KI-67 AND OSTEOCALCIN IMMUNOSTAINING EXPRESSION IN OSTEOGENIC TUMORS; OSTEOBLASTOMA AND OSTEOSARCOMA

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SUMMARY

Background/Aims: *Osteoblastoma and osteosarcoma are primary bone forming tumors that show different radiological/pathological features and clinical behavior. This study aims to specify the expression profile of Ki-67 and osteocalcin in osteoblastomas and osteosarcomas.* ***Methods:*** *Ki-67 and osteocalcin immunoexpression were studied in 9 osteoblastomas and 30 osteosarcomas; including 27 osteoblastic osteosarcomas.* ***Results:*** *There was gradual significant increase ($p<0.01$) in Ki-67 labeling index (Ki-67-LI) from osteoblastomas (23.7%), low grade osteosarcomas (58.8%), to high grade osteosarcomas (74.9%). Ki-67-LI in osteosarcomas was significantly higher than that in osteoblastomas ($p<0.001$). Osteocalcin was found in tumor 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:*** *Ki-67 increased ongoing from osteoblastomas through low grade osteosarcomas, to high grade osteosarcomas and its estimation may help in the distinction between osteoblastomas and osteosarcomas. Intensity and distribution of osteocalcin may indicate the degree of osteoblastic differentiation.*

Keywords: *Ki-67, osteocalcin, osteoblastomas and osteosarcomas*

Abbreviation: *Ki-67-label index (LI), hematoxylin and eosin (H&E), Catalogue number (Cat #), phosphate buffered saline (PBS), minutes (min), normal goat serum (NGS), phosphate buffered saline (PBS), percentage of positive cells (PP), staining intensity (SI), average weighted score (AWS).*

INTRODUCTION

Osteoblastoma and osteosarcoma are primary osteogenic bone tumors. Osteoblastoma is an uncommon neoplasm accounting in about 1% of all primary bone tumors [1, 2]. It usually affects young patients in the age range of 10-30 years [3]. On the other hand, osteosarcoma is the commonest, non-hematopoietic, malignant primary tumor of bone [4]. Its incidence peaks in those aged 10-20 years. A second small peak is seen in those older than 60 years [5]. Variants of osteosarcoma include conventional types (ie, 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 [6].

The most common sites for osteoblastoma are vertebral column and long bones [7]. It has a distinctive predilection for metaphysis and diaphysis. Epiphyseal location is rare [8]. Osteosarcoma arising in bones distal to the wrists and ankles is extremely unusual [9, 10].

Microscopically, osteoblastoma is composed of proliferating osteoblasts along

with anastomosing woven bone trabeculae and rich vascular fibrous stroma [11]. It is important to examine the edges of an osteoblastoma because the tumor does not infiltrate and isolate pre-existing lamellar bone structures as osteosarcoma does. In addition, no sheets of spindle cells are seen in osteoblastomas. The osteoblasts may show some mitoses but they are not atypical [12].

On the other hand, 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.

Osteoblastoma-like osteosarcoma is a low-grade osteosarcoma with characteristic histopathological features [13, 14]. It has to be recognized by the pathologist to achieve the right treatment which is wide surgical procedure. Differential diagnosis may be very difficult or even impossible on a small biopsy [15, 16].

Osteoblastomas may also 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 [17].

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 [18, 19].

Ki-67 is a proliferation marker that is involved in the growth, local invasion, and metastatic spread of osteosarcoma[20, 21]. The expression of Ki-67 in osteoblastoma was not previously studied. On the other hand, 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 [22].

This study aims to:

Study the proliferative index (Ki-67-LI) and osteocalcin immunoexpression in osteoblastomas and osteosarcomas.

PATIENTS AND METHODS

This study included excisional and incisional biopsies sent to the Pathology Laboratories from the Orthopedic Departments of Sohag and Assiut Universities Hospitals through the period from January 2003 to June 2006. Cases include 9 osteoblastomas and 30 osteosarcomas; 27 osteoblastic osteosarcomas, two chondroblastic and one fibroblastic osteosarcoma.

