Evaluation of some histochemical and immunohistochemical criteria of round cell tumors of bone

Eman M. S. Muhammad[a], Zeinab H. El-badawi[a], Sana S. Kroosh[c]
and Hassan H. Noaman[b]

Departments of [a]Pathology, [b]Orthopedic Surgery,
Faculty of Medicine, University of Sohag, Sohag and
[c]Department of Pathology, Faculty of Medicine,
University of Assiut, 82524 Assiut, Egypt

Correspondence to Eman M. S. Muhammad,
Department of Pathology, Faculty of Medicine,
University of Sohag, Sohag, Egypt
Tel: + 02 093 460 2963; e-mail: e_salah@yahoo.com

Background/aims
The differential diagnosis of round cell tumors of bone (RCTB), Ewing sarcoma, small-cell osteosarcoma, mesenchymal chondrosarcoma, osteoblastoma, chondroblastoma, primary bone lymphoma, and multiple myeloma still remains a challenge. Given the significant differences in treatment, an accurate diagnosis is crucial. This study aimed to evaluate some histochemical and immunohistochemical criteria of RCTB.

Participants and methods
Periodic acid-Schiff (PAS), CD99, CD138, osteocalcin, and leukocyte common antigen (LCA) were evaluated in 113 patients with RCTB.

Results
PAS was positive in neoplastic cells of all Ewing sarcomas, 27% of osteosarcomas, 92% of chondrosarcomas, all osteoblastomas and chondroblastomas, and the osteoid tissue of all osteosarcomas and osteoblastomas. CD99 was positive in all Ewing sarcomas, in 11, 4, and 11% of osteoblastomas, multiple myelomas, and bone lymphomas, respectively. CD99 was higher in Ewing sarcoma than in other RCTB ($P < 0.0001$). Osteocalcin was positive in neoplastic cells of all osteosarcomas, osteoblastomas, and 20% of chondroblastomas, 84, and 78% of osteoid of osteosarcomas and osteoblastomas, respectively. CD138 was positive in all multiple myelomas, 12% of Ewing sarcomas, 20% of osteosarcomas, 44% of osteoblastomas, 8% of chondrosarcomas, and 40% of chondroblastomas. CD138 was higher in multiple myeloma ($P < 0.0001$) than in other RCTB. LCA positivity was higher ($P < 0.01$) in bone lymphomas (100%) than in multiple myelomas (73%).

Conclusion
PAS negativity excludes multiple myeloma and bone lymphoma from other RCTB that could be differentiated by LCA and CD138. CD99 positivity confirms the diagnosis of Ewing sarcoma. PAS could detect areas of osteoid in osteosarcoma and osteoblastoma. Osteocalcin suggests an osteogenic tumor origin: osteosarcoma/osteoblastoma. Double negativity of CD99 and osteocalcin suggests a chondrogenic tumor origin: chondrosarcoma/chondroblastoma.

Keywords:
CD138, CD99, leukocyte common antigen, osteocalcin, periodic acid-Schiff, round cell tumors of bone

Introduction

Round cell tumors of bone (RCTB) are a heterogeneous group of neoplasms with morphologically, biologically, and clinically specific features [1]. They include Ewing sarcoma, multiple myeloma, primary bone lymphoma, mesenchymal chondrosarcoma, and small-cell osteosarcoma [2,3]. In addition, osteoblastoma and chondroblastoma may show high cellularity and atypical features, simulating RCTB [4].

The diagnosis of these tumors is made mainly on the basis of clinical data, radiographic image, and histological features of hematoxylin and eosin (H&E)-stained sections. For example, the presence of osteoid in small-cell osteosarcoma and cartilage in mesenchymal chondrosarcoma are highly specific and diagnostic [5]. However, the identification of these specific features may be difficult in some cases of RCTB. A panel of immunohistochemical markers may be necessary to establish an accurate diagnosis [3].

Ewing sarcoma is a highly malignant primary bone tumor composed of small round cells with regular round nuclei. The combined use of CD99 immunostaining and periodic acid-Schiff (PAS) staining may be highly useful in the differentiation of Ewing sarcoma from other RCTB [6].

Small-cell osteosarcoma is a rare variant of osteosarcoma that is composed of small cells resembling those of Ewing sarcoma with recognizable osteoid. This type of tumor often results in difficulty in establishing a diagnosis when tissue samples do not include osteoid [7]. The micro-

scopic appearance of small-cell osteosarcoma may also mimic that of mesenchymal chondrosarcoma, which is a rare but aggressive, high-grade malignancy of primitive cartilage-forming mesenchyme [8]. Osteocalcin is a human bone protein that is sensitive and specific for both osteoid matrix and bone cells. It may be highly useful in the differentiation of small-cell osteosarcoma from mesenchymal chondrosarcoma and other RCTB [9].

Multiple myeloma is characterized by the accumulation of malignant plasma cells in bone [2]. CD138 is an excellent marker of plasmacytic differentiation and can be considered as a special antigen for myeloma cells [10]. Bone lymphoma is composed of malignant lymphoid cells, producing an infiltrative lesion within the bone. The differentiation of primary bone lymphoma from other RCTB may be difficult and leukocyte common antigen (LCA) immunostaining may be highly useful in the diagnosis of primary bone lymphoma and its differentiation from other RCTB [11].

In addition, osteoblastoma and chondroblastoma may show high cellularity and atypical features, simulating RCTB [4,12,13].

In this study, the evaluation of the histopathological criteria, histochemical (PAS), as well as the immunohistochemical detection of CD99, osteocalcin, CD138, and LCA was highly useful in the differentiation between different types of RCTB.

Participants and methods

This study included excisional and incisional biopsy specimens sent to the Pathology Department Laboratories from the Orthopedic Departments of Sohag and Assiut Universities Hospitals through the period from January 2003 to June 2006. All patients were informed about the study, which was approved by the Local Ethics Committee. Clinical evaluation included history taking, age, sex, and clinical presentation, and the site and size of bone lesions that were obtained from the referral clinical reports. These cases include 30 osteosarcomas, 22 multiple myelomas, 16 Ewing sarcomas, 15 chondroblastomas, 12 chondrosarcomas, nine osteoblastomas, and nine primary bone lymphomas. Five-micrometer tissue sections were prepared from the formalin-fixed, paraffin-embedded tissues and stained with H&E and examined using different magnifications of a light microscope to confirm their final diagnosis.

