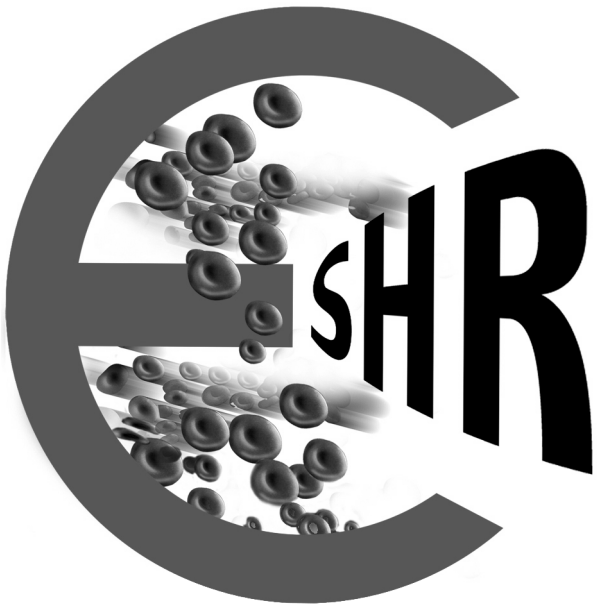
The Journal of the Egyptian Society of Haematology & Research



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A Suggested Algorithm for Detection of Bone Marrow Involvement in Diffuse Large B-Cell Lymphoma (DLBCL)

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ABSTRACT

Diffuse large B-cell lymphoma (DLBCL) is defined by the World Health Organization (WHO) as a heterogeneous entity, encompassing morphologic and genetic variants, and variable clinical presentations and outcomes. It accounts for approximately 30% of NHL and 80% of all aggressive lymphomas. In Egypt, DLBCL is the most common subtype of NHL; it represents about 49% of NHL presenting to the National Cancer Institute, Cairo University. In DLBCL patients, bone marrow (BM) involvement portends a poor prognosis and correlates with poor survival. Thus, assessment of BM status in patients with DLBCL is critical for proper staging and planning of optimal therapeutic strategies.

Although the traditional light microscopic examination of the BM aspirate (BMA) and trephine BM biopsy (BMB) has been considered the gold standard technique for routine BM evaluation in NHL patients, additional laboratory techniques have been recruited like flowcytometric immuno-phenotyping, immunohistochemical staining and/or molecular studies to improve their assessment capabilities. In this work we tried to re-stage DLBCL patients by assessment of BM status, by morphology / immunohistochemictry as well as molecular testing, to detect possible occult BM involvement, hence verifying the value of incorporating this technique in the initial routine testing for the BM.

Patients and Methods: A total of 45 cases newly diagnosed DLBCL patients were included in the study, during the time period from July 2004 to June 2005. They were referred from the Medical Oncology Department to the Clinical Pathology Department in Menofeyia University Hospital for staging, then to Clinical Pathology Department National Cancer Institute, Cairo University for BM morphology/Immunohistochemistry re-evaluation and molecular testing for Immunoglobulin heavy chain (IgH) gene rearrangements on DNA. The main inclusion criterion in this study was a negative bone marrow aspirate for evidence of infiltration by lymphoma cells; 41 cases were BMA negative. The 4 BMA positive cases were used as positive controls for both techniques.

Results: Cases were 18 females and 23 males with an age range of 21-84 with a median of 55 years. BMB/IHC showed an overall detection rates of 11/41 (26.8%) versus molecular testing that showed 26/41(63.4%). Twenty four cases were negative by BMB/IHC, when molecularly tested 10/24 cases were positive (41.7%).

A total of 10/41 cases were negative by both methods. Two cases showed IgH rearrangements in the peripheral blood while the BM was negative. In respect to stage: Stage I cases, IgH rearrangements were detected in (63.2%) of cases, in Stage II in 50% and in Stage III in 75% of cases.

