Imunopheno typing as an adjuvant technique in the diagnosis of hematological malignancies

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ABSTRACT

Background: **Hematological malignancies are neoplasms that arise through malignant transformation of bone marrow derived cells. World Health Organization classification system (2008) has recognized over 60 different clinical and pathological disease subtypes. The current World Health Organization classification is based on lineage as demonstrated by antig en expression into lymphoid or myeloid malignancies. Within each lineage, distinct subtypes are defined based on clinical and morphologic features in conjunction with immunophenotyping.**

Aim of the study: **To define, classify and reach the proper diagnosis of the hematological malignancies, and to compare the results obtained by immunophenotyping with the established diagnostic techniques.**

Methods: **Patients included in this study are newly diagnosed cases as hematological malignancies (leukemias and lymphomas). The immunophenotyping analysis is done at the pathology laboratory of Al Sadir Teaching Hospital in Basrah. All cases were subjected for immunostaining for both myeloid and lymphoid markers to confirm the nature of the disease. In the present study, twelve monoclonal antibodies were used to recognize various hematopoietic cells based on their surface and cytoplasmic antigens.**

Results: **Sixty three cases of newly diagnosed patients with hematological malignancies were investigated prior to treatment. By using leishman's stain, hematoxyline and Eosin stains and selected cytochemical stains, the included cases were diagnosed morphologically as lymphoid malignancies (acute and chronic), myeloid malignancies (acute type) and a group of undifferentiated hematological malignancies. Based on the pattern of reactivity to a panel of lineage associated antibodies (CD markers) the hematological malignancies were classified into two major types; lymphoid (precursor and mature type) and myeloid (acute myeloid type) with further sub classification.**

Conclusions: **The diagnosis of the hematological malignancies is mainly based on morphology, but diagnostic yield is highly increased by the use of the immunophenotyping analysis.**

Key word: HM, Leukemias, lymphoma, diagnosis of the HM1, Immunophenotyping of the HMs

الصبغة المناعية كتقنية تشخيصية مساعدة في تشخيص أورام الدم في البصرة

خلفية الدراسة: اورام لدم هي الاورام التي تنشأ اثناء التحول الخبيث للخلايا المشتقة من نخاع العظم. ان هناك تنوع كبير في هذه المجموعة من الاضطرابات الذي يعكس التعقيد في العملية الطبيعية لتكوين خلايا الدم. يعتقد ان الخلل الاساسي يعود الى الانحراف الوراثي في مستوى خلايا الدم الجذعية. بعض اورام الدم تكون حادة وممكن ان تؤدي الى الموت اذا لم تعالج و هناك الكثير من الاورام تتطور بمكر وتصبح مزمنة تؤدي الى الموت المحتم. النوع الحاد يكون غالبا اورام نابعه من الخلايا الطليعية بينما النوع المزمن تكون غالبا اورام ناشئة من الخلايا الناضجة كليا او جزئيا. أورام الدم تعتبر تقليديا كسرطان الدم، سرطان الغدد اللمفاوية و سرطان الخلايا البلازمية. ان برنامج تصنيف منظمة الصحة العالمية ٢٠٠٨ ميز اكثر من ٦٠ نوع مرضي مختلفة سرسريا و مرضيا. التصنيف الحالي لمنظمة الصحة العالمية لأورام الدم والنسيج اللمفي مبنيه على سلالات كما ظهرت بواسطة اظهار الانتيجين (مولد المضاد) الى اورام اللمف و الدم. ضمن كل سلاله هناك انواع مختلفة عرفت بناء على الصفات السريرية والشكلية مع النمط الظاهري المناعي باستخدام الصبغات المناعية الخلوية، الصبغات المناعية النسيجية مع او مقياس مجرى الخلية. النوع المناعي الشكلي اصبح واحدا من الطرق الضرورية للتنصيف الصحيح لأورام الدم .

الهدف من الدراسة: الهدف من هذه الدراسة هو لتعريف، تصنيف و الوصول للتشخيص الصحيح لأورام الدم وايضا لمقارنة النتائج التي حصلنا عليها بواسطة النمط الظاهري المناعي مع التقنيات التشخيصية الثابتة و تشمل صبغة ليشمان صبغة الهيماتوكسيلين والايوسين وبعض الصبغات الخلوية الكيميائية المختارة.

طريقة اجراء الدراسة: المرضى المشمولين في هذه الدراسة هم المرضى المشخصين حديثا بإصابتهم بأورام الدم (سرطان الدم وسرطان الغدد اللمفاوية). تحاليل الصبغة المناعية اجريت في مختبر الباثولوجي في مستشفى الصدر التعليمي في البصرة. كل الحالات اخضعت لاجراء الصبغة

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المناعية الخلوية لاورام الدم والاورام اللمفاوية لتاكيد نوع المرض. في هذه الدراسة ١٢ اضداد وحيدة المنشأ استخدمت لتمييز مختلف خلايا الدم بناء على الانتيجين السطحي والسايتوبلازمي. اثنان خاصان لانواع اورام الدم **CD33** و **MPO** واثنان خاصان لخلايا من نوع **B** **CD19** و **CD20** وجسم مضاد احادي النوع **CD3** يستخدم للخلايا من نوع **T**. الاجسام المضادة السبعة تستخدم للتصنيف الثانوي للأورام اللمفاوية.

نتائج الدراسة: ثلاثة وستون حاله من المرضى المشخصين حديثا بأورام الدم اجريت لهم التحاليل قبل العلاج. بأستخدام صبغة ليشمان، الهيماتوكسيلين والايوسين وبعض الصبغات الخلوية الكيمائية المختارة، الحالات المشمولة شخصت شكليا كأورام لمفاوية حاده و مزمنة و اورام دم حاده فقط و مجموعه من اورام الدم اللامتمايزه.

