

A morphological and immunohistochemical study to assess bone marrow involvement in Egyptian Non Hodgkin lymphoma patients

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Abstract: Background and aim of the work: Non Hodgkin Lymphoma is a heterogeneous group of malignancies which has predilection for bone marrow infiltration. Bone marrow biopsy is performed in an attempt to evaluate stage IV disease. This study aimed to evaluate the features of bone marrow trephine biopsy involvement in Egyptian NHL patients. **Patients and methods:** BM Biopsies of 100 NHL patients were assessed for percentage of marrow involvement, patterns of involvement (Interstitial, diffuse, nodular and paratrabecular), histological and phenotypic subtypes of the infiltrate. **Results:** BM biopsy showed involvement by lymphoma cells in 18 cases (18%). The incidence of involvement was higher in B-cell lymphomas 15/18 (83%), compared to T-cell lymphomas 3/18 (17%). The predominant pattern of involvement was interstitial infiltration 14/18 (77.8%). Diffuse Large B-Cell Lymphoma had the highest incidence of BM involvement in all the lymphomas 8/18 (44%). A discordant histology between BM and primary anatomic site was found in only one case. **Conclusion:** Proper staging of NHL using a reliable trephine biopsies improves the prognosis of patients by increasing or even changing the protocol of management.

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Key words: NHL, Bone Marrow Biopsy, Immunohistochemistry, BM involvement.

1. Introduction

Non-Hodgkin Lymphoma (NHL) is a group of heterogeneous malignancies of the lymphoid cells. NHL histological subtypes have different patterns of involvement and different underlying etiologies (1). NHL involves uncontrolled clonal expansion of B and T cells. B-cell NHL constitutes the majority of cases and, of these, diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL) are the two major subtypes (2). NHL is the sixth leading type of new cancer cases among men and women, accounting for 4% of new cancer cases and it is also the sixth leading cause of cancer deaths among men and the seventh among women. In Egypt, lymphoma represented 11.66% of all diagnosed cancer cases at the National Cancer Institute (NCI), Cairo University during the period from January 2003 to December 2004 according to the Cancer Pathology Registry. The B-phenotype comprised 81.1% while T-phenotype represented 9.8% and 9.1% of the cases were non-specified (3). There is a slight male-to-female predominance and a higher incidence for Caucasians than for African Americans. The incidence rises steadily with age, especially after the age of 40 years. There are striking differences in the age-dependent incidence of NHL by histological subtype. In children, Burkitt's and lymphoblastic are the most common. Histological subtypes commonly diagnosed in adults, specifically the indolent lymphomas (small

lymphocytic and follicular lymphomas) are extremely rare in children. With increasing age, the incidence of DLBCL, FLs and other aggressive lymphomas continues to rise. Small lymphocytic and FLs are most commonly diagnosed in patients over 60 years (4). BM involvement by malignant lymphoma indicates stage IV disease and trephine biopsy is the preferred method for detecting marrow infiltration (5).

In this study, we aimed to assess the characteristics of BM involvement in Egyptian NHL patients regarding incidence, histological pattern, morphology of infiltrating Lymphoma cells and presence of discordance, if present with the primary site.

2. Patients and Methods

The present study was carried in the Clinical Pathology Department, National Cancer Institute (NCI), Cairo University during the period between August 2011 and January 2013.

Patients:

One hundred NHL patients presented to the Hemato-Oncology department. CLL and Hairy Cell Leukemia patients were excluded from this study. Patients were evaluated for BM infiltration by Bone Marrow Aspirate (BMA) and Bone Marrow biopsy (BMB). Written informed consent was obtained from every patient. Study was carried after approval of the

institutional review board according to declaration of Helsinki.

All patients were subjected to complete history and physical examination with particular attention to age, gender, presenting symptoms, performance status, constitutional symptoms, presence of fever, signs of infections, bleeding manifestations, hepatomegaly, splenomegaly, and lymphadenopathy. In addition to whole body CT.