We present an algorithm for investigating stages I, II and III aiming at stressing the importance of ancillary testing and probably restaging of a sizable number of DLBCL cases.

In Conclusion, our results confirm that PCR based IgH gene rearrangement analysis is a sensitive and specific method for demonstrating B cell clonality in BMA and PB. It should be considered as an additional tool to properly stage DLBCL cases according to the suggested algorithm.

Key Words: *DLBCL – IgH gene rearrangement.*

INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is defined by the World Health Organization (WHO) as a heterogeneous entity, encompassing morphologic and genetic variants, and variable clinical presentations and outcomes **[1]**. It accounts for approximately 30% of NHL **[2]** and 80% of all aggressive lymphomas **[1]**. In Egypt, DLBCL is the most common subtype of NHL; it represents about 49% of NHL presenting to the National Cancer Institute, Cairo University **[3]**. In patients with DLBCL, bone marrow (BM) involvement portends a poor prognosis and correlates with poor survival **[4,5]**. Thus, assess-

ment of BM status in patients with DLBCL is critical for proper staging and planning of optimal therapeutic strategies. Several previous studies on DLBCL patients showed variable frequencies (11-27%) of BM involvement at diagnosis time **[4-8]**. This discrepancy may partially reflect differences in study populations but also may be partially attributed to variability in laboratory techniques used in these studies for BM assessment. Although the traditional light microscopic examination of the BM aspirate (BMA) and trephine BM biopsy (BMB) has been considered the gold standard technique for routine BM evaluation in NHL patients, several centers are recruiting additional laboratory techniques like flowcytometric immunophenotyping, immunohistochemical staining and/or molecular studies to improve their assessment capabilities **[9-16]**. However, in the routine practice converse results, potential pitfalls and technical limitations are still reported on using these ancillary techniques for BM assessment **[17-19]**; thus many of them are not yet appropriately validated.

In this respect, this work tried to re-stage DLBCL patients by assessment of BM status, using IgH chain rearrangement as a molecular technique, to detect possible occult BM involvement, hence verifying the value of incorporating this technique in the initial routine testing for the BM rather than using it in the morphologically ambiguous cases only. Also, an algorithm of using standard and ancillary laboratory testing for staging DLBCL patients is suggested to be introduced.

PATIENTS AND METHODS

A total of 45 newly diagnosed DLBCL patients were included in the study, they were referred from the Medical Oncology Department to the Clinical Pathology Department in Menofyia University Hospital for staging, then to Clinical Pathology Department National Cancer Institute, Cairo University for morphologic re-evaluation and molecular testing during the time period from July 2004 to June 2005. They included 19 females and 26 males with an age range of 21-84 with a median of 55 years. Patients were diagnosed according to the standard routine clinical, radiologic and laboratory tests. Standard staging work-up included thoracic, abdominal, and pelvic computed tomography scans, as well as bone marrow biopsy and aspiration. The main inclusion criteria in this study was a negative bone marrow aspirate for evidence of infiltration by lymphoma cells; 41 cases fulfilled this criterion (BMA negative) but 4 cases showed frank bone marrow involvement by BM aspiration (BMA positive) and were used as positive control. They comprised one female, 60 years, stage I and three males, age 27, 53 and 62 years, stage III.

BM morphology:

BM biopsies were performed for all DLBCL BMA negative cases at first diagnosis. All trephines were fixed in buffered 10% formal-saline for 24 hours and then decalcified using formic acid-sodium citrate for 48 hrours. Samples were then embedded in paraffin, routinely processed and sections stained with Haematoxylin and Eosin (H&E). The adequate trephine length was in the range of 1.5cm to 2.0cm; shorter than 1.2 cm in section was considered inadequte **[20]**.

Standardized criteria were used to classify trephine biopsy samples as positive, negative or suspicous **[21]**.