بناء على النسق التفاعلي لهيئة السلالات المترابطة الاجسام المضادة (واسمات **CD**) اورام الدم تصنف الى نوعين رئيسين: اللمفاوية (السلفية و الناضجة النوع) والنقويه النخاعية من النوع الحاد مع تنصيف ثانوي اخر الى سلفيه من نوع خلية **B** وسلفيه من نوع خلية **T** وناضجه من نوع **B** و ناضجة من نوع **T** و سرطان الدم النقوي.

استنتاجات الدراسة: ان تشخيص اورام الدم يعتمد بشكل رئيسي على الفحص الشكلي ولكن الناتج التشخيصي يتزايد باستخدام تحليل النمط الظاهري المناعي. التشخيص لا يكتمل الا اذا جهز الفحص المظهري بالنمط الظاهري المناعي. سرطان الدم وسرطان الغدد اللمفاويه لايمكن اكتشافه بواسم مناعي مفرد بدلا عن ذلك استخدام هيئة الاضداد الوحيدة المنشأ لأجسام مضادة متعددة مطلوب ليساعد التشخيص المشروط مبنيا على النتائج الشكلية.

INTRODUCTION

Hematological Malignancies (HMs) are heterogeneous group of diverse incidence, prognosis and etiology; derived from a single cell in the bone marrow or peripheral lymphoid tissue which has undergone a genetic alteration.[1,2] Hematological malignancies are the fourth most frequently diagnosed cancer in both males and females in developed regions of the world[3] and the second leading cause of cancer-related death.[4] Hematological malignancies may derive from either of the two major cell lineages; myeloid and lymphoid cell lines.[5] The Myeloid cell-line normally produces a number of morphologically, phenotypically and functionally distinct cell types including granulocytes (neutrophils, basophils, eosinophils), monocytes, macrophages, erythrocytes, megakaryocytes, and mast cells. The Lymphoid cell line produce B- cell, T- cell and Natural Killer cells (NK).[6] Lymphomas, lymphocytic leukemias and myelomas are from lymphoid line while acute and chronic myelogenous leukemia, myelodysplastic syndromes and myeloproliferative diseases are myeloid in origin.[7] The Classification systems of the HMs have changed over the years from a largely morphology-based system to one that incorporates clinical features, morphology, immunophenotyping, and cytogenetic/molecular genetic data into a single coherent diagnosis.[3] The current classification system was established by the World Health Organization (WHO) in the 2001 and more recently modified in 2008[8] and updated in 2011.[9] The basic principle of the WHO system is that the classification of hematopoietic and lymphoid neoplasm should utilize not only morphologic findings but also all available information including genetic, immunophenotypic, biologic and clinical features to define specific disease entities.[10] The WHO classification of HMs stratifies these neoplasms primarily according to lineage: myeloid neoplasms, lymphoid neoplasms, mast cell disorders and histiocytic neoplasms, within each category distinct diseases are defined according to a combination of morphology, immunophenotype, genetic features and clinical syndromes.[11] The diagnosis of the hematological malignancies is not complete unless morphological examination is supplemented by further studies like immunophenotyping, karyotyping (conventional cytogenetic) and/or molecular studies.[12]

Morphology remains to be important and is the key front-line diagnostic technique which must not be ignored, in addition it is the foundation upon which decision about further scientific assessment are based.[13] The standard sequence to morphological review is peripheral blood film, bone marrow aspiration smear and biopsy.[14] Laboratory diagnosis in patients with a hematological malignancies has three major applications: establishing the diagnosis, prognostic classification and evaluation of treatment effectiveness. Immunophenotypic evaluation is currently recognized to provide essential information for all three applications.[15] Immunophenotyping utilizes the use of primary antibodies that are targeted against certain antigens in the cell. The primary antibodies can be divided into two categories polyclonal and monoclonal antibodies.[16] Antigens may be expressed on the surface of the cells within the cytoplasm or within the nucleus. Many monoclonal antibodies react with lymphoid or myeloid antigens have been characterized and are described by cluster of differentiation (CD) numbers. The CD number refers to a group of antibodies that recognize the same antigen and also refers to the antigen expressed.[17] Immunophenotyping has become the essential method for proper classification of hematopoietic neoplasms, a wide range of monoclonal antibodies are available to recognize various hematopoietic cells based on their surface and cytoplasmic antigens. Leukemia and lymphoma cells usually cannot be detected with a single immunological marker so the use of a monoclonal antibodies panel consisting of multiple antibodies is required for supporting the provisional diagnosis.[18]

