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Immunophenotyping of acute lymphoblastic leukemia using immunohistochemistry in bone marrow biopsy specimens

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Summary. Flow cytometry is the preferred method of diagnosing and immunophenotyping acute lymphoblastic leukemia (ALL). However, there are situations in which immunohistochemical staining (IH) of bone marrow trephine biopsy specimens can be used to provide immunophenotypic information. To evaluate the use of IH and to confirm its value in diagnosing and typing of ALL, we studied 50 cases of denovo ALL that were previously classified into pre B, T and B by morphologic, cytochemical and FC methods. Paraffin embedded bone marrow trephine biopsies sections were stained using a panel of antibodies,namely, myeloperoxidase (MPO), terminal deoxynucleotidyl transferase (TdT), CD10, CD20, CD79a, CD3. The cases included 37 pre BALL, 10 T ALL and 3 mature BALL. TdT was the most commonly expressed antibody and was positive in 41 of 50 cases of ALL (82%) and in 95% of pre B ALL cases. CD79a and CD10 were positive in 68% and 65% of pre B ALL cases, respectively. CD79a showed similar positivity in B ALL cases (66%). CD 20 was positive in 66% of mature B ALL cases but less positive in pre B ALL (22%). CD3 was positive in 70% of T ALL cases and negative in other ALL subtypes. All of the cases were negative for MPO. Diagnosis and immunophenotyping of acute lymphoblastic leukemia is possible using immunohistochemical staining of bone marrow trephine biopsies.

Key words: Acute lymphoblastic leukemia, Immunophenotyping, Immunohistochemistry, Bone marrow biopsy

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Introduction

Morphologic examination of bone marrow aspiration smears in conjunction with cytochemical staining and immunophenotyping are the methods routinely used for the diagnosis and classification of acute lymphoblastic leukemia (ALL) (Bene et al., 1959; Neame et al., 1986; Farahat et al., 1994). Flow cytometry (FC) is the method of choice for immunophenotyping (Jennings and Foon, 1997), because it has several advantages, such as rapid analysis and high sensitivity due to better preservation of antigens.

Although Immunohistochemical staining (IH) of bone marrow trephine biopsy specimens has become an essential part of diagnosing hematopathologic malignancies, e.g. lymphoma, acute leukemia has been the subject of relatively few IH studies. This was due to the inability to detect many lineage related antigens in paraffin sections and the availability of flow cytometry for immunophenotyping. However, with the introduction of new antibodies these limitations are now reduced and IH of bone marrow trephine biopsy sections is a possible alternative method. Its advantages include identification of morphologic features, the possibility of retrospective analysis and cost effectiveness, and in situations such as "dry tap", it is the only available source of material for the diagnosis and classification of acute leukemia. Furthermore, recently more cell lineage-specific antibodies that can be applied to paraffin sections have become available.

Few studies (Kurec et al., 1990; Orazi et al., 1994; Arber and Jenkins, 1996; Chuang and Li, 1997; Toth et al., 1999) have addressed the use of IH in the diagnosis of ALL. Although in general they have confirmed the ability to distinguish between myeloid and lymphoid leukemia, they have shown variable and conflicting results for the panel of antibodies required.

We studied 50 immunologically confirmed and subtyped cases of ALL using a panel of immunohistologic antibodies to evaluate the adequacy of IH performed on bone marrow trephine biopsies for the diagnosis and sub-classification of ALL, to determine whether a correlation exists between immunophenotyping by FC and IH and to establish a minimum panel of antibodies for the diagnosis and subtyping of ALL especially for centers with limited resources.

Materials and methods

Routinely processed bone marrow biopsy specimens from 50 adult patients with ALL diagnosed and subtyped on the basis of conventional criteria based on morphologic examination of bone marrow aspirate smears, cytochemical tests and flow cytometric analysis were obtained. Only *de novo* cases of ALL with adequate biopsies were selected. All cases were retrieved from the files of the Division of Hematopathology, Department of Pathology, at King Khalid University Hospital, Riyadh, Saudi Arabia. The 50 cases were subtyped into pre B, T, and B ALL. Bone marrow trephine biopsy sections were studied.