H&E staining:

Five-micron tissue sections were prepared from the formalin-fixed, paraffin-embedded tissues, stained with Hematoxylin and Eosin (H&E) and examined using light microscope. Histological subtypes of osteosarcomas were defined as osteoblastic, chondroblastic, or fibroblastic.

Immunohistochemistry:

Immunostaining using peroxidase-labelled strepavidin-biotin technique to detect Ki-67 and osteocalcin was done for all cases. The following primary antibodies were used: Rabbit polyclonal antibody against human Ki-67 gene product (Catalogue; Cat # RB-9043-P0, 0.1ml, LABVISION Corporation) and mouse monoclonal antibody to Human/Bovine osteocalcin (Clone BD1152, Cat # H95152M, 0.1ml, BioDesign Corporation).

Staining procedure:

Five-micron tissue sections mounted on Poly-Lysine coated slides, deparaffinized and rehydrated. Endogenous peroxidase activity was blocked using peroxidase blocking reagent (Cat # TP-012-HD, LabVision Corporation). Unmask the antigen sites by immersing the slides in sufficient amounts of antigen retrieval solution (10 mmol sodium citrate buffer, pH 6.0) was done. 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 nonspecific interactions.

Tissue sections were incubated overnight at 4 C° in a humid chamber with 1/150 Ki-67 and 1/75 osteocalcin. The resulting immune-complex was detected by a universal staining kit (Cat # TP-012-HD, LabVision Corporation). Tissue sections were treated with biotinylated goat anti-polyvalent, and 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 controls were prepared from previously diagnosed breast carcinoma and normal bone tissue for detection of Ki-67 and osteocalcin staining respectively. ***Negative controls:*** Negative control was done by omitting the primary antibody from the staining procedure. The positive and negative controls were consistently immunoreactive and lacking reactivity. These findings therefore confirm the validity of our staining results.

Evaluation of immunostaining:

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

For quantitative evaluation of Ki-67 immunoreactivity, the number of Ki-67 positive nuclei in relation to the total number of tumor cells was counted in three different high power fields, and the average was calculated. Ki-67-LI was defined as the ratio of Ki-67 positive nuclei to the total number of tumor cells, and was expressed as a percentage.

Osteocalcin positivity was expressed as the mean percentage (%) of positive cells and the staining intensity in at least three different fields. Cells positive for osteocalcin were identified by the presence of both membranous and cytoplasmic

brownish staining. Semi-quantitation of osteocalcin immunoreactivities were calculated with a 12-point weighted score system.

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%.
2. Second, the staining intensity (SI) of positive signal was scored with a 3-point scale: 0 for negative, 1 for weak, 2 for medium, and 3 for intense staining [22-24].
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) [23,24].

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 Ki-67 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 were presented with a swelling or mass and/or an osteolytic bone lesion.

H&E staining:

Examination of the H&E (Figure 1 A-B) stained sections obtained from the lesions revealed the presence of marked nuclear pleomorphism and hyperchromatism in most cases of osteosarcoma (90% of cases). Cases of osteoblastoma showed mild to moderate pleomorphism with minimal or no hyperchromatism. Mitotic activity was prominent in cases of osteosarcoma, with abnormal mitoses found in 73% of cases of osteosarcoma. Many tumor giant cells were present in 43% of cases of osteosarcoma. About half of cases of osteosarcoma (47%) showed moderate to large areas of necrosis. Cases of osteosarcoma were classified as 27 osteoblastic osteosarcomas, two chondroblastic and one fibroblastic osteosarcoma.