PATIENTS AND METHODS

Patients included in this study are newly diagnosed cases as hematological malignancies (HMs), leukemias and lymphomas, by hematological examination of peripheral blood, bone marrow aspirate and/or histopathological examination of tissue biopsy in Al Sadir Teaching Hospital, Basrah Specialized Children Hospital and Private Laboratory in Basrah. Bone marrow aspirate with or without biopsy from posterior iliac crest was performed for further evaluation. In acute leukemias bone marrow biopsy was made for cases with dry or diluted aspirate. Regarding myeloid leukaemias, chronic myeloid leukemia (CML) was not included in the present study since its diagnosis is based on the detection of the Philadelphia (Ph) chromosome by cytogenetic or molecular genetic studies.[19] The morphological and immunophenotypic analysis was done in the laboratory of Pathology in Al Sadir Teaching Hospital in Basrah. Bone marrow aspirates and/or biopsies of all cases were subjected for immunostaining for both myeloid and lymphoid markers to confirm the myeloid or lymphoid nature of the disease. Immunostaining was performed on the peripheral blood smears when bone marrow aspirate is dry or diluted, like in some cases of hairy cell leukemia, and also in cases of leukemias with good number of leukemic cells in the peripheral blood. Fresh blood and bone marrow aspirate with or without bone marrow biopsy were taken from cases of hematological malignancies prior to the initiation of treatment. Peripheral blood sample (2.5 ml) was withdrawn, 2 ml were transferred to Ethylene diaminetetra-acetic acid (EDTA) tube for complete blood counts, while 0.5 ml was used directly to prepare smears for routine hematology staining (Leishman's stain) and immunocytochemical staining. Bone marrow aspirate (0.5 ml) was directly used for preparation of marrow smears for Leishman's stain, cytochemical stains and immuocytochemical stains. Bone marrow biopsy was taken and sent for processing in the histopathology laboratory. For patients with lymph node biopsy, the histopathological slides were reviewed and the paraffin blocks were used to prepare slides for immunostaining. All samples for immunocytochemistry (ICC) staining were fixed with absolute methanol and

all the tissues for immunohistochemistry (IHC) staining were fixed by formalin and then paraffin embedded (FFPE). All samples were stained according to Leica microsystems staining protocol (NovoLink Polymer detection system 250 tests, product No: RE7140-K,Wetzlar, Germany); with some modification. Twelve monoclonal antibodies (CD3, CD5, CD10, CD19, CD20, CD33, CD34, Kappa, Lambda and anti-Myeloperoxidase (anti-MPO) from Leica microsystems, Wetzlar, Germany; and CD45 and Bcl2 from Dako A/S, DK-2600 Glostrup, and Denmark) were used for immunostaining.

In the present study, only ten cases, who are suitable for flowcytometric immunophenotyping (fresh and good sample size with good percentage of pathological cells) were referred for flowcytometry immunophenotypic analysis (BD Accuri™ C6 FlowCytometer, from BD Biosciences, USA) in a private laboratory to confirm the results of immunocytochemistry and immunohistochemistry. An informed consent was obtained from patient and / or one of parents before enrollment in the study. The work was approved by the Ethical committee of Basrah Medical College.

Figure-1, explains how we dealt with different cases and how we used our panel of antibodies to reach the diagnosis. We followed a scheme that is similar to the WHO classification system of HMs in which we first tried to identify whether the case is myeloid, B-lymphoid or T-lymphoid.

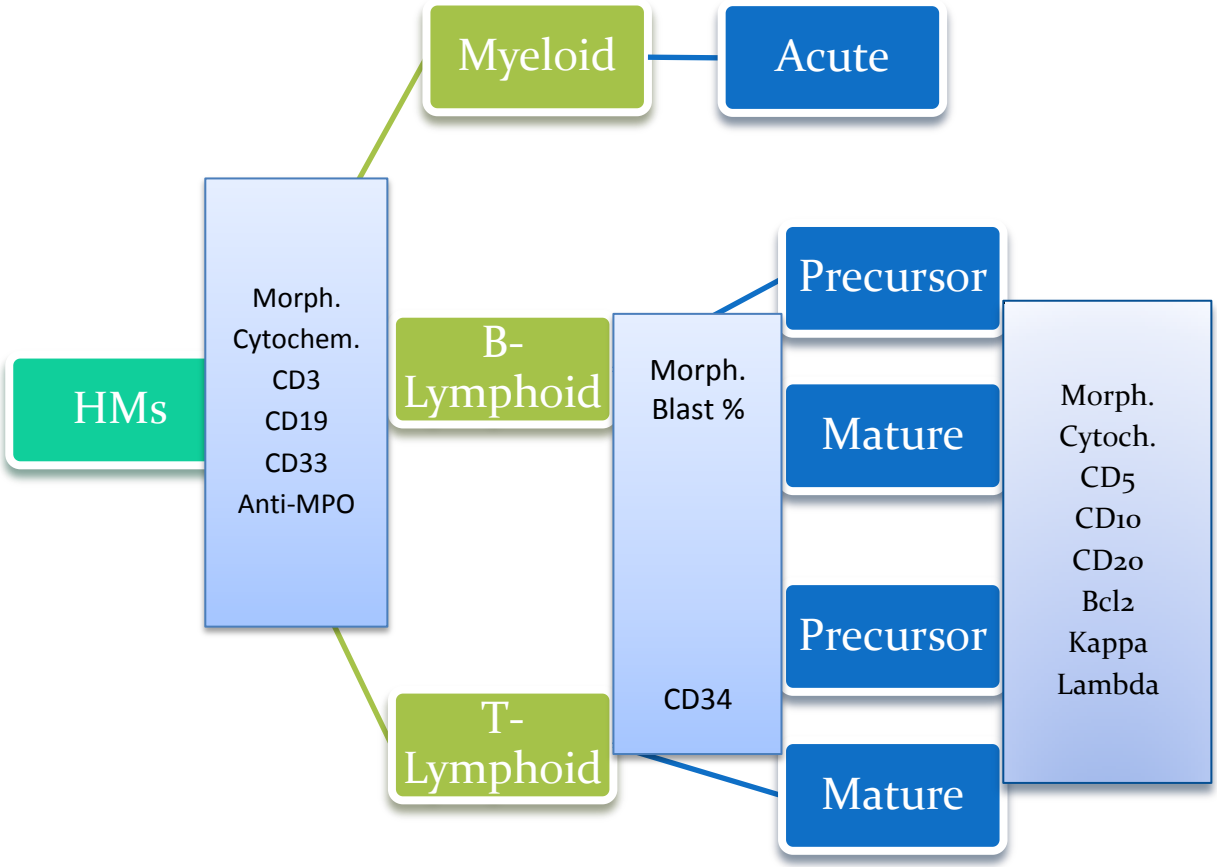


Fig 1. The suggested scheme for the classification of the HMs.