Representative sections were chosen for immunostaining after review of tissue sections that confirmed the diagnosis.

Bone marrow biopsies were fixed in formalin or B5 for 2 hours before being transferred to neutral buffered formalin and processed routinely following acid decalcification. Sections were cut 2-3 μm thick and mounted on poly-L-lysine coated glass slides and allowed to dry at room temperature overnight.

IH using the heat induced epitope retrieval technique was performed by avidin biotin peroxidase complex (ABC) method according to previously published data (Kurec et al., 1990; Chuang and Li, 1997). Appropriate control tissue was used for each antibody. The panel of antibodies studied included MPO, TdT, CD10, CD20, Cd3, and CD79a (Table 1).

The criteria for antigen expression were based on the recommendations of the general hematologic task force of the British Committee for Standards in Haematology (Hematology Task Force of BCSH). Staining for each individual antigen was considered positive if more than 10% of leukemia cells were stained. The percentage of positive blast cells was based on a 200 cell differential count and was examined under oil immersion.

Flow cytometric immunophenotyping was performed by standard technique (Lacombe et al., 1978; Borowitz et al., 1993) by gating on CD45 and included a panel of lymphoid and myeloid associated monoclonal antibodies. These antibodies included CD13, CD33, HLADR, CD3, CD7, CD20, CD22, CD10 as a minimal panel. A cell population was considered positive for an antibody by FC if 20% of the gated cells stained positively with the antibody in accordance with previously published criteria (Khalidi et al., 1998). Although all cases were immunophenotyped, the panel of antibodies used varied slightly with time.

Results

The results of IH are summarized in Table 2. TdT was the most commonly expressed lymphoid antibody and was positive in 82% of ALL cases. It was the most frequent antibody in preB ALL (95%) and the intensity was most prominent in CALLA cases. CD10 and CD79a were the second most common. CD10 was positive in 65% of pre B ALL. It was negative in T ALL. CD79a

Table 1. Panel of antibodies used.

Antibody	Clone	Source
MPO	Polyclonal	Dako
TdT		Dako
CD10	56C6	Novocastra
CD20	L26	Dako
CD3	Polyclonal	Dako
CD79a	JCB117	Dako

MPO: Myeloperoxidase; TdT: Terminal deoxynucleotidyl Transferase.

Table 2. Immunophenotypic profile using immunohistologic examination in 50 cases of acute lymphoblastic leukemia.

Subtype/Antibody	No. (%) positive cases
T (n =10)	
MPO	0 (0%)
TdT	6 (60%)
CD3	7 (70%)
CD20	0 (0%)
CD79a	1 (10%)
CD10	0 (0%)
Precursor B-cell (n=37)	
MPO	0 (0%)
TdT	35 (95%)
CD3	0 (0%)
CD20	8 (22%)
CD79a	25 (68%)
CD10	24 (65%)
B (n=3)	
MPO	0 (0%)
TdT	0 (0%)
CD3	0 (0%)
CD20	2 (66%)
CD79a	2 (66%)
CD10	1 (33%)
All cases n = 50	
MPO	0 (0%)
TdT	41 (82%)
CD3	7 (14%)
CD20	10 (20%)
CD79a	28 (56%)
CD10	25 (50%)

MPO: Myeloperxidase; TdT: Terminal deoxynucleotidyl Transferase.

was positive in mature B ALL (66%) and in pre B ALL (68%). CD20 showed similar positivity to CD79a in B ALL (66%) but less positivity in pre B ALL (22%). CD3 was very specific for T cells AL with 100% specificity and a relatively high sensitivity for T cells (70%) with strong positivity.

Comparing IH results with FC results for CD3, CD10 and CD20 there was a high concordance for CD1 0 and CD3 but not for CD20 (see table 3).

Table 3. Comparison of CD3, CD20 and CD10 detection by flow cytometry and immunohistochemical stain.