Immunohistochemistry:

Immunohistochemical analysis was performed on sections from formalin-fixed decalcified paraffin-embedded (FFDPE) trephine biopsies which were immunostained using the monoclonal antibodies CD20, CD45RO, LCA, and the immunoperoxidase staining kit [DAKO Envision™+System (HRP Horse raddish peroxidase),, Peroxidase (DAB), Dako Cytomation, Inc. 6392 Via Real. Carpinteria, California 9013 USA].

The presence of B-cell aggregates was classified as abnormal or malignant when there were large numbers of aggregates, aggregates were large-sized, or contained disproportionate numbers of larger cells. Control slides were prepared for comparison.

Molecular studies:

Samples for molecular studies were obtained from BMA and/or PB.

DNA extraction was performed by salting out technique **[22]**. To verify the quantity and integrity of the DNA, all samples were, measured on the nano drop spectrophotometer (ND

1000, nanodrop technology, Thermo Fisher Scientific Inc.) at 260 and 280 wavelengths, run on gel and amplified with the house keeping gene ß-globin (Table 1).

Immunoglobulin heavy chain IgH gene rearrangement analysis:

Analysis of IgH gene rearrangements was performed by semi-nested PCR (sn-PCR), using framework regions (FR), FRII-V_H and FRIII-V_H-specific primer amplifications (Table 1). In addition, the consensus FRIc primer directed against a highly conserved region in the FRI region shared by the 7 V_H families was employed for the FRI-JH amplification using the same methodology described for the two other VH framework-specific primers.

For each initial amplification, 0.5µg of extracted DNA template, was used in a total of 25µL PCR reaction mixture that contained 0.5µmol of primers, 100µmol dNTPs, 10mmol Tris-HCl (pH 8.3), 1.5mmol $MgCl_2$, 50mmol KCl, and 2U Native Taq DNA polymerase (Finnzymes) and was subjected to 45 cycles of PCR amplifications. For all PCR amplifications, an initial step was set for 6 minutes at 94°C to denature the DNA templates and to activate the Taq enzyme. Then the PCR cycles consisted of denaturation at 94°C for 60 sec; annealing, for 60 sec, at 63°C for FR1c/LJH, at 50°C for FR2a/LJH and at 55°C for FR3a/LJH; followed by extension at 72°C for 60 sec with a 10 minutes final extension step. For reamplification of the IgH gene PCR products, one µl of the first round was used as a template and subjected to another 35-cycles PCR amplification using VLJH primer. Then, 10µL of each amplified product was separated on 3% agarose gel at 100 volt for 30 minutes and visualized by staining with ethidium bromide.

Clonal rearrangements of IgH chain genes resulted in one or two predominant amplification products within the expected range of size; FR1c/JH 330 to 350 bp, FR2a/JH 230 to 270 bp, and FR3a/JH 70 to 110 bp, *ß*-globin gene: HBG7-F, HBG7-R was at 300 bp. respectively **[23-26]**, (Fig. 1A,B,C).

All standard precautions were taken to guard against cross-contamination of amplified DNA. In each run, a negative (sterile water [blank]) control was systematically included. Positive control sample (Pre-B acute lymphobalstic leukemia) was systematically included in each run.

Treatment protocol:

Patients were treated with Cyclophosphamide, Doxorubicin, Vincristine and Prednisolone (CHOP) **[27]** or variations of CHOP chemotherapy protocols **[28,29]**. Response was assessed according to conventional criteria; complete response was confirmed by the disappearance of all lesions, determined by two observations, not less than 4 weeks apart. Partial response was defined as 50% or more decrease in total tumor size that has been measured to determine the effect of therapy by two observations, not less than 4 weeks apart. No response was defined as <50% decrease in total tumor size, while progressive disease was reported by ≥25% increase in the size of the tumor.

Follow-up period ranged from 16-80 months with a median of 34 months.

RESULTS

The efficiency of BMB morphology and immunohistochemistry in detection of marrow involvement in BMA negative cases is presented in (Table 2).