First, we assessed morphology (Morph.), cytochemistry (cytochem.) in combination with primary panel of antibodies; CD3, CD19 for lymphoid lineage and CD33 and anti-MPO for myeloid lineage. Once the lineage has been determined, the second panel of antibodies was used. Each one of the cases will then be sub classified into precursor and mature depending on blast percentage in the peripheral blood or bone marrow (cut off point of 20% determined by morphology and/or reaction to CD34). If blasts cells constitute 20% of all nucleated cells or more the case is assigned as precursor or acute. Later on further sub-classification of cases is made according to the morphology, cytochemistry in addition to immunophenotyping (CD5, CD10, CD20, Bcl2, Kappa, Lambda), Figure (1).

Figure (2) shows how sub classification of cases is made according to the morphology and immunophenotyping by immunocytochemistry and immunohitochemistry. Precursor B-lymphoid malignancies can be classified according to the positivity and pattern of reactivity to CD10 and immunoglobuline light chain. Morphologically primitive cells that exhibit positive reaction to CD34 as well as CD10 and negative for immunoglobuline are usually acute lymphoblastic leukemia (ALL) of the common type. Morphologically lymphoblast with characteristic features of Burkett type show positive reaction to immunoglobuline with surface expression pattern. This particular entity is regarded as mature type B lymphoid disorder according to the WHO classification.

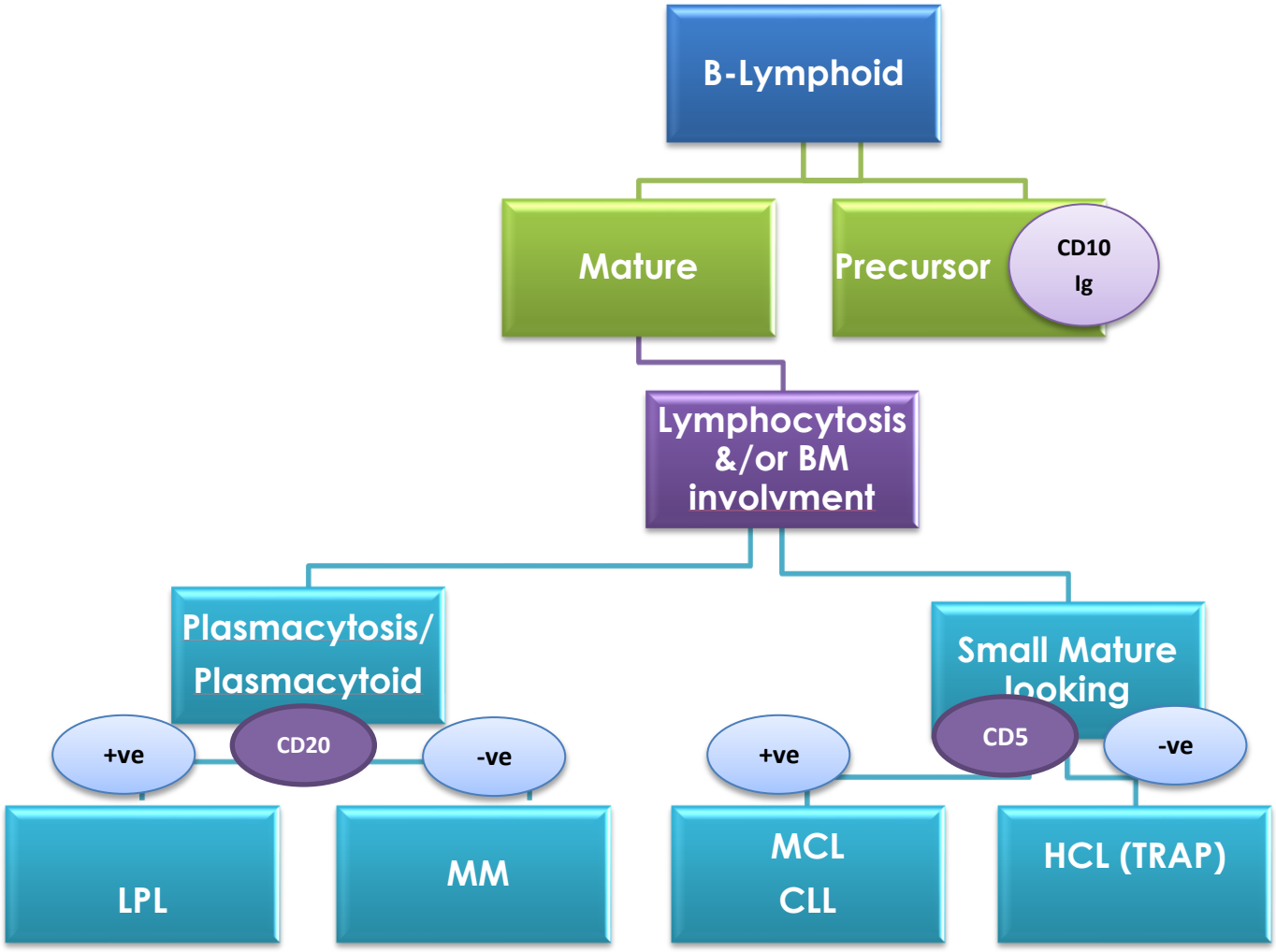


Fig 2. The suggested scheme for the sub classification of the HMs.