	+IH +FC	+IH -FC	-IH +FC	-IH -FC
CD3	7	0	3	40
CD10	22	3	7	18
CD20	10	0	22	18

IH: Immunohistochemical staining; FC: Flow cytometry.

Table 4. Proposed minimal immunohistochemical panel for diagnosis and immunophenotyping of acute lymphoblastic leukemia.

Antibody	T	Pre B	PreB CALLA	B
MPO	–	–	–	–
TdT	+	+	+	–
CD3	+	–	–	–
CD79a	–	+	+	+
CD10	–	±	+	–

MPO: Myeloperxidase; TdT: Terminal deoxynucleotidyl Transferase.

Discussion

In most cases diagnosis and subtyping of ALL can be performed utilizing bone marrow aspiration specimen. When the aspirate is insufficient for various reasons, paraffin embedded bone marrow trephine biopsy is the only material available for diagnosis and immunophenotyping of ALL.

Although few groups (Kurec et al., 1990; Orazi et al., 1994; Arber and Jenkins, 1996; Chuang and Li, 1997; Toth et al., 1999) have studied the feasibility of paraffin embedded bone marrow biopsy specimen in immunophenotyping of ALL, the procedure is not widely accepted for diagnosis and subtyping of ALL as in other hematologic malignancies, e.g. lymphoma. IH of acute leukemia was commonly thought to be useless because of poor preservation of many antigens and the limited number of antibodies available. The recent development of antibodies against fixative-resistant epitopes and of new antigen retrieval techniques, however, has expanded the possibility of accurately

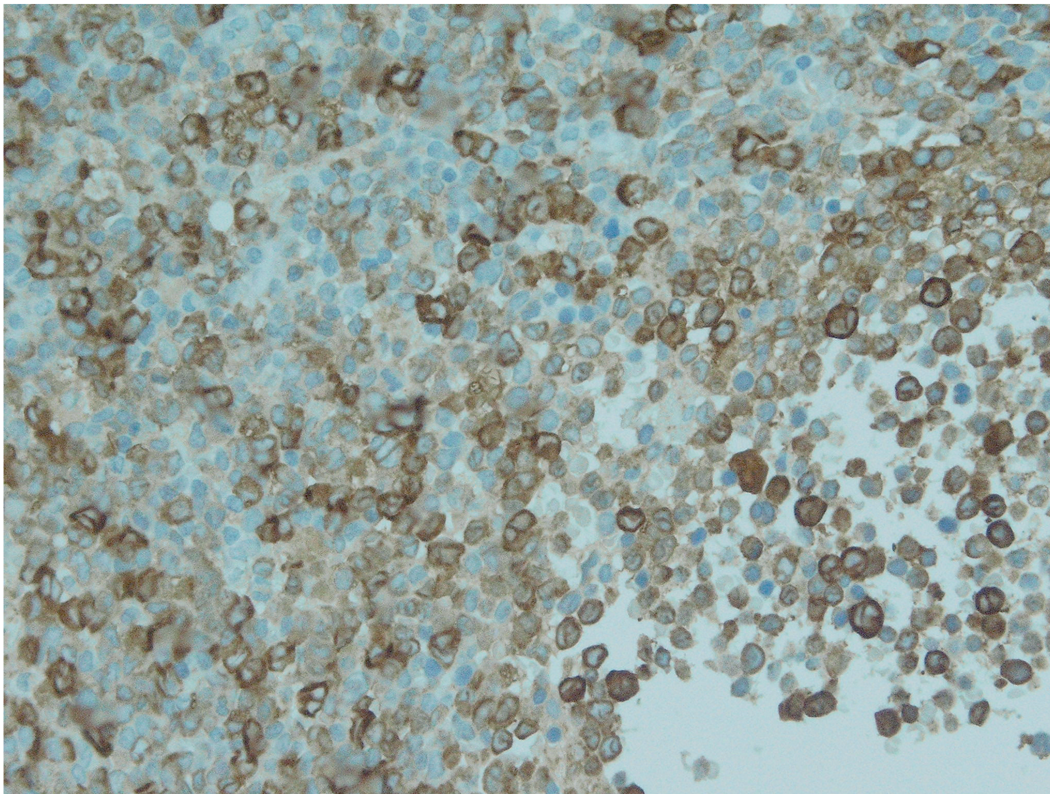

Fig. 1. Representative case of acute lymphoblastic leukemia (T type). Positive immunostaining for CD3. x 1000.