Periodic acid-Schiff staining

PAS staining was performed for all cases as follows: Schiff's reagent was prepared by adding 1 g of basic fuchsin to 200 ml of boiling distilled water and was left to cool. Potassium metabisulfite (1.5 g) was added to the solution, stored in a dark bottle, and left overnight in a dark cupboard. Activated charcoal (2.5 g) was added, shaken for 1–2 min, filtered, and stored in a dark bottle at 4°C temperature. Tissue sections were deparaffinized, hydrated, washed in distilled water, and oxidized in 1% aqueous periodic acid for 10–15 min and washed again.

Tissue sections were placed in Schiff's reagent for 20–30 min, and then placed in 1% potassium metabisulfite, stained with Myer's hematoxylin, dehydrated, and cleared in xylol. Sections were mounted and cover slipped using a drop of canada balsam. The positivity was visualized as bright Magenta-colored PAS-positive granules.

Immunohistochemistry

Immunostaining using the peroxidase-labeled streptavidin–biotin technique to detect CD99, osteocalcin, CD138, and LCA (CD45) was performed for all cases. The following primary antibodies were used: mouse monoclonal antibodies against human CD99 (Clone HO36-1.1, Catalogue # MS-294-R7; LabVision Corporation, Missouri, USA), human/bovine osteocalcin (Clone BD1152, Catalogue # H95152M, 0.1 ml; BioDesign Corporation, Thailand Science Park Inc., Thailand), human CD138 (Clone 5F7, Catalogue # MS-1769-S0, 0.1 ml; LabVision Corporation), and human CD45/LCA (Clone PAN-LCA, Catalogue # MS-413-P0, 0.1 ml; LabVision Corporation).

Staining procedure

Five-micrometer tissue sections were mounted on poly-lysine-coated slides, deparaffinized, and rehydrated. Endogenous peroxidase activity was blocked using a peroxidase blocking reagent (Catalogue # TP-012-HD; LabVision Corporation). The antigen sites were unmasked by immersing the slides in sufficient amounts of an antigen retrieval solution (10 mmol sodium citrate buffer, pH 6.0). Sections were microwaved at a power 750 W for 10 min, allowed to cool down for 15 min, and washed in distilled water, followed by PBS (pH 6.0). Tissue sections were incubated in normal goat serum to block nonspecific interactions.

Tissue sections were incubated overnight at 4°C in a humid chamber with sufficient amounts of ready-to-use CD99, 1/75 osteocalcin, and 1/100 CD138, and for 1 h at room temperature with 1/500 LCA. The resulting immune complex was detected using a universal staining kit (Catalogue # TP-012-HD; LabVision Corporation). Tissue sections were treated with biotinylated goat antipolyvalent and then peroxidase-labeled streptavidin was applied for 10–15 min at room temperature, rinsed in PBS, incubated with 14-diaminobenzidine and 0.06% H_2O_2 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, mounted with Canada balsam, and cover slipped.

Positive controls

Positive control slides were prepared from previously diagnosed Ewing sarcoma, normal bone tissue, tonsils, and reactive hyperplasia of the lymph node for the detection of CD99, osteocalcin, CD138, and LCA staining, respectively.

Negative controls

For the negative control, the primary antibody was excluded from the staining procedure.

Evaluation of immunostaining

Sections were histologically examined using a bright-field microscope at a low-power magnification (×40 and ×100) to detect the sites of antibody positivity, and then by higher power magnification (×200 and ×400) to evaluate the immunostaining.

CD99, osteocalcin, CD138, and LCA positivity were expressed as the mean percentage (%) of positive cells and staining intensity in at least three different fields. Only membranous brownish staining for CD99 was considered as true positivity. Positive osteocalcin brownish staining was present in both the bone matrix and the cytoplasm of osteoid cells. Cells positive for CD138 were identified by the presence of both membranous and cytoplasmic brownish staining. Positivity for LCA was identified as a brownish-stained ring covering the cell surface membrane.

Semiquantification of CD99, osteocalcin, CD138, and LCA immunoreactivities was carried out using a 12-point weighted score system [14].

First, the percentage of positive cells was scored using a five-point scale: 0 for less than 5%, 1 for 5–25%, 2 for 25–50%, 3 for 50–75%, and 4 for over 75%.

Second, the intensity of positive staining was scored using a three-point scale: 0 for negative, 1 for weak, 2 for medium, and 3 for intense staining [15,16].

Then, the average weighted score was calculated by multiplying the percentage of positive cells by their staining intensity. The results were scored as negative (0–1), weak (2–3), moderate (4–6), and strong (8–12) [14,17].

Statistical analysis

Results were analyzed statistically using the statistical package for social sciences for Windows (Chicago, USA). The χ^2-test and analyses of variance were used to assess the statistical significance of the relationships of CD99, osteocalcin, CD138, and LCA expression among the different types of RCTB.

Results

Clinical features

Histopathological features

The study group included 113 patients, with 64/113(57%) males and 50/113(43%) females, ranging in age from 4 to 70 years. The initial histological examination of H&E-stained sections showed the following: 30 osteosarcomas, 22 multiple myelomas, 16 Ewing sarcomas, 15 chondroblastomas, 12 chondrosarcomas, nine osteoblastomas, and nine primary bone lymphomas (Fig. 1a–h).

Periodic acid-Schiff staining

All cases of Ewing sarcomas showed positive cytoplasmic PAS staining that was strongly diffuse in 69%, moderately diffuse in 6%, moderately focal in 13%, and mildly focal in 12%. Neoplastic osteoblasts showed PAS-positive cytoplasmic material in 27% of osteosarcoma and in all cases of osteoblastoma. Neoplastic chondrocytes showed positive cytoplasmic PAS staining in all cases of chondroblastoma and chondrosarcoma, except for a single case of dedifferentiated chondrosarcoma. The osteoid matrix in all cases of osteoblastoma and osteosarcoma and the chondroid matrix in 80% of chondroblastomas and 33% of chondrosarcomas indicated positive PAS staining. No PAS-positive material was found in all cases of multiple myeloma and of bone lymphoma (Table 1, Fig. 2a–f).

