grade osteoblastic osteosarcomas and aggressive osteoblastomas. We found that, in contrast to low grade osteoblastic osteosarcomas, all aggressive osteobastomas (three cases) showed COX-2 positivity and this difference is statistically significant ($p<0.01$). These findings suggest that COX-2 expression would confirm the diagnosis of aggressive osteoblatomas versus low grade osteosarcomas. Further studies are recommended on a large number of cases to confirm these results.

Consistent with many studies that demonstrate the high specificity and sensitivity of osteocalcin

for osteoblasts,[31,32] all cases of osteosarcoma and osteoblastoma in the current study showed positive osteocalcin immunostaining with varying degrees of immunoreactivity.

The staining intensity of osteocalcin in the osteoid was variable in both osteosarcomas and osteoblastomas, with positive osteocalcin immunoreactivity in the osteoid of most of cases (81%). This is in agreement with others who demonstrate high expression of osteocalcin in both the tumour cells and osteoid tissue in osteogenic bone tumours; osteosarcoma and osteoblastoma. [33]

In the current study, the percentage of cases having strong osteocalcin immunoexpression was significantly higher ($p<0.02$) in osteoblastoma (78%) than in osteosarcoma (27%). This finding suggests that osteocalcin is more expressed in the well-differentiated osteoblasts of osteoblastoma than in the less differentiated osteoblasts of osteosarcoma. In addition, it was found that most of the osteocalcin-positive cells of osteosarcoma were the relatively well-differentiated osteoblasts that are rimming and surrounding areas of osteoid production. This suggestion is supported by the fact that osteocalcin is a marker of late osteoblast differentiation and is induced only after the expression of other osteoblastic markers such as alkaline phosphatase and type I collagen.[34, 35] Many authors considered osteocalcin as an osteoblast differentiation marker.[36-39] It is suggested that the intensity and distribution of osteocalcin immunostaining may give a clue to the degree of differentiation of the tumour cells (osteoblasts), and hence whether it is benign (osteoblastoma) or malignant (osteosarcoma).

It is concluded that the immunohistochemical analysis of COX-2 may be a valuable marker in the distinction between osteosarcomas and osteoblastomas. Negative COX-2 expression could confirm the diagnosis of low grade osteosarcoma versus aggressive osteoblastoma. The intensity and distribution of osteocalcin immunostaining may indicate the degree of osteoblastic differentiation.

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