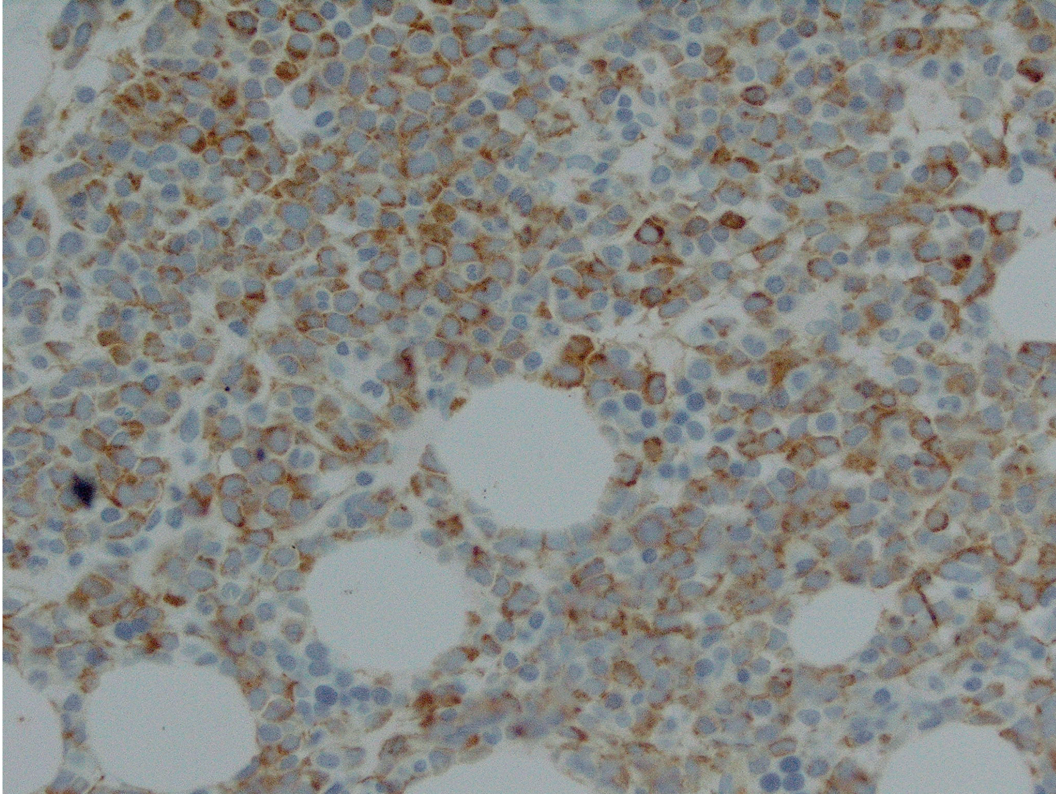

Fig. 2. Representative case of acute lymphoblastic leukemia (pre B type). Positive immunostaining for CD10. x 1000