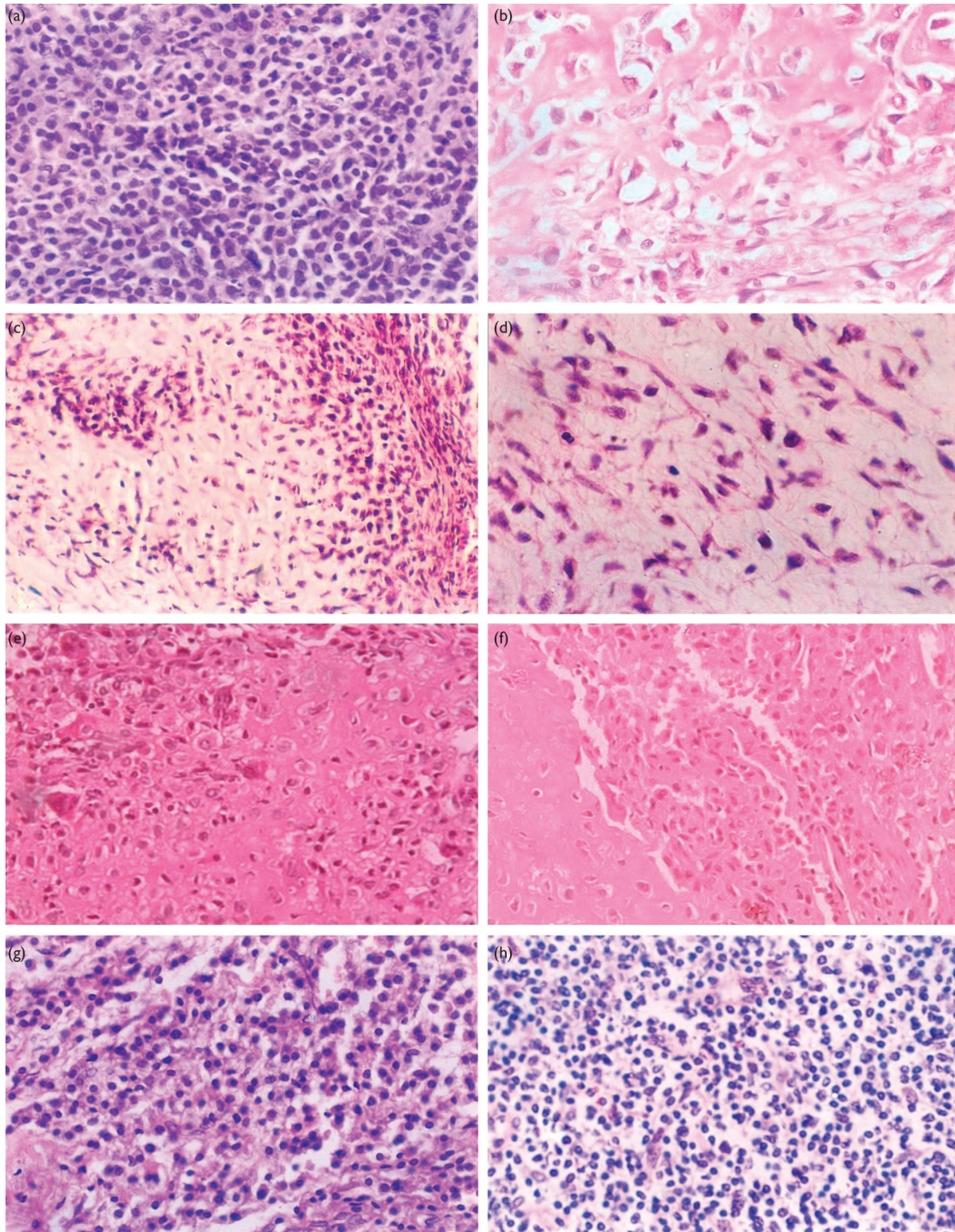### Immunohistochemical examination

CD99 expression

All cases of Ewing sarcomas showed positive CD99, with varying degrees of immunoreactivity: strong in 50%, moderate in 31%, and weak in 19%. Weak CD99 immunoreactivity was found in 11% of osteoblastoma, 4% of multiple myeloma, and 11% of bone lymphoma. However, all cases of osteosarcomas, chondrosarcomas, and chondroblastomas showed no immunoreactivity to CD99. CD99 expression in Ewing sarcomas (100%) was significantly higher ($P < 0.0001$) than that in other RCTB (3%) (Table 2, Fig. 3a).

Osteocalcin expression

All cases of osteosarcomas were positive for osteocalcin in malignant osteoblasts, with varying degrees of immunoreactivity: strong in 27%, moderate in 43%, and weak in 30% of cases. Osteocalcin was strong in 47% and mild to moderate in 37% in osteoid tissue of osteosarcomas. All cases of osteoblastomas were positive for osteocalcin in their neoplastic osteoblasts, which was strong in 78%, moderate in 11%, and weak in 11% of cases. Strong osteocalcin was found in 33% and mild to moderate in 45% of osteoid of osteoblastoma. The percentage of strong osteocalcin expression was significantly higher ($P < 0.02$) in osteoblastomas (78%) than that in osteosarcomas (27% of cases). The expression of osteocalcin in osteosarcomas and osteoblastomas (100%) was significantly higher ($P < 0.0001$) than that in other RCTB (4%). All cases of chondrosarcomas showed no osteocalcin immunoreactivity. Alternatively, osteocalcin was weak in 13% and moderate in 7% of chondroblastoma (Table 3, Fig. 2b and c).

CD138 expression

All cases of multiple myelomas were positive for CD138, with varying degrees of immunoreactivity: strong in 36%, moderate in 46%, and weak in 18% of the cases. CD138 expression in multiple myelomas (100%) was significantly higher ($P < 0.0001$) than that in other RCTB (21%). CD138 immunoreactivity was weak in 12% of cases of Ewing sarcoma, whereas CD138 was moderate in 7% and weak in 13% of cases of osteosarcoma. CD138 was weak in 11%, moderate in 22%, and strong in 11% of cases of osteoblastoma. Only a single case (8%) of chondrosarcoma showed weak CD138 immunoreactivity, whereas 13% of chondroblastoma showed weak CD138 and 27% showed moderate CD138 (Table 4, Fig. 2d).

Figure 1

Hematoxylin and eosin staining: (a) Ewing sarcoma (uniform small round cells with regular round nuclei, inconspicuous nucleoli, a narrow rim of a clear or a pale eosinophilic cytoplasm, and indistinct cytoplasmic membranes, × 400). (b) Osteosarcoma (pleomorphic neoplastic cells with areas of osteoid production, × 400). (c) Chondrosarcoma (part of a lobule of malignant cartilage, × 200). (d) Chondrosarcoma (atypical chondrocytes with considerable cellular pleomorphism and hyperchromatic nuclei, × 400). (e) Osteoblastoma (proliferating benign-appearing osteoblasts with osteoid production, × 200). (f) Chondroblastoma (round to polygonal tumor cells with an area of chondroid differentiation, × 200). (g) Multiple myeloma (neoplastic plasma cells with eccentric hyperchromatic nuclei, × 400). (h) Non-Hodgkin's primary bone lymphoma (mixture of neoplastic small, medium, and large lymphocytes, × 400).