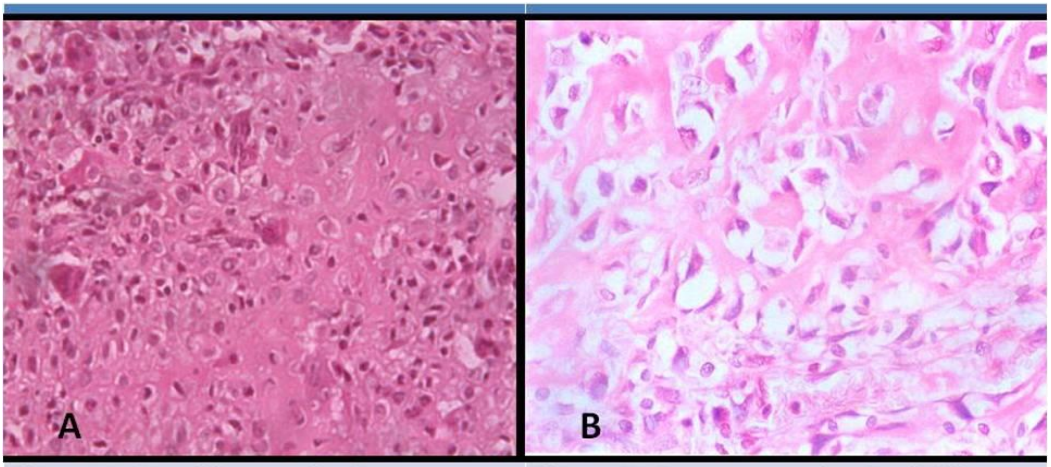


Figure 1 A: Osteoblastoma showing proliferating benign-appearing osteoblasts with osteoid production (H&E X200).

Figure 1 B: Osteosarcoma showing highly pleomorphic neoplastic cells with areas of osteoid production (H&E X400).

Immunohistochemical features

Ki-67 expression

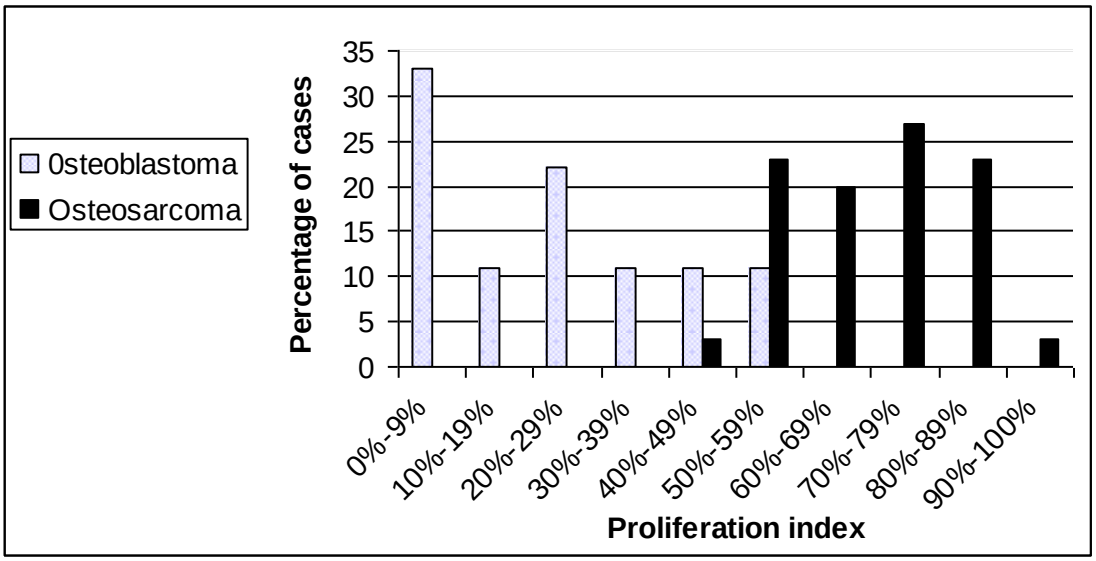
Ki-67-LI of the 9 cases of osteoblastomas ranged from 1% to 58%, with a mean of 23.7%. Ki-67-LI of the 30 cases of osteosarcoma ranged from 44% to 90%, with a mean proliferation index of 69.6%. The 10 cases of low grade osteosarcoma (including two chondroblastic and one fibroblastic ones) showed a mean Ki-67-LI of 58.8%. The mean Ki-67-LI of the 20 cases of high grade osteosarcomas was 74.9%. Ki-67-LI of osteosarcomas was significantly higher than that of osteoblastomas ($p<0.001$) as shown in Table 1, Figure 2 A-C and Graph 1.