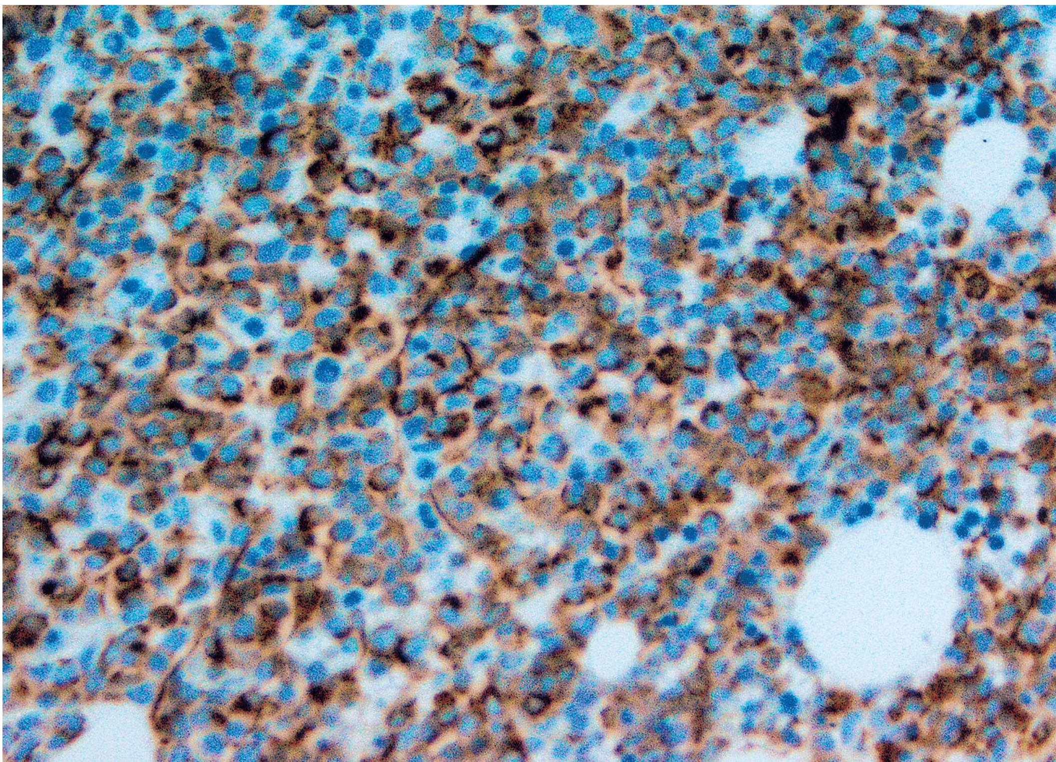

Fig. 3. Representative case of acute lymphoblastic leukemia (Mature B type). Positive immunostaining for CD79a. x 1000

testing routine samples.

In addition, several new antibodies that immunoreact with leukemia-related antigen in paraffin section have been described and are commercially available. These include antibodies that detect MPO, TdT and CD79a.

We used a panel of commercially available antibodies on 50 cases of ALL. We found the most sensitive and lineage specific markers were MPO, TdT, CD10, CD79a (for B lineage) and CD3 (for T ALL). This is similar to the finding by (Arber and Jenkins, 1996).

MPO is an enzyme in the primary granules of neutrophilic granulocytes and is not present in lymphoid cells. MPO has been demonstrated to be a specific marker for myeloid cells in paraffin sections (Pinkus and Pinkus, 1991). All our cases were negative for MPO. No aberrant MPO expression was detected. This is in contrast to the finding by (Arber and Jenkins, 1996) who reported 17% of pre B ALL to be MPO positive by IH.

TdT is a nuclear protein widely used as a marker for the diagnosis and classification of acute leukemia. It is a highly sensitive marker for immature lymphoid cells. It showed a distinct nuclear positivity in 82% of the cases. (Orazi et al., 1994) described Tdt positivity in 97% of pre Band T ALL by IH, while (Arber and Jenkins, 1996) reported 100% positivity in B-cell ALL.

CD79a detects an intracellular epitope of the a-chain of the B-cell receptor. It is a functional protein in B-lymphocytes, which forms part of the B cell antigen receptor. CD79a reportedly reacts with normal and neoplastic B cells and is highly specific for B lymphoid lineage, although there have been reports describing CD79a expression in T- lineage ALL (Polizzi et al., 1998; Lai et al., 2000). In our study one case of T ALL showed positive CD79a as well as strong expression of CD3, while (Chuang and Li, 1997) described 100% specificity of CD79a for B cell lineage.

We demonstrated CD79a positivity in 68% of precursor B cell cases and it was more sensitive than CD20 (23%). However, the latter was equally sensitive to CD79a in detecting mature B ALL cells (66%). CD20 molecule is a membrane embedded non-glycosylated phospho-protein that appears in the precursor B-cell differentiation after the expression of CD19 and just before the expression of the cytoplasmic μ chains.