Table 1 Periodic acid-Schiff staining in round cell tumors of bone

	ES ($n=16$)	MM ($n=22$)	PBL ($n=9$)	OS ($n=30$)	CS ($n=12$)	OB ($n=9$)	CB ($n=15$)
Tumor cells							
Positive	16/16 (100%)	–	–	8/30 (27%)	11/12 (92%)	9/9 (100%)	15/15 (100%)
Negative		22/22 (100%)	9/9 (100%)	22/30 (73%)	1/12 (8%)	–	
Stroma							
Positive				30/30 (100%)	4/12 (33%)	9/9 (100%)	12/15 (80%)
Negative	16/16 (100%)	22/22 (100%)	9/9 (100%)	–	8/12 (67%)	–	3/15 (20%)

CB, chondroblastomas; CS, chondrosarcomas; ES, Ewing sarcomas; MM, multiple myelomas; OB, osteoblastomas; OS, osteosarcomas; PBL, primary bone lymphomas.

Leukocyte common antigen expression
All cases of primarybone lymphomas were positive for LCA, which was strong in 89% of cases and moderate in a single case (11%). About 73% of cases of multiple myeloma showed mild scarcely found to moderate immunoreactivity to LCA. The average percentage of tumor cells positive for LCA was significantly higher in bone lymphomas than in multiple myelomas ($P<0.01$). All cases of Ewing sarcomas, osteosarcomas, osteoblastomas, chondrosarcomas, and chondroblastomas were negative for LCA. The expression of LCA in primary bone lymphoma (100%) was significantly higher ($P<0.0001$) than that in other RCTB (15%) (Table 5, Fig. 2e and f).

Discussion

In this study, it was hypothesized that the evaluation of the histopathological criteria, as well as the detection of CD99, osteocalcin, CD138, and LCA in RCTB may be highly useful in their accurate diagnosis and in the differentiation of the different types. To test this hypothesis, a total of 113 cases representing different types of RCTB were examined for their histopathological and histochemical PAS staining, as well as the immunohistochemical expression of CD99, osteocalcin, CD138, and LCA. Because of the rarity of small-cell osteosarcoma and mesenchymal chondrosarcoma, their conventional counterparts were examined to assess the immunohistochemical features of their malignant cells.

In agreement with Goto and colleagues [18,19], bright red PAS-positive cytoplasmic materials were found in all cases of Ewing sarcoma because of the presence of glycogen granules in the cytoplasm of Ewing sarcoma cells. Positive PAS staining was also evident in the osteoid of all cases of osteosarcoma and osteoblastoma. The neoplastic osteoblasts showed PAS-positive cytoplasmic materials in eight cases of osteosarcoma and all cases of osteoblastoma. This finding is in agreement with other studies, which showed positive PAS staining in the newly formed bone [20]. This PAS positivity is because of the presence of many bone matrix glycoproteins, such as osteonectin, osteopontin, fibronectin, fibrillin, thrombospondin, bone sialoprotein, and collagens II, III, V, and XI [21]. Thus, PAS staining may be highly useful in detecting areas of osteoid production in cases of osteosarcoma and osteoblastoma, especially in cases with minimal osteoid production or in those cases in which the nature of the tumor matrix cannot be identified on H&E-stained sections.

PAS-positive material was observed in the cytoplasm of the neoplastic chondrocytes in 92% cases of chondrosarcomas and all chondroblastomas. This is in agreement with Chikata *et al.* [22], who observed PAS-positive glycogen granules in the cytoplasm of chondrocytes. The chondroid matrix stained positively with PAS in 33% of cases of chondrosarcoma and in 80% of cases of chondroblastoma. This indicates the presence of glycoproteins in the chondroid matrix [23,24].
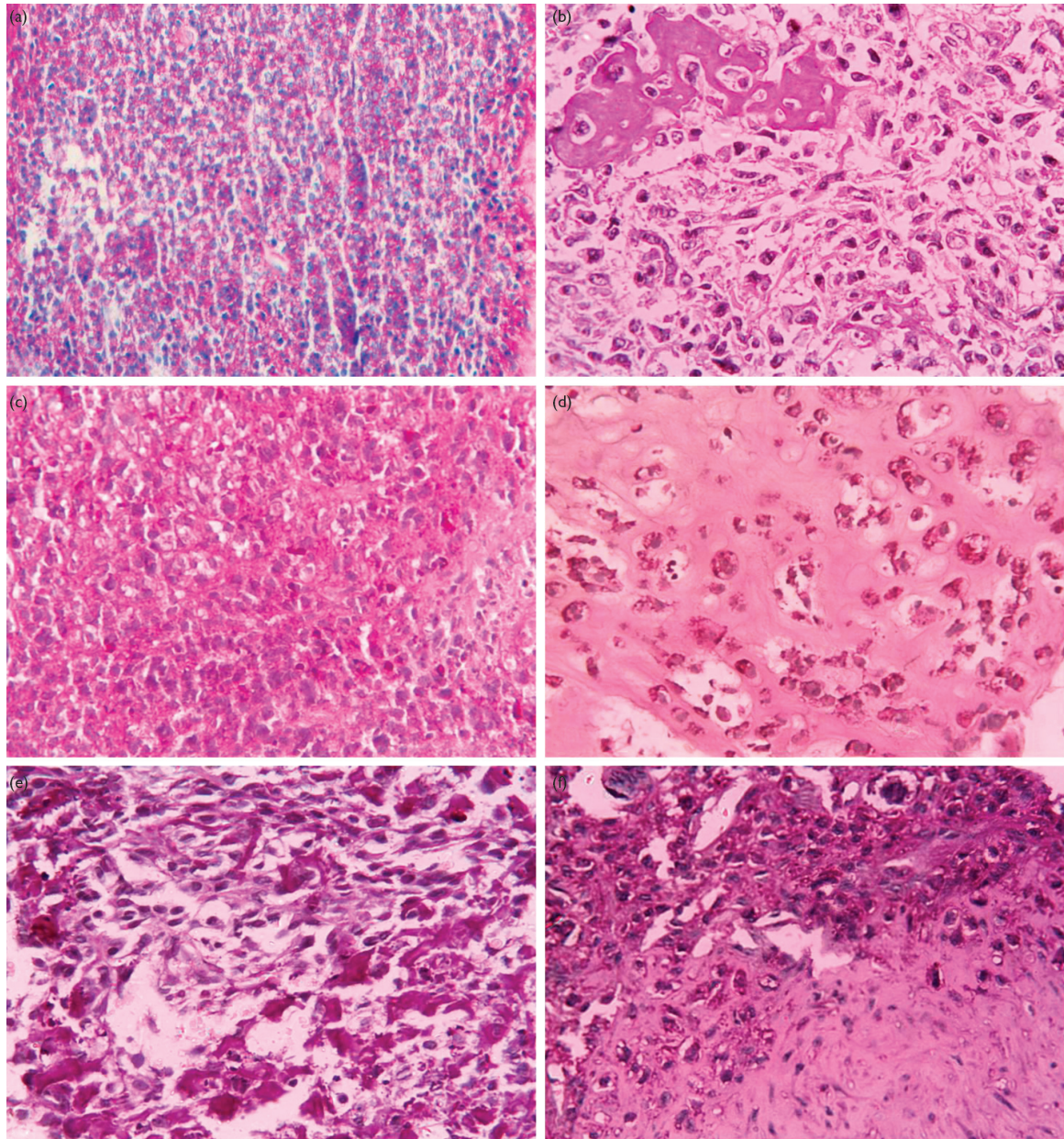
No PAS-positive material was found in all cases of multiple myeloma and bone lymphoma. These findings suggest that PAS staining of RCTB may be useful in the exclusion of multiple myeloma and bone lymphoma from the differential diagnosis of RCTB. However, the neoplastic cells of Ewing sarcoma, osteosarcoma, osteoblastoma, chondrosarcoma, and chondroblastoma may stain positively with PAS, and therefore, immunohistochemistry is recommended to differentiate between these types of RCTB in the absence of their specific histological features.