Methods:

Laboratory investigations performed included complete blood count (CBC) with TLC, lymphocytes percentage, blasts percentage, hemoglobin level and platelet count. Biochemical analysis included serum urea, creatinine, uric acid, liver profile [serum bilirubin, alkaline phosphatase, AST, ALT, serum albumin], fasting blood sugar and 2 hrs postprandial and serum LDH. **Bone marrow aspirate smears** were examined for assessment of cellularity and lymphocytes percentage. **Bone Marrow Trepphine Biopsy** was examined to assess BM infiltration and its pattern and histological subtype.

Bone Marrow Biopsy Preparation:

The obtained BMB core was immediately preserved in screw capped plastic tube, containing 10% Formol-Saline for 24-48 hours in room temperature. The fixed specimen was then washed in slowly running tap water for a minimum of 30 minutes. BMB core was placed in Formic Acid-Sodium Citrate for 2 days. When decalcification was completed, the core was transferred directly to 70% alcohol. The decalcified core (placed into tissue embedding cassette) was transported into a wire-mesh basket, where it was dehydrated in ascending grades of (70- 80- 90- 100%) alcohol, cleared in Xylene, impregnated and embedded in paraffin wax. On a rotating microtome, the paraffin blocks were trimmed and 4-5 μ -thick sections were obtained and mounted onto glass slides using a floating water bath (40-45°C).

Haematoxylin & Eosin Staining:

Sections were deparaffinized in 3 changes of Xylene, 10 minutes each then rehydrated in 2 changes of 100% ethyl alcohol, 5 minutes each then 90% alcohol, then 80% alcohol, then 70% ethyl alcohol for 2 minutes each and were rinsed briefly in Distilled Water (DW). Sections were stained with Harris haematoxylin solution for 2-5 minutes then were washed in running tap water for 2-3 minutes. Counter-stain in Eosin Y working solution was used for 1 minute. Over stain was washed off in DW. Dehydration through 95% alcohol & 2 changes of 100% alcohols, 5 minutes each was done. Then sections were cleared in 2 changes of Xylene, 5 minutes each. Xylene-based mounting medium was used.

Immunohistochemical Staining:

To detect the type of infiltration and discordance with the primary site, panel of Monoclonal Antibodies (DAKO, USA) was performed including CD20, CD3, CD10, BCL2, BCL6, Cyclin D1, AIK, Kappa and Lambda light chains.

Procedure: Ag Retrieval was done by: heat induced epitope retrieval (HIER):

- Buffer was prepared e.g. Citrate, Tris, and EDTA (as recommended in McAb data-sheet). Dako Target Retrieval sol. Conc. 10x (code: S-1700) was diluted 1:10, then 1 ml conc. Sol. to 10ml D.W. (or 5 ml conc. + 50 ml DW.).

- In "Microwave", the slides were immersed in a plastic coplin jar filled with the prepared diluted buffer and the jar's cap was sealed tightly, switched on the microwave for one minute, then opened the jar cap, then the decreased buffer level was completed till cover all sections level, then closed cap, then microwave was switched for a 2nd minute, then buffer level was adjusted again, then microwave was switched for a 3rd minute.

The preparation was rinsed gently in 3 changes of PBS (pH 7.2-7.4); 10-20 dipping each, then excess buffer was tapped off and wiped carefully around the sections. Blocking endogenous peroxidase was done by applying enough blocking reagent to fully cover the sections for 10 minutes. The preparation was rinsed gently in 3 changes of PBS; 10-20 dipping each, then excess buffer was tapped off and wiped carefully around. 1st Antibodies were diluted by freshly prepared clean PBS, according to dilution ranges recommended in the MoAb data sheet, then each Ab Eppendorf was mixed well by Vortexing (3 times), then enough diluted Ab was applied on its corresponding labeled section then incubated in closed humid chamber for 40-45 minutes. The preparation was rinsed gently in 3 changes of PBS; 10-20 dipping each, then excess buffer was tapped off and wiped carefully around the sections. Enough "labeled Polymer" was applied, conjugated with 2nd Ab, to cover the whole sections, then incubated for 30-40 minutes in same chamber. The preparation was rinsed gently in 3 changes of PBS; 10-20 dipping each, then sections were leaved in the 3rd change of PBS till preparation of DAB. Freshly prepared substrate-chromogen solution by adding 20 μ l DAB-chromogen to 1000 μ l buffered-substrate was done, then mixing well by Vortex was done, and then left in Dark. Returning to sections in the 3rd change of PBS excess buffer was tapped off and wiped carefully around the sections, and then enough of prepared DAB chromogen-substrate solution was applied, and then incubated in closed Dark chamber, for 5-10 minutes. The preparation was rinsed gently in 3 changes of PBS; 10-20 dipping each, then excess buffer was