RESULTS

The age and sex distribution of cases are shown in (Table-1). The age of the patients involved in this study ranged from 1-81 years. The mean age and the median were 38.63 ± 20.96 years and 40 year respectively. The cases were diagnosed by morphological examination as lymphoid malignancies in 43 (68.25%), the majority of which were of chronic type 28(65.12%) while acute lymphoid malignancies were 15(34.88%) cases. The second type of HMs was the acute myeloid leukemia (AML) in 14 (22.22%) patients. Six cases were diagnosed as undifferentiated HMs (uddx) (9.52%), Figure (3).

Table 1. Age and sex distribution.

Age groups in years	Sex		No.	%
	Male	*female*		
< 10	2	3	5	7.94
10-20	8	3	11	17.46
21-30	4	1	5	7.94
31-40	7	3	10	15.87
41-50	8	4	12	19.05
51-60	8	0	8	12.70
>60	4	8	12	19.05
Total	41	22	63	100

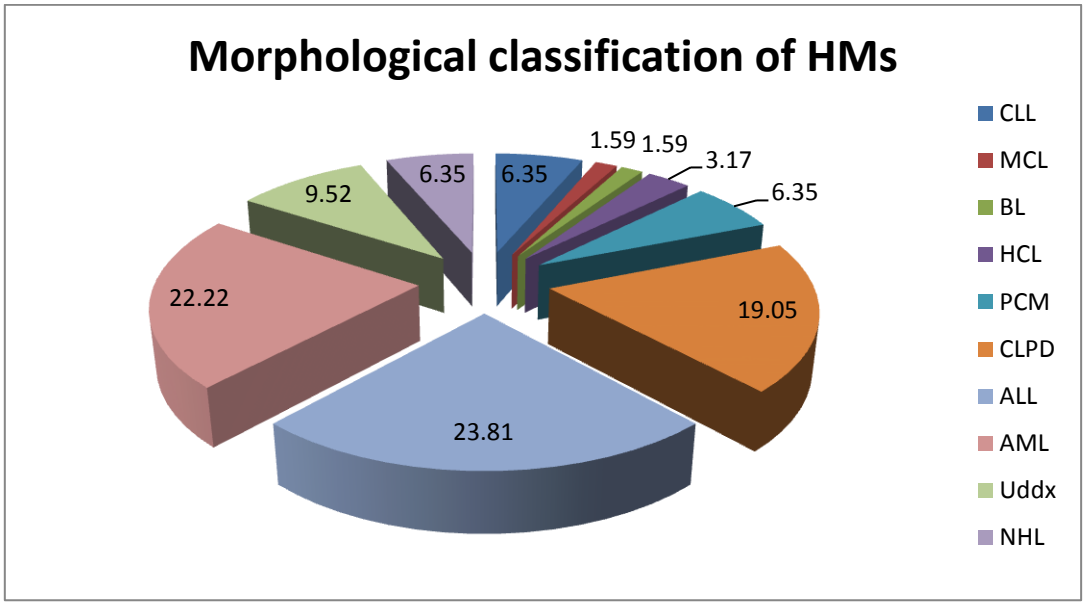


Fig 3. Morphological classification of HMs

Based on the pattern of reactivity to a panel of lineage associated antibodies (CD markers), is shown in (Table-2), the HMs were classified into two major types; lymphoid 44 (69.84%) (precursor and mature type) and myeloid (acute myeloid type) in 15 (23.81%). The precursor lymphoid type represents 17 cases (38.64%) while mature type represents 27 cases (61.36%). Out of the 17 precursor lymphoid type, precursor B-acute lymphoblastic leukemia (B-ALL) are 16 in number (94.12%) and only one (5.88%) precursor T-acute lymphoblastic leukemia (T-ALL) diagnosed in this study. The mature B-type are 27(61.36%) that show reactivity to pan-B markers e.g. CD19 and CD20, Picture, (1 and 2). No mature T- type malignancies diagnosed in this study, Figure-4.

Table 2. Immunostaining results in major types of HMs

	CD3	CD10	CD19	CD20	CD33	Anti-MPO	CD45 (LCA)
B- Lymphoid malignancies N=43	-ve (43)	+ve(5) -ve(25)	+ve (31)	+ve (37)	-ve (43)	-ve (43)	+ve (43)
T- Lymphoid malignancies N=1	+ve (1)	-ve (1)	-ve (1)	-ve (1)	-ve (1)	-ve (1)	+ve (1)
Myeloid malignancies N=15	-ve (15)	-ve (15)	-ve (15)	-ve (15)	+ve (9)	+ve (7)	**Not done**
Undifferentiated malignancies N=2	-ve (2)	-ve (2)	-ve (2)	-ve (2)	-ve (2)	-ve (2)	+ve (2)
Mixed(lymphoid/ myeloid) N=1	-ve (1)	+ve (1)	+ve (1)	+ve (1)	+ve (1)	-ve (1)	+ve (1)
Non hematological malignancies N=1	-ve (1)	-ve (1)	-ve (1)	-ve (1)	-ve (1)	Not done	-ve (1)