Chuang and Li (1997) reported similar results with CD79a being more sensitive in pre B ALL with 100% positivity. This is in contrast to CD20 which showed only 17% positivity. However, both markers were equally sensitive in mature B ALL. CD20 appears to be more sensitive in the more differentiated B lineage ALL cases and strongest in mature B ALL. (Perkins and Kjeldsberg, 1993; Arber and Jenkins, 1996) reported a higher positivity of CD20 in pre B ALL of 50% and 30%, respectively. CD20, however, had more specificity for B cell lineage.

CD10 or the common acute lymphoblastic leukemia antigen (CALLA) is a 90 to 110 Kd monomeric integral membrane glycoprotein that functions as a neutral endopeptidase and is expressed in a variety of non-neoplastic and neoplastic cell types. In this study CD10 is proven to be reliable on paraffin sections showing concordance with FC in 80% of cases (Bavikatty et al., 2000) reported a 97% concordance with FC in ALL. Twenty four out of the 25 positive cases in our study were precursor B cells while one case was a mature B ALL. None of the T ALL cases were positive (Toth et al., 1999) reported high sensitivity with CD10 in 95% of cases.

Polyclonal anti-CD3 is a paraffin section reactive antibody that specifically stains T lymphocytes and is essentially non-reactive with non-hematopoietic tissues, B lymphocytes and granulocytes and their precursors. In our study CD3 showed a high specificity of 100% and 70% sensitivity for T ALL. Higher sensitivity of 100% was reported by (Arber and Jenkins, 1996; Chuang and Li, 1997) even in cases that were negative by FC. Similarly 100% specificity was reported by (Rousselet et al., 1995; Chuang and Li, 1997) while (Toth et al., 1999) reported aberrant T cell antigen expression in 30% of pre B ALL cases.

The variation in sensitivity of various antibodies in different studies can be attributed to many factors that influence the utility of IH in immunophenotyping. These include the type and specificity of antibodies, the sensitivity of the staining procedure used and the adequacy and type of fixative used. The role of the fixative used is controversial. (Arber and Jenkins, 1996) found no difference in immunoreactivity when different fixatives were compared. This is disputed by others who reported that most of the antibodies,namely, anti CD3, anti CD20, anti CD79a, and MPO work better in specimens fixed in formalin (Mason et al., 1988, 1995; Chuang and Li, 1997). Decalcification was considered as a possible factor, since many antigens analyzed by monoclonal antibodies used in diagnostic hematopathology might be destroyed by routine decalcification and processing.

Comparing IH results with FC results for 3 antibodies, namely, CD3, CD20 and CD10 showed high concordance for CD10 and CD3 but not for CD20. Based on these results we suggest a minimum panel of 5 monoclonal antibodies namely, MPO, CD3, TdT CD79a, CD10 for the preliminary diagnosis and immuno-phenotyping of ALL especially in small centers or centers in developing countries with limited resources (Toth et al., 1999) suggested a panel of CO10, CO79a, CO20, Beta F, MPO, CO34, TdT and lysozyme. MPO can identify all cases of AML with a myeloblastic component (M1, M4 and M6). Most cases of ALL of T cell or B cell lineage can be identified by CD3 and CD79a. Tdt and CD10 can help in distinguishing ALL from AML and help in diagnosing the pre B ALL.

Conclusion

Our findings indicate IH on routinely processed bone marrow biopsy specimens is a relatively reliable

alternative to FC for the diagnosis and subtyping of ALL when only bone marrow biopsy specimen is available and bone marrow aspirate and/or FC are unavailable for analysis. Also, it is a feasible method in small centers with limited resources. Antibodies against MPO, Tdt, CD10, CD3 and CD79a seem to be the the most helpful.

Based on our study we propose a minimum panel (Table 4) for IH antibodies that are useful for diagnosis and sub-typing in the majority of cases of ALL. By using this panel we believe that cases of ALL can be diagnosed and possibly subtyped especially in hospitals with limited resources.

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