tapped off and wiped carefully around the sections. Counter stain was performed by immersing sections, in light green stain, for 15-20 min. Washing in D.W was done, 2 changes, 10 dipping each. Dehydration by short fast (continuous 2 dipping) in ascending grades of ethyl alcohols (70 -80-90-100%) was done, then clearing by 2 dipping in 2 changes of Xylene, then the preparation was left to dry. Mount with DPX was done, and then clean glass cover was used, then left to dry (6-7).

Statistical Methods:

Data was analyzed using SPSS win statistical package version 15 (SPSS Inc., Chicago, IL). Numerical data were expressed as mean and standard deviation (SD) or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test was used to examine the relation between qualitative variables. Comparison between two groups regarding quantitative variables was done using non-parametric t-test. All tests used were two-tailed. P -value ≤ 0.05 was considered significant (8).

3. Results

This work was carried out on 100 NHL patients: (60 males and 40 females) with a median age of 40 years (1-74 years). Table (1) represents major laboratory parameters while table (2) shows clinical data and table (3) shows BMB Findings of 100 NHL patients.

BM Infiltration in relation to organomegaly:

Four/16 (25%) patients with splenomegaly showed BM infiltration. Four/15 (26.7%) of patients with Hepatomegaly showed BM infiltration, while 13/69 (18.8%) of patients with lymphadenopathy had BM infiltration. Difference between patients with or without organomegaly was statistically non-significant regarding BM infiltration ($p= 0.426$, $p= 0.343$, $p= 0.744$, respectively).

Table (4): Correlation between BM infiltration and median hematological parameters

BM Infiltration		TLC ($\times 10^3$)/L	PLT ($\times 10^6$)/L	HB gm/dl	Lymphocytes %
Positive	Median	6.16	249.5	10.8	37.5
	Range	1.86-28.2	11.0-716	7.5-14.2	5-67
Negative	Median	7.37	304.5	11.8	31.5
	Range	0.9-33.10	45-950	3.6-15.9	4 - 80
P Value		0.276	0.116	0.13	0.137

BM Infiltration in relation to BM cellularity:

13/76 (17%) of normocellular marrows showed BM infiltration versus 4/20 (19%) of hypercellular marrows and 1/4 (25%) of hypocellular marrows.

BM Infiltration:

18/100 (18%) cases only showed BM infiltration. Interstitial infiltration was the most

Table (1): Laboratory data of 100 NHL patient

Parameters	Median (range)
TLC ($\times 10^9$ /L)	7.27 (0.9-33.1)
Hb (g/dL)	11.4 (3.6-15.9)
Platelets ($\times 10^9$ /L)	300 (11-950)
Lymphocytes%	33 (4-80)
BM blast % at diagnosis	1 (1-6)

Table (2): Clinical data of 100 NHL patients

Parameter	Number (%)
Hepatomegaly	15 (15%)
Splenomegaly	16 (16%)
Lymphadenopathy	69 (69%)
Constitutional Symptoms	29 (29%)

Table (3): BMB Findings of 100 NHL patients

Parameter	Number (%)
BM cellularity:	
-Hypercellular	20/100 (20%)
-Normocellular	76/100 (76%)
-Hypocellular	4/100 (4%)
BM Infiltration	18/100 (18%)
-CD 20 +ve	15/18 (83%)
-CD 3 +ve	3/18 (17%)

BM Infiltration in relation to constitutional symptoms:

A total of 5/29 (17%) patients showed BM infiltration versus 24/29 (83%) patients showed no infiltration ($p = 0.9$)

BM Infiltration in relation to major hematological parameters:

Table (4) represents correlation between BM infiltration and median TLC, PLT, HB and Lymphocytes%.

common pattern detected among all BM involvements. Table (5) B-cell phenotype NHL had a higher percent of BM infiltration than T-cell. Table (6) Incidence of BM infiltration in different NHL histological subtypes is represented in table (7).