In agreement with previous reports, our results indicated that all cases of Ewing sarcoma showed positive CD99, with varying degrees of immunoreactivity. This means that CD99 is 100% sensitive for Ewing sarcoma [25–27]. Only a single case of multiple myeloma showed weak CD99 immunoreactivity. This result is comparable with the findings of Kumar *et al.* [28], who detected CD99 positivity in five of 30 (17%) patients with multiple myeloma.

In agreement with Sung *et al.* [29], who showed that CD99 is infrequently expressed in mature B-cell and T-cell lymphomas, we found weak CD99 immunoreactivity in a single case of primary bone lymphoma. This indicated that CD99 is a valuable marker in the distinction between Ewing sarcoma and mature B-cell and T-cell lymphomas. However, a number of studies have shown a high expression of CD99 in lymphoblastic lymphoma/leukemia [29,30].

No CD99 immunoreactivity was found in all cases of osteosarcoma, whereas only a single case of osteoblastoma showed weak CD99 immunoreactivity. This is in agreement with Manara *et al.* [31], who found high CD99 expression in normal osteoblasts and very low CD99 expression in neoplastic osteoblasts of osteosarcoma.

Figure 2

Periodic acid-Schiff staining: (a) Ewing sarcoma (positive cytoplasmic granules in tumor cells, × 200). (b) Osteosarcoma (positive staining in areas of osteoid production, × 400). (c) Osteosarcoma (positive staining in the cytoplasm of malignant osteoblasts, × 400). (d) Chondrosarcoma (positive staining in the cytoplasm of neoplastic chondrocytes, × 200). (e) Osteoblastoma (positive staining in both the cytoplasm of osteoblasts and areas of osteoid production, × 200). (f) Chondroblastoma (positive granules in the cytoplasm of tumor cells, × 200).

In addition, Manara *et al.* [31] showed that the level of expression was higher in normal osteoblasts that line the bone surface compared with less differentiated osteoblasts included in immature bone matrix, proliferating, and migrating osteoblasts. This pattern of expression is in agreement with the role of CD99 in cell adhesion processes. This suggests that CD99 expression is down-regulated in osteosarcoma and it would be highly useful in the differentiation of Ewing sarcoma from small-cell osteosarcoma. This finding needs to be confirmed in a larger study.

All cases of chondrosarcoma and chondroblastoma showed no immunoreactivity to CD99. However, many other studies reported strong membranous expression of CD99 in the round cell component of mesenchymal

Table 2 CD99 expression in round cell tumors of bone

	ES (n=16)	MM (n=22)	PBL (n=9)	OS (n=30)	CS (n=12)	OB (n=9)	CB (n=15)
Staining intensity							
0 (negative)	–	12/22 (55%)	7/9 (78%)	30/30 (100%)	11/12 (92%)	8/9 (89%)	11/15 (73%)
1 (mild)	3/16 (19%)	10/22 (45%)	2/9 (22%)	–	1/12 (8%)	1/9 (11%)	4/15 (27%)
2 (moderate)	9/16 (56%)	–	–	–	–	–	–
3 (strong)	4/16 (25%)	–	–	–	–	–	–
% of positive cells							
0 (0–5%)	–	15/22 (68%)	7/9 (78%)	30/30 (100%)	12/12 (100%)	8/9 (89%)	14/15 (94%)
1 (5–25%)	–	6/22 (27%)	1/9 (11%)	–	–	–	1/15 (6%)
2 (25–50%)	4/16 (25%)	1/22 (4%)	1/9 (11%)	–	–	1/9 (11%)	–
3 (50–75%)	6/16 (37%)	–	–	–	–	–	–
4 (>75%)	6/16 (37%)	–	–	–	–	–	–
AWS							
0–1 (negative)	–	21/22 (96%)	8/9 (89%)	30/30 (100%)	12/12 (100%)	8/9 (89%)	15/15 (100%)
2–3 (weak)	3/16 (19%)	1/22 (4%)	1/9 (11%)	–	–	1/9 (11%)	–
4–6 (moderate)	5/16 (31%)	–	–	–	–	–	–
8–12 (strong)	8/16 (50%)	–	–	–	–	–	–

AWS, average weighted score; CB, chondroblastomas; CS, chondrosarcomas; ES, Ewing sarcomas; MM, multiple myelomas; OB, osteoblastomas; OS, osteosarcomas; PBL, primary bone lymphomas.

chondrosarcoma, but not in its cartilaginous component [32–34]. This discrepancy may be attributed to the small sample size of chondrosarcoma in this study (12 cases). In addition, all cases of chondrosarcoma included in this study were of the conventional type, as no cases of mesenchymal chondrosarcoma were included in this work. It is suggested that mesenchymal chondrosarcoma cannot be distinguished from Ewing sarcoma on the basis of CD99 immunoreactivity [35]. In agreement with the current study, Hong et al. [36] observed negative CD99 expression in five cases of chondroblastoma.

On examination of CD99 expression in Ewing sarcoma in relation to other RCTB, it was significantly higher ($P < 0.0001$) in cases of Ewing sarcoma (100%) than in other cases of RCTB (3%). This finding indicates that CD99 is highly useful in the differentiation of Ewing sarcoma from other RCTB, except mesenchymal chondrosarcoma and lymphoblastic lymphoma. In addition, PAS negativity in lymphoblastic lymphoma and the histological features of the cartilage in mesenchymal chondrosarcoma are important in the differentiation of these tumors from Ewing sarcoma [5,37].