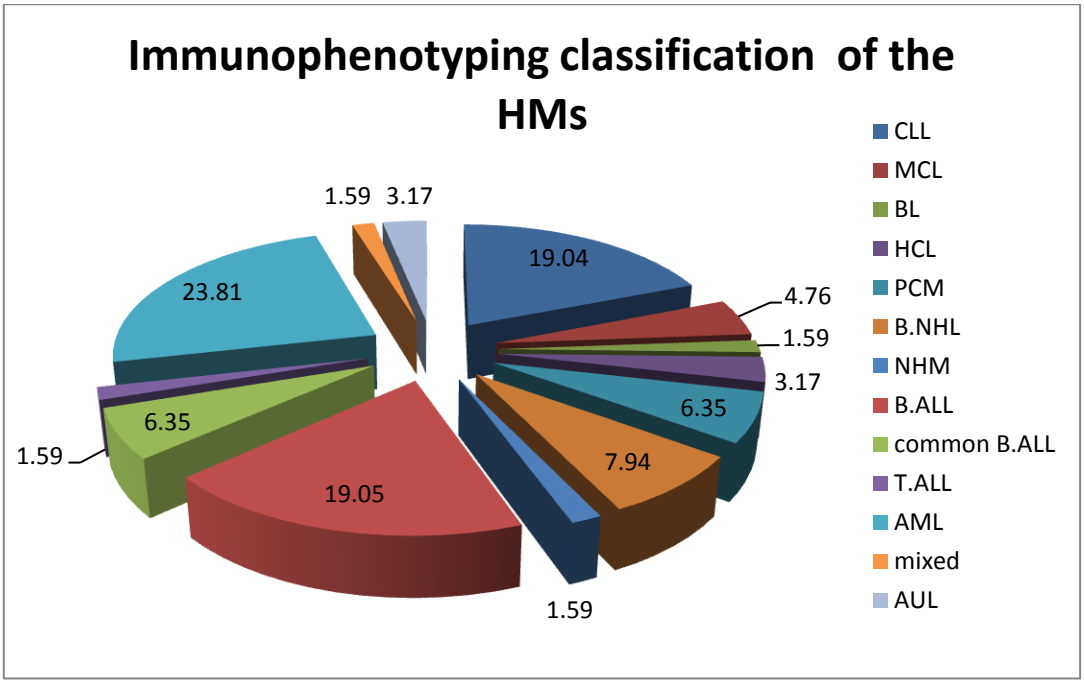
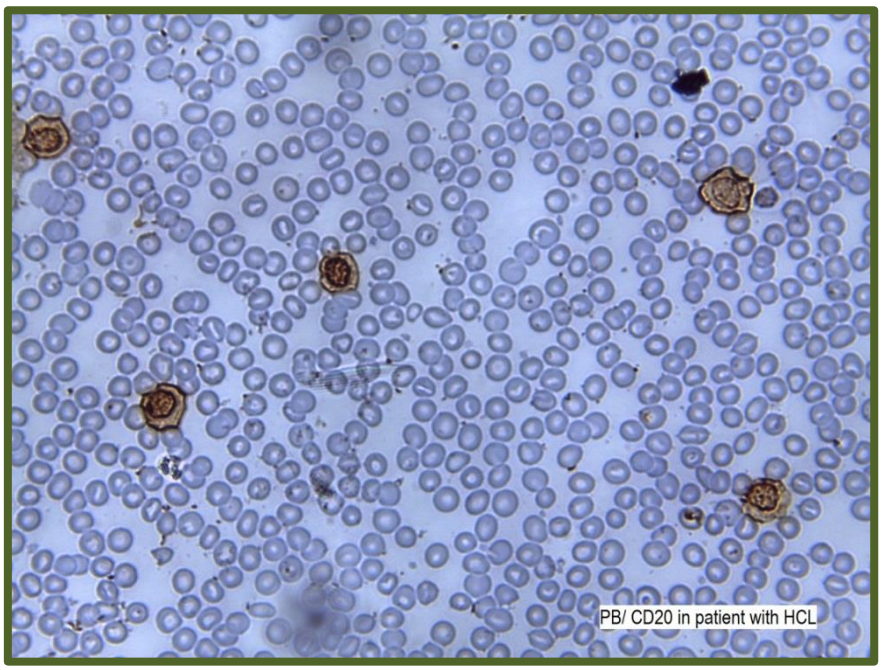
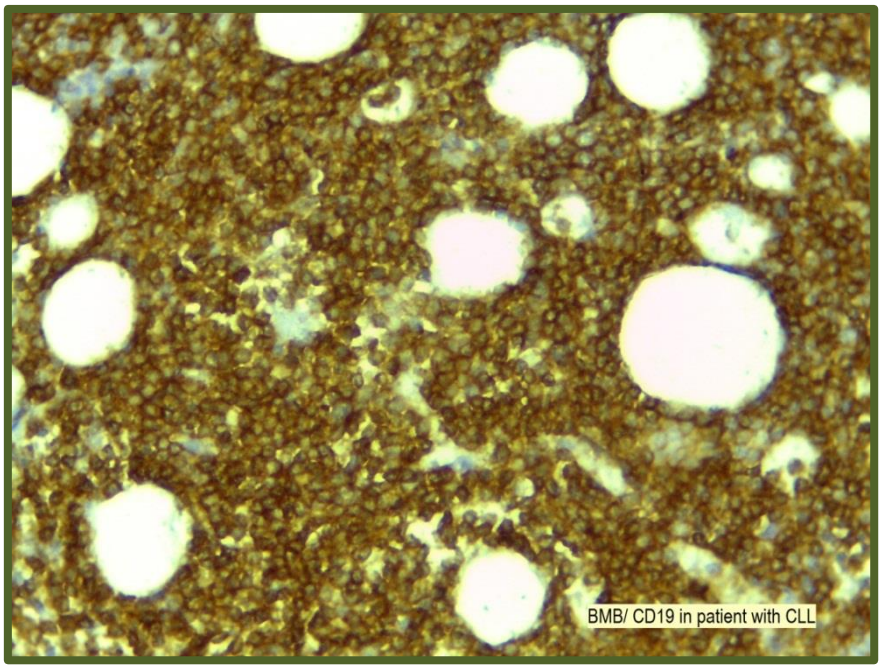