Table (5): Patterns of BM Infiltration

Pattern	Frequency	%
Interstitial	14	77.8%
Diffuse	2	11.1%
Nodular	1	5.6%
Paratrabeular	1	5.6%

Table (6): Relation between NHL major phenotypes and pattern of BM infiltration

	Bone Marrow Infiltration Pattern			
	Interstitial	Diffuse	Paratrabeular	Nodular
B-cell Type 15/18 (83%)	12	1	1	1
T-cell Type 3/18 (17%)	2	1	-	-

Table (7): Incidence of BM infiltration in different NHL histological subtypes

Pathological subtypes n (%)	Positive Infiltration n (%)	Negative Infiltration n (%)
DLBCL 59 (59%)	8 (13.5%)	51 (86.5%)
Burkitt's lymphoma 9(9%)	3 (33.3%)	6 (66.6%)
Follicular lymphoma 8(8%)	2 (25%)	6 (75%)
Splenic Marginal Zone lymphoma 4(4%)	-	4 (100%)
MALT lymphoma 2(2%)	1 (50%)	1 (50%)
Mantle lymphoma 2(2%)	1 (50%)	1 (50%)
Lymphoplasmacytic lymphoma 2(2%)	-	2 (100%)
Tcell rich B lymphoma 2(2%)	-	2 (100%)
Peripheral T-Cell lymphoma 8(8%)	2 (25%)	6 (75%)
Angioimmunoblastic T-Cell Lymphoma 2(2%)	-	2 (100%)
NK T-Cell Lymphoma 1(1%)	1 (100%)	-
Anaplastic Lymphoma ALK +ve 1(1%)	-	1 (100%)

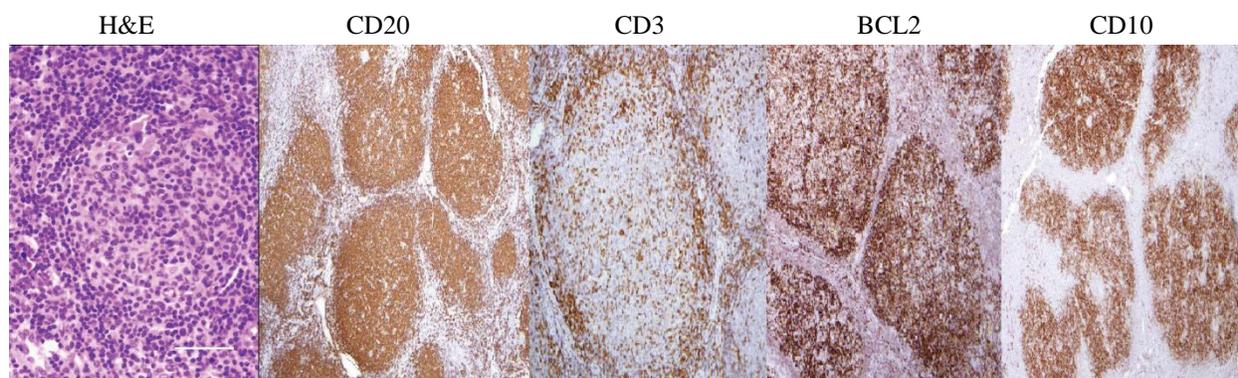


Figure (1): Follicular lymphoma case showing nodular bone marrow infiltration by H&E. Positive CD20, BCL2, CD10 and negative CD3.