However, some cases of lymphoblastic lymphoma may be PAS positive [38], and therefore, other immunohistochemical markers may be required in the differentiation of lymphoblastic lymphoma and Ewing sarcoma such as LCA (CD45), CD43, and TdT.

On examination of the expression of osteocalcin in RCTB, negative immunoreactivity was found in all cases of Ewing sarcoma, multiple myeloma, and bone lymphoma. Similarly, all cases of chondrosarcoma showed no immunoreactivity for osteocalcin. However, all cases of osteosarcoma and osteoblastoma showed positive immunostaining for osteocalcin in the cytoplasm of the osteoblasts, with varying degrees of immunoreactivity. These findings are in agreement with many other studies that show high specificity and sensitivity of osteocalcin for osteoblasts [39–42].

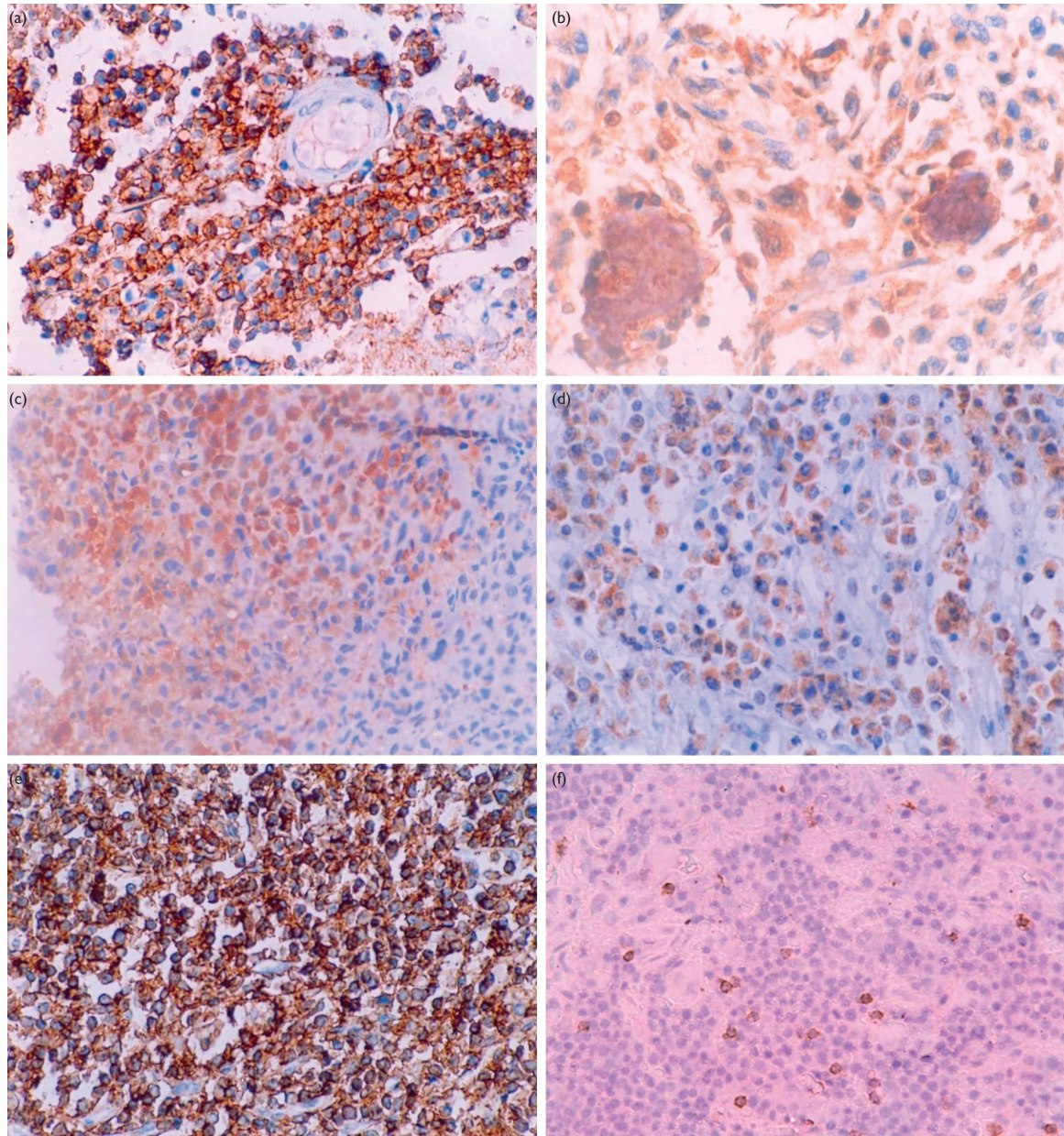Osteocalcin staining intensity in the osteoid of osteosarcoma and osteoblastoma was variable, with most cases (about 81%) showing positive osteocalcin immunoreactivity in the osteoid. This is in agreement with many studies that show a high expression of osteocalcin in both the tumor cells and the osteoid tissue in osteogenic bone tumors such as osteosarcoma and osteoblastoma [43,44]. However, no immunostaining for osteocalcin was found in the osteoid of about 19% of cases of osteosarcoma and osteoblastoma. This lack of osteocalcin expression could not be explained, and there is no explanation for this in the available literature.

The current study showed that the percentage of cases with strong immunostaining for osteocalcin was significantly higher ($P < 0.02$) in osteoblastoma (78%) than in osteosarcoma (27%). Many authors consider osteocalcin as an osteoblastic differentiation marker [45–50]. Our finding suggests that osteocalcin is more expressed in well-differentiated osteoblasts of osteoblastoma than in less differentiated osteoblasts of osteosarcoma. In addition, we 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 finding indicates 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, in agreement with the study of Takahashi et al. [51]. Our finding suggested that the degree of osteocalcin immunopositivity may indicate the degree of differentiation of tumor cells (osteoblasts), and hence whether it is benign (osteoblastoma) or malignant (osteosarcoma).

Osteocalcin immunoreactivity was weak in two cases and moderate in a single case of chondroblastoma. In agreement with this finding, Park et al. [52] found focal expression of osteocalcin in 90% of studied chondroblastomas. This suggests that chondroblastoma may show histological diversity with the focal coexpression of chondroid and osteoid markers.