Fig 4. Immunophenotyping classification of the HMs



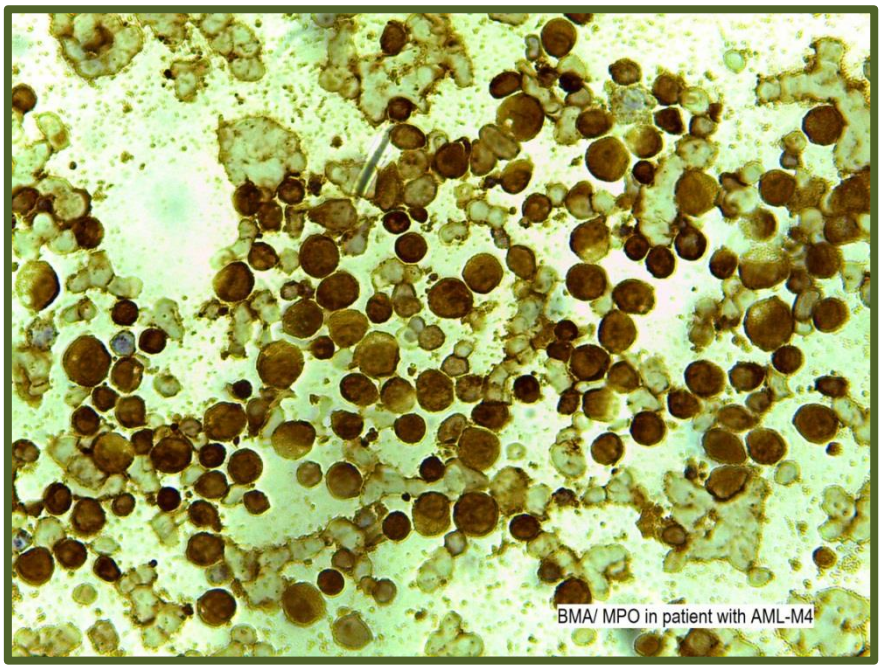
Pic 1. Immunocytochemical stain of PB for CD20, positive cells in case of HCL. x400.



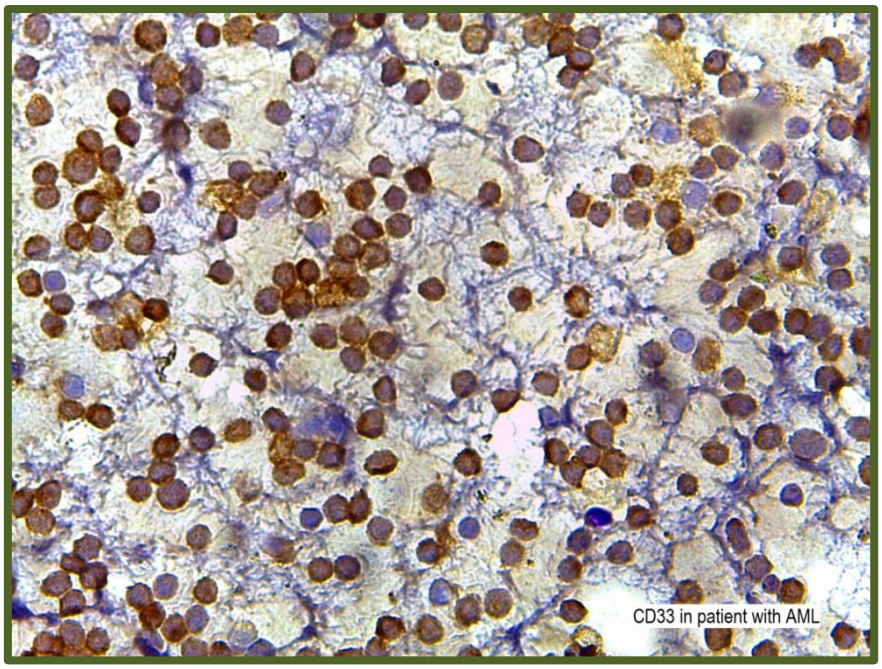
Pic 2. Immunohistochemical stain of BM biopsy for CD19, strong positive cells in patient with CLL. x400.

The immunostaining for CD33 and anti-myeloperoxidase (myeloid cells markers) was positive in 15 patients (23.81%), as shown in Picture (3, 4). Correlations among morphology, cytochemistry and immunophenotyping of leukemic cells results in sub-classification of myeloid type of HMs (AML) into M1 through M5. The majority of cases were M4 (33.33%) and M5 (26.67%) followed by M2 (20%) while M1 and M3 represents (6.67%) and (13.33%) respectively. During the period of the study, no cases of M0, M6 and M7 were diagnosed. The panel of available monoclonal antibodies were used to recognize and subtype cases that were

diagnosed as undifferentiated HMs by morphology and cytochemistry (six cases). By immunophenotyping two cases were found to be precursor B lymphoid malignancy (B-ALL) and one was assigned as acute myeloid leukemia. One case showed positive immunostaining for both B-lymphoid markers and one myeloid marker (CD33), this could be B-ALL with aberrant expression of myeloid marker or bilineage/biphenotypic leukemia. Two cases remained undifferentiated despite the use of immunological methods and were regarded as undifferentiated (Uddx), Figure (4).



Pic. 3. Immunocytochemical stain of BM with anti-MPO in patient with AML.x400.