Table (1): Proliferation index in osteosarcoma and osteoblastoma

	Osteoblastoma n = 9	**Osteosarcoma n = 30**
Mean Proliferation index (± SD)	23.7 (± 19.4)	69.6 (± 12.7)

($p<0.001$)

Graph (1): Showing the Proliferation index in osteoblastomas and osteosarcomas



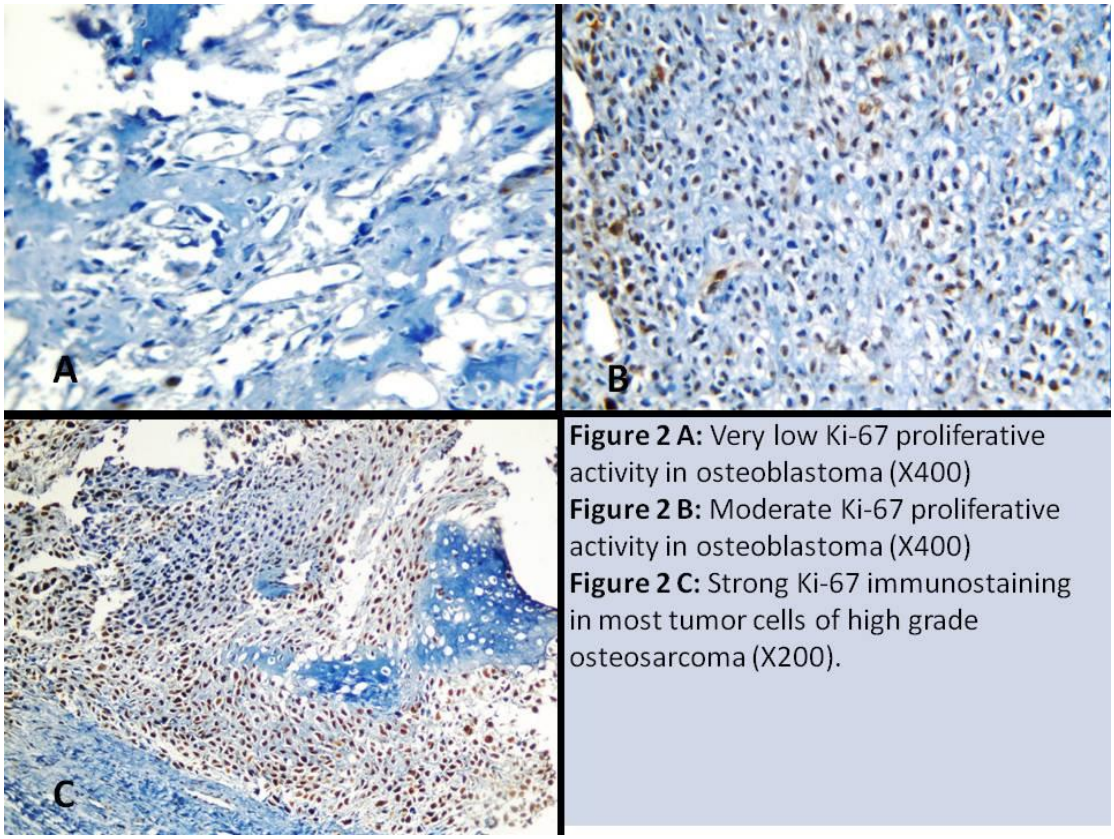


Figure 2 A: Very low Ki-67 proliferative activity in osteoblastoma (X400)
Figure 2 B: Moderate Ki-67 proliferative activity in osteoblastoma (X400)
Figure 2 C: Strong Ki-67 immunostaining in most tumor cells of high grade osteosarcoma (X200).