The expression of osteocalcin in osteosarcoma and osteoblastoma (100%) is significantly higher ($P < 0.0001$) than that in other RCTB (4%). This finding confirms that

Figure 3

Immunostaining of (a) CD99 in Ewing sarcoma (strong membranous immunostaining, × 400). (b) Osteocalcin in osteosarcoma (strong immunostaining appearing as a brownish color in the cytoplasm of both osteoblasts and the osteoid tissue, × 400). (c) Osteocalcin in osteoblastoma (positive in the cytoplasm of neoplastic osteoblasts, × 200). (d) CD138 in multiple myeloma (brownish membranous and cytoplasmic immunoreactions of tumor cells, × 400). (e) leukocyte common antigen (LCA) in Primary Non-Hodgkin's bone lymphoma (strong membranous staining for LCA, × 400). (f) LCA in multiple myeloma (focal positive immunoreactivity in some scattered tumor cells, × 200).

the detection of osteocalcin could be important in the differential diagnosis of RCTB, especially in small biopsies, in which osteoid production cannot be identified. In addition, the intensity and distribution of osteocalcin immunostaining may indicate the degree of osteoblastic differentiation.

The current study showed positive CD138 immunostaining in all cases of multiple myeloma, with varying degrees of immunoreactivity. CD138 in multiple myeloma (100%) is significantly higher ($P < 0.0001$) than that in other RCTB (21%). This finding is in agreement with other studies, in which CD138 has been reported to be a highly

Table 3 Osteocalcin expression in round cell tumors of bone

	ES (*n*=16)	MM (*n*=22)	PBL (*n*=9)	OS (*n*=30)	CS (*n*=12)	OB (*n*=9)	CB (*n*=15)
Staining intensity							
0 (negative)	16/16 (100%)	21/22 (95%)	7/9 (78%)	–	8/12 (67%)	–	4/15 (27%)
1 (mild)	–	1/22 (5%)	2/9 (22%)	10/30 (33%)	4/12 (33%)	1/9 (11%)	9/15 (60%)
2 (moderate)	–	–	–	14/30 (47%)	–	3/9 (33%)	2/15 (13%)
3 (strong)	–	–	–	6/30 (20%)	–	5/9 (56%)	–
% of positive cells							
0 (0–5%)	16/16 (100%)	22/22 (100%)	8/9 (89%)	–	11/12 (92%)	–	11/15 (73%)
1 (5–25%)	–	–	1/9 (11%)	1/30 (3%)	1/12 (8%)	–	2/15 (13%)
2 (25–50%)	–	–	–	6/30 (20%)	–	1/9 (11%)	2/15 (13%)
3 (50–75%)	–	–	–	15/30 (50%)	–	2/9 (22%)	–
4 (>75%)	–	–	–	8/30 (27%)	–	6/9 (67%)	–
AWS							
0–1 (negative)	16/16 (100%)	22/22 (100%)	9/9 (100%)	–	12/12(100%)	–	12/15 (80%)
2–3 (weak)	–	–	–	9/30 (30%)	–	1/9 (11%)	2/15 (13%)
4–6 (moderate)	–	–	–	13/30 (43%)	–	1/9 (11%)	1/15 (7%)
8–12 (strong)	–	–	–	8/30 (27%)	–	7/9 (78%)	–

AWS, average weighted score; CB, chondroblastomas; CS, chondrosarcomas; ES, Ewing sarcomas; MM, multiple myelomas; OB, osteoblastomas; OS, osteosarcomas; PBL, primary bone lymphomas.

Table 4 CD138 expression in round cell tumors of bone

	ES (*n*=16)	MM (*n*=22)	PBL (*n*=9)	OS (*n*=30)	CS (*n*=12)	OB (*n*=9)	CB (*n*=15)
Staining intensity							
0 (negative)	11/16 (69%)	–	8/9 (89%)	–	–	–	–
1 (mild)	5/16 (31%)	4/22 (18%)	1/9 (11%)	19/30 (63%)	9/12 (75%)	5/9 (56%)	6/15 (40%)
2 (moderate)	–	14/22 (64%)	–	9/30 (30%)	3/12 (25%)	1/9 (11%)	5/15 (33%)
3 (strong)	–	4/22 (18%)	–	2/30 (7%)	–	3/9 (33%)	4/15 (27%)
% of positive cells							
0 (0–5%)	11/16 (69%)	–	8/9 (89%)	19/30 (63%)	9/12 (75%)	5/9 (56%)	6/15 (40%)
1 (5–25%)	3/16 (19%)	–	1/9 (11%)	5/30 (17%)	2/12 (17%)	–	3/15 (20%)
2 (25–50%)	–	6/22 (27%)	–	4/30 (13%)	1/12 (8%)	1/9 (11%)	5/15 (33%)
3 (50–75%)	2/16 (12%)	9/22 (41%)	–	2/30 (7%)	–	2/9 (22%)	1/15 (6%)
4 (>75%)	–	7/22 (32%)	–	–	–	1/9 (11%)	–
AWS							
0–1 (negative)	14/16 (88%)	–	9/9 (100%)	24/30 (80%)	11/12 (92%)	5/9 (56%)	9/15 (60%)
2–3 (weak)	2/16 (12%)	4/22 (18%)	–	4/30 (13%)	1/12 (8%)	1/9 (11%)	2/15 (13%)
4–6 (moderate)	–	10/22 (46%)	–	2/30 (7%)	–	2/9 (22%)	4/15 (27%)
8–12 (strong)	–	8/22 (36%)	–	–	–	1/9 (11%)	–

AWS, average weighted score; CB, chondroblastomas; CS, chondrosarcomas; ES, Ewing sarcomas; MM, multiple myelomas; OB, osteoblastomas; OS, osteosarcomas; PBL, primary bone lymphomas.

Table 5 Leukocyte common antigen expression in round cell tumors of bone

	ES (*n*=16)	MM (*n*=22)	PBL (*n*=9)	OS (*n*=30)	CS (*n*=12)	OB (*n*=9)	CB (*n*=15)
Staining intensity							
0 (negative)	16/16 (100%)	1/22 (5%)	–	30/30 (100%)	12/12 (100%)	9/9 (100%)	15/15 (100%)
1 (mild)	–	4/22 (18%)	–	–	–	–	–
2 (moderate)	–	12/22 (54%)	4/9 (44%)	–	–	–	–
3 (strong)	–	5/22 (23%)	5/9 (56%)	–	–	–	–
% of positive cells							
0 (0–5%)	16/16 (100%)	5/22 (23%)	–	30/30 (100%)	12/12 (100%)	9/9 (100%)	15/15 (100%)
1 (5–25%)	–	13/22 (59%)	–	–	–	–	–
2 (25–50%)	–	4/22 (18%)	–	–	–	–	–
3 (50–75%)	–	–	1/9 (11%)	–	–	–	–
4 (>75%)	–	–	8/9 (89%)	–	–	–	–
AWS							
0–1 (negative)	16/16 (100%)	6/22 (27%)	–	30/30 (100%)	12/12 (100%)	9/9 (100%)	15/15 (100%)
2–3 (weak)	–	13/22 (59%)	–	–	–	–	–
4–6 (moderate)	–	3/22 (14%)	1/9 (11%)	–	–	–	–
8–12 (strong)	–	–	8/9 (89%)	–	–	–	–

AWS, average weighted score; CB, chondroblastomas; CS, chondrosarcomas; ES, Ewing sarcomas; MM, multiple myelomas; OB, osteoblastomas; OS, osteosarcomas; PBL, primary bone lymphomas.

sensitive and specific marker of multiple myeloma cells in bone marrow biopsies. Therefore, CD138 is a marker that allows the unequivocal identification of plasma cells among other hematopoietic cells [15,53]. In addition, it allows an excellent assessment of plasma cell numbers and distribution in bone marrow biopsies [54].