Pic. 4. Positive CD33 in BM aspirate of patient with AML, Immunocytochemical stain. x400.

DISCUSSION

The cases of Hematological malignancies included in this study were divided into seven age groups. As with most other cancers, the possibility of being diagnosed with HMs increases markedly with age. However, HMs can be diagnosed at any age, with different subtypes predominating at different ages.[22]. In the present study the highest frequency of patients with HMs was recorded in the age group 41-50 years and more than 60 years (19.05% each). Cases among the age group below 10 years constitute 7.94% of all cases of HM, and are of acute leukemias, mostly of lymphoid type. These results are in agreement with Mckenzie SB and what was reported about the peak incidence of acute leukemias in the first decade and that chronic leukemias are rare in children.[23] The percentage of males was higher than females in all types of HMs (65.07% compared to 34.92%) in this study. This finding is in agreement with Sant, et al who found that HMs incidence in Europe was generally lower in women than men; this is a well-known phenomenon and could be in part the result of lower exposure to environmental and occupational risk factors.[1] This study has focused on the use of the manual immunocytochemistry and immunohisto-chemistry in the diagnosis of the HMs. Once the diagnosis of leukemia or lymphoma has been established, antigen expression is evaluated with the available panel of monoclonal antibodies. The morphological results were correlated with immunophenotyping using the available monoclonal antibodies. Through identification of lineage-associated antigens, all mature lymphoid malignancies included in this study are of B- cell lineage. Abnormal mature B-lymphoid cells can be distinguished from normal B-cells by the identification of 3 main types of immunophenotypic abnormalities: immunoglobulin light chain class restriction (expression of one class of immunoglobulin light chain; kappa or lambda), abnormal antigen expression (presence of antigens not normally expressed by B cells) or asynchronous expression pattern of markers (e.g. CD10 with late marker e.g. CD20). Depending on morphology only, 27(64.28%) cases were diagnosed as chronic (mature) lymphoid malignancies with no further categorization, figure (3). According to the reactivity to available panel of monoclonal antibodies in conjunction with morphology and cytochemistry, several diseases under the category of mature B-lymphoid HMs were recognized. These include: chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL), Burkett lymphoma (BL), hairy cell leukemia (HCL), plasma cell myeloma (PCM) and B-cell type non-Hodgkin lymphoma (B-NHL). Precursor B-cell and T-cell lymphoid malignancies are derived from immature lymphocytes. The malignant precursor lymphoid cells (lymphoblasts) show aberrant marker expression and deviation from the normal arrangement of maturation that can help to differentiate them from normal progenitor cells depending on the pattern of expression of these markers.[24] The lineage assignment and the level of differentiation was determined by the presence of the pan-lineage markers e.g. CD19 and CD20 for B-cell lineage and CD3 for the T-cell lineage. Accordingly the lymphoid malignancies are diagnosed as precursor (Acute) B-cell and T-cell lymphoid malignancies. In correlation to the morphological classification, the cases diagnosed morphologically as acute lymphoid type of hematological malignancies without determination of the cell lineage. The use of monoclonal antibodies as a diagnostic aid in acute myeloid leukemia (AML) has improved the diagnosis of cases in which the definitive diagnosis can't be reached by using morphological and cytochemical stains alone. In the current study, immunological markers are used to identify acute myeloid leukemia (AML) and distinguish it from acute lymphoblastic

leukemia (ALL) including reactivity with antibodies CD33 and anti-myeloperoxidase (anti-MPO). These markers show little difference between AML subtypes. So for more precise sub classification of AML, monoclonal antibodies that show some selectivity for immature cells, mature cells, granulocytic, monocytic, erythroid and megakaryocytic differentiation should be available. However, it was possible to make differentiation between the subtypes of AML using the CD33 and anti-MPO in correlation with morphology and cytochemical stains in study group. Of the 63 cases included in the study, 6 (9.52%) cases are classified morphologically as undifferentiated hematological malignancies, in which blast cells are determined morphologically and are further analyzed by immunophenotyping. The immunological analysis results in establishing the diagnosis of four cases; acute myeloid leukemia, B-cell type acute lymphoblastic leukemia, common B-ALL and one case shows positive immunostaining for both lymphoid (B-cell markers) and myeloid marker (CD33). This case could be either B-ALL with aberrant expression of myeloid marker (CD33) or bilineage / biphenotypic leukemia. Biphenotypic leukemia should not be diagnosed on the basis of the expression of a single in appropriate marker. A scoring system is usually followed in such cases. In this system, proposed by the WHO classification of hematological malignancies, the reactivity to several antibodies should be considered with points being counted according to the specificity of that antibody.[25] Unfortunately this system could not be applied in this study because of the unavailability of some of these markers **(TdT, CD13, CD79a, CD22, IgM, anti-TCR, CD2, CD8, CD117, CD65, CD24, CD7, CD1a, CD14, CD15 and CD64).** So the immunological analysis results in reduction of the undifferentiated hematological malignancies from six to two cases. These two cases remain undifferentiated and are classified as acute undifferentiated leukemia. However, markers for rare leukemias, e.g. acute megakaryoblastic leukemia or M7, were not available, and hence these rare entities cannot be excluded with certainty depending on morphological assessment alone. From this study it can be concluded that although the diagnosis of hematological malignancies is mainly based on morphology, but the diagnostic yield is highly increased by the use of the immunological methods. Leukemia and lymphoma cells cannot, usually, be detected with a single immunological marker. Instead, the use of monoclonal antibodies panels consisting of multiple antibodies is required for supporting the provisional diagnosis based on morphological findings.

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