4- Discussion

NHL has propensity to disseminate especially to the bone marrow. BM involvement by lymphoma indicates stage IV disease and trephine biopsy is the most preferable method to detect marrow infiltration. In this study, BMB was examined to assess incidence, morphology, pattern of infiltration and discordance with the primary diagnostic site.

In this work, median age was 40 years ranging from 1 to 74 years with a male to female ratio of 1.5:1. In similar studies, median age reported by Sovani *et*

al., 2013 was slightly higher, 45 years, while male to female ratio was comparable, M:F=1.4:1. In another study by Kumar S *et al.*, 2009 median age was 42 years which is comparable to our study while male to female ratio was slightly higher, M:F=2:1.

In the present study, 76% of patients had normocellular marrows while 20% had hypercellular marrow and 4% of patients showed hypocellular BM. Patients with positive BM infiltration were normocellular and hypercellular except for one patient which showed hypocellular BM 1/18 (5%). This result

was similar to Kumar *et al.*, 2009, where all infiltrated BMBs were normo to hypercellular except one, which was hypocellular, and Vishakha *et al.*, 2013, reported 8% of patients with positive BM infiltration showed hypocellularity.

BM was infiltrated by lymphoma in 18% of patients which was comparable to Chen *et al.*, 2000 where 21.4% had bone marrow involvement, but was obviously lower than the study by Kumar *et al.*, 2009 who reported 55.1% infiltration. This variation can be attributed to inclusion of unequal proportions of patients with early and advanced disease. In addition, the inclusion of different proportions of various histological subtypes of lymphomas in the different studies may account for this variation as the incidence of BM pathology varies greatly according to the histological subtypes. Kumar *et al.*, included CLL/SLL patients which resulted in a higher percent of infiltration.

The incidence of the B cell lymphomas was 88% and T cell lymphomas was 12%, which was the same as observed by Sovani *et al.*, 2013 and Kumar S *et al.*, 2009 where the incidence of B cell lymphomas was 90%, 85% and T cell lymphomas 10%, 15% respectively.

The predominant histological pattern of involvement by a lymphomatous infiltrate was interstitial 77.8%, followed by diffuse pattern 11.1%, paratrabeular 5.6% and nodular pattern 5.6%, which was comparable to that reported by S P Sah *et al.*, 2003, where commonest pattern of infiltration was interstitial 42% followed by diffuse pattern 20%. But the results in the present study varied from the findings of Kumar *et al.*, 2009, where the predominant histological pattern observed was mixed 52%, followed by nodular 22%, paratrabeular 11%. Increased mixed pattern of (nodular & interstitial) in Kumar *et al.*, study can be also attributed to the inclusion of CLL patients.

In our study, 8/59 (13.5%) of DLBCL patients showed positive marrow infiltration. This was comparable to Campbell *et al.*, 2006 who reported that (11%) of DLBCL involved the marrow, but it was less than Chung *et al.*, 2007 who reported involvement in (27%) of patients.

DLBCL was the most common type to invade the marrow, 8/18 (44%) followed by Burkitt's lymphoma 3/18 (16%) and Follicular Lymphoma 2/18 (11%). However, Sovani *et al.*, 2013 reported the FL as the commonest lymphoma to invade the marrow 26.2% followed by Lymphoplasmacytic 19.2% and DLBCL 12.5%. Another study conducted by Arber DA & George TI, 2005 reported that follicular lymphoma (37.5%) was the most common type to involve the marrow, followed by diffuse large B-cell lymphoma (16.0%). This difference can be attributed

due to the high number of FLs included in Sovani and Arber studies.

In the present work a discordant histology between the BM and the primary anatomic site was encountered in one case (5.5%), while discordant histology in Kumar *et al.*, 2009, was found in 29.63% (8/27) of the cases, Arber DA & George TI 2005 reported discordance in 24.9% of cases.

In conclusion, different lymphomas often demonstrate reliably characteristic architectural patterns of marrow involvement, which can help differentiate them even when cytological features do not permit this. BM morphology and Immunohistochemical staining remain the corner stone for proper staging in NHL patients to increase the diagnostic and prognostic accuracy, for appropriate treatment protocols.

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