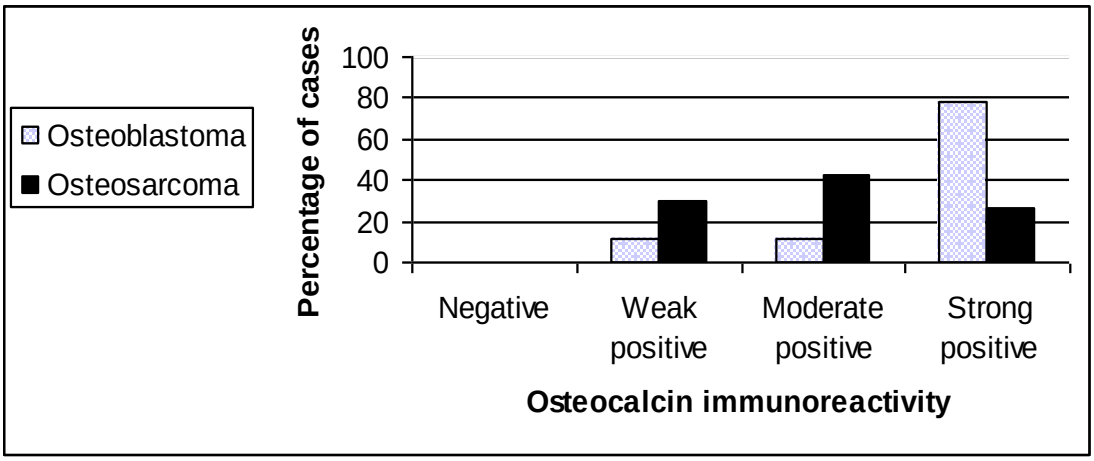
Osteocalcin expression

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 2 & Graph 2). 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. 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-B). 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 (2). 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 2 C-D). 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) as shown in Table (2).