However, all cases of primary bone lymphoma showed no CD138 immunoreactivity. This finding is consistent with that of other researchers, who found negative CD138 immunostaining in all cases of non-Hodgkin's lymphomas lacking plasmacytoid differentiation [10,55] and in 98% of cases of diffuse large B-cell lymphomas [56]. Alternatively, other studies have reported positive CD138 immunoreactivity in many Hodgkin lymphomas with classic Reed–Sternberg cells [57,58], plasmoblastic lymphomas [59,60], and certain AIDS-related lymphomas [61]. In addition, positive CD138 immunoreactivity was found to be a poor prognostic marker in AIDS-related B-cell lymphoma [61] and in diffuse large B-cell lymphomas [62].

In this series, moderate CD138 immunoreactivity was observed in four cases of chondroblastoma, two cases of osteosarcoma, and two cases of osteoblastoma. In addition, weak CD138 was found in four cases of osteosarcoma, two cases of Ewing sarcoma, two cases of chondroblastoma, and a single case of osteoblastoma and chondrosarcoma. These findings are in agreement with other studies that show CD138 immunoreactivity in many nonhematopoietic neoplasms, including prostate adenocarcinoma [63], renal cell carcinoma [64], squamous cell carcinoma [65], and some cases of breast carcinoma [66,67]. In addition, it was found that a variety of undifferentiated epithelial and mesenchymal neoplasms may also express CD138 [65].

It is obvious that CD138 is an excellent marker of plasmacytic differentiation within the hematolymphoid system. However, on the basis of its broad staining profile, CD138 reactivity for neoplastic cells is not a definitive marker for plasmacytic derivation, unless a hematolymphoid origin has been established [10]. Therefore, the expression of CD138 should be interpreted with extreme caution in the setting of an undifferentiated neoplasm [65].

Immunoreactivity for LCA was found in all cases of primary bone lymphoma that was strong in 89% of cases. However, all cases of Ewing sarcoma, osteosarcoma, osteoblastoma, chondrosarcoma, and chondroblastoma were negative for LCA. The expression of LCA in primary bone lymphoma is significantly higher ($P < 0.0001$) than that in other RCTB. These findings are in agreement with those of other studies, which reported that LCA is highly specific for non-Hodgkin's lymphomas and that LCA-expressing nonhematopoietic tumors are very rare [67–70].

Mild to moderate immunoreactivity for LCA was found in only scattered tumor cells of about 73% of cases of multiple myeloma, in agreement with Moreau et al. [71] who observed positive LCA immunoreactivity in a small population of myeloma cells, which correspond to the most proliferative myeloma cells. LCA expression decreases, but may remain detectable (CD45-positive myeloma) or becomes undetectable (CD45-negative myeloma). This can be attributed to the fact that LCA is usually expressed in normal immature proliferative plasma cells, but is weakly expressed in mature resting plasma cells o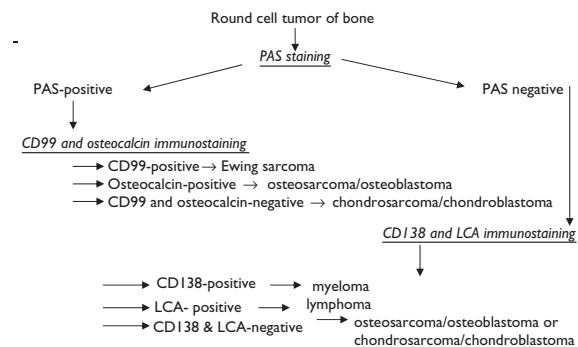f the bone marrow [72]. It has been shown that CD45-negative myeloma cells have a greater capacity to circulate, disseminate, and clone [71].

There are clinical and experimental studies suggesting that the CD45-negative phenotype is the phenotype of progressive myeloma and is of negative prognostic value when present at diagnosis [73]. The poor outcome of CD45-negative myeloma patients could be related to the capacity of CD45-negative myeloma cells to take advantage of multiple growth factors involved in promoting myeloma cell growth [74,75]. Conversely, it has been reported that CD45-positive myeloma cells are associated with a longer survival [71].

To differentiate between plasmablastic lymphoma (which may be both LCA and CD138 positive) and myeloma (which may show some LCA-positive cells), the percentage of LCA-positive cells could be useful (more in lymphomas). In addition, CD19 and CD56 immunostaining may be also useful, as plasma cells in lymphoma are more likely to express CD19 and less likely to express CD56 than those in myeloma [76].

The average percentage of tumor cells positive for LCA was significantly higher ($P < 0.01$) in primary bone lymphoma than in multiple myeloma. It is obvious that LCA is highly specific for non-Hodgkin's lymphoma, and examination of LCA immunoreactivity could be useful in the differentiation between primary bone lymphoma and other RCTB. However, very rare cases of anaplastic large cell lymphoma and T-cell lymphoma may occasionally be LCA-negative [77,78].

On the basis of the current study, the following scheme is highly recommended for the accurate diagnosis of RCTB:

Conclusion

PAS staining is useful in the exclusion of multiple myeloma and primary bone lymphoma from other RCTB that could be differentiated by LCA and CD138 immunostaining. Double negativity for LCA and CD138 suggests an osteogenic origin of the tumor, osteosarcoma/osteoblastoma, which can be confirmed using osteocalcin immunostaining. In cases of PAS positivity, CD99 positivity confirms the possibility of Ewing sarcoma; osteocalcin suggests an osteogenic origin of the tumor: osteosarcoma/osteoblastoma. Double negativity for CD99 and osteocalcin suggests a chondrogenic origin of the tumor: chondrosarcoma/chondroblastoma. PAS staining is

also highly useful in detecting areas of osteoid production in cases of osteosarcoma and osteoblastoma. Osteocalcin is a useful marker for the detection of osteoid matrix and osteoblastic differentiation. Moreover, CD138 is an excellent marker of plasmacytic differentiation only within the hematolymphoid system. LCA is very useful in the diagnosis of primary bone lymphoma.

Acknowledgements

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