Table (2): Osteocalcin expression in osteosarcoma and osteoblastoma

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

Graph 2: Showing osteocalcin expression in osteosarcoma and osteoblastoma



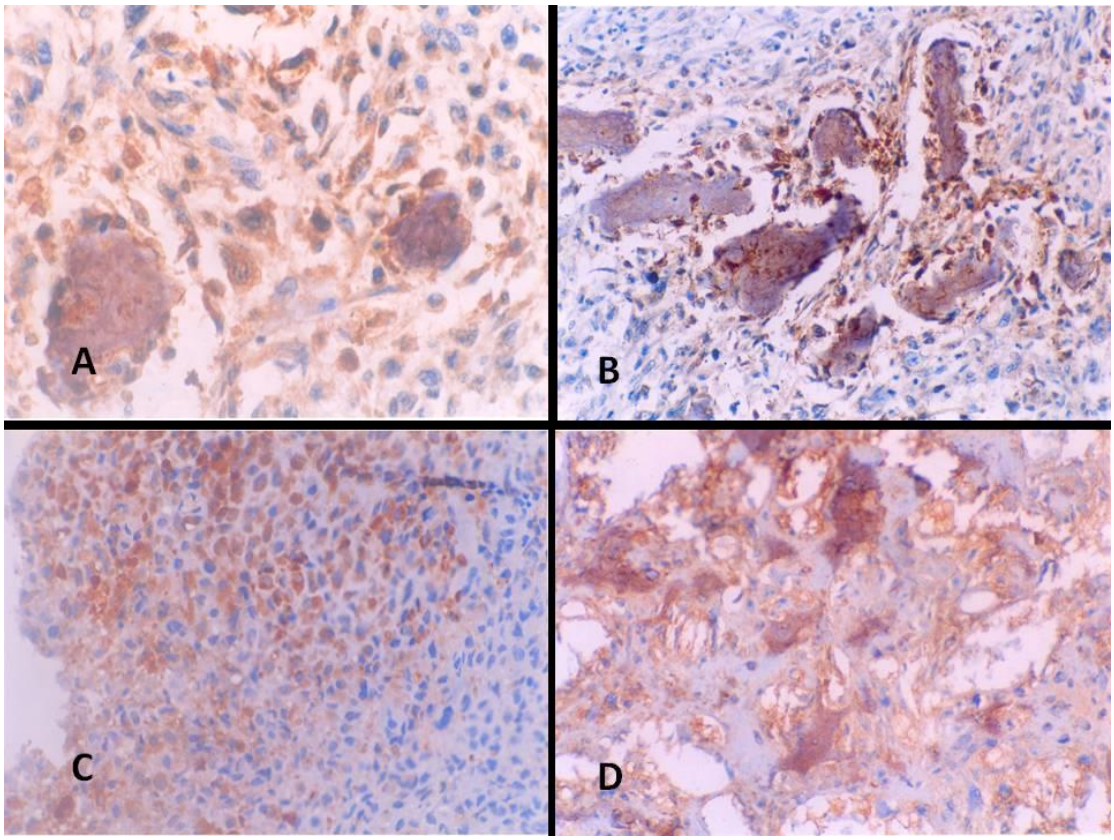


Figure (3): A- Osteosarcoma showing strong positive osteocalcin in both the cytoplasm of the osteoblasts and the osteoid tissue (X400). **B-** Lower power view showing osteocalcin-positive cells rimming and surrounding the areas of osteoid production in a case of osteosarcoma (X200). **C-** Osteoblastoma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

DISCUSSION

To achieve our aim, a total of 39 cases representing different types of bone forming tumors; osteoblastomas and osteosarcomas were examined for detection of Ki-67 and osteocalcin expression.

Osteoblastoma is a benign tumor that is composed of proliferating osteoblasts along with small trabeculae of woven bone and rich vascular fibrous stroma [11]. These neoplastic osteoblasts may occasionally have atypical features, with high cellularity and epithelioid appearances, making its distinction from osteosarcoma and other bone tumors difficult [18,19].

The current study showed a significant higher proliferation index in cases of osteosarcomas than in osteoblastomas. Many other studies showed high proliferation index in osteosarcomas, especially the high grade ones [25, 26]. However, to our knowledge, Ki-67 expression in osteoblastoma was not previously assessed. Our results suggest that estimation of the proliferation index may help in the distinction

between problematic cases of osteoblastomas and osteosarcomas, such as aggressive osteoblastomas and osteoblastoma-like osteosarcoma. This needs to be confirmed by further larger studies.

Consistent with many studies that demonstrate the high specificity and sensitivity of osteocalcin for osteoblasts [27, 28], 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 tumor cells and osteoid tissue in osteogenic bone tumors; osteosarcoma and osteoblastoma [29].

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 [30, 31]. Many authors considered osteocalcin as an osteoblast differentiation marker [32-35]. It is suggested that the intensity and distribution of osteocalcin immunostaining may give a clue to the degree of differentiation of the tumor cells (osteoblasts), and hence whether it is benign (osteoblastoma) or malignant (osteosarcoma).

Taking the results of immunoexpression of Ki-67 and osteocalcin together, it is concluded that the proliferation index is related to the degree of osteoblastic differentiation; being higher in the less differentiated osteoblasts of osteosarcoma (which showed lower degree of osteocalcin immunoreactivity than the more differentiated osteoblasts of osteoblastomas). In addition, estimation of the proliferation index may help in the distinction between osteoblastomas and osteosarcomas.

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