Molecular studies:

IgH rearrangements were detected in all 4 BMA positive cases as well as 26/41 BMA negative cases (63.4%). FRI was the most commonly encountered in 21 cases, whether alone or in combination with other FRs, followed by FRIII (12 cases), then FRII (6 cases). The most frequent combination was FRI with FRIII (Table 3). In the BMA positive group, 3 cases had the FRI FRII FRIII combination while one case had the FRI FRII combination.

All BMB positive cases were positive for IgH rearrangement (8/8), as well as all BMB/IHC cases (11/11). In the BMB suspicious group, 7/11 (63.6%) were positive, in BMB negative group, 6/16, (37.5%) were positive; while in the BMB inadequate group, 5/6 (83.3%) were positive.

Molecular results in relation to disease stage are shown in (Table 4).

Stage I cases were 19/41 (46.3%), with 7 cases stage IE (extra nodal disease). IgH rear-

rangements were detected in 12/19, (63.2%) of whom 5/7 (71.4%) were extra nodal cases. Only 4/19 cases were negative by both morphologic/IHC and molecular methods.

Stage II were 10/41(24.4%) cases with 1/10 case stage IIE. IgH rearrangements were detected in 5/10 (50%). In this group, 3/10 cases were negative by both methods including the stage IIE case.

Stage III cases presented 12/41 cases, none were extranodal. IgH rearrangements were detected in 9/12 (75%) and the remaining 3/12 were negative by both methods.

A total of 10/41 cases were negative by both methods.

Comparing detection rates of BMB/IHC versus molecular testing showed an overall detection rates of 11/41 (26.8%) versus 26/41 (63.4%) respectively. Twenty four cases were negative by BMB/IHC, when molecularly tested 10/24 cases were positive (41.7 %). (In view of stage, in stage I, it was 15.7% vs. 63.7%), stage II 10% vs. 50% and in stage III 41.7% vs. 75%).

Two cases showed IgH rearrangements in the peripheral blood while the BM was negative. One of them was stage II with a suspicious BMB but IHC showed polyclonality. The other was stage III with also suspicious by morphology but showed B clonality by IHC.

Here we present an algorithm for investigating stages I, II and III aiming at stressing the importance of ancillary testing and probably restaging of a sizable number of DLBCL cases (Fig. 2).

Clinical follow-up was available for 32/45 cases. All 4 BMA positive cases relapsed over a variable period of time (7-30 months). The 28/41 BMA negative did not show a particular treatment response pattern as regards the clinical, standard lab, BMB/IHC or molecular testing results. Follow-up period ranged from 16 to 80 months. There were 18 relapses (R), 5 partial response (PR), and 3 in complete remission (CR). Their distribution among the stages was as follows: Stage I: 10 R, 1 PR and 1CR (12/19cases), stage II: 5 R, 3 PR, 1CR (9/10 cases) and stage III: 3R, 1PR, 1CR (5/12cases).

Table (1): Oligonucleotide primer sequences used for amplification of the IgH and ß-globin genes.

Primer	Primer sequence	Ref
FRIc	5'-AGG TGC AGC TG (G/C) (A/T) G (G/C) AGT C (G/A/T) G G-3´	23
FRIIa	5´-TGG (A/G) TC CG (C/A) CAG (G/C) C (T/C) (T/C) CN GG-3´	24,25
FRIIIa	5´-ACA CGG C (C/T) (G/C) TGT ATT ACT GT-3´	
LJH	5´-TGA GGA GAC GGT GAC C-3´	
VLJH	5´-GTG ACC AGG GTN CCT TGG CCC CAG-3´	
HBG7-F	5´-GAAGAGCCAAGGACAGGTAC-3´	26
HBG7-R	5´-CAACTTCATCCACGTTCACC-3´	

Table (2): Detection rate of bone marrow involvement by biopsy in aspirate negative diffuse large B-cell lymphoma cases.

Detection Method	Positive	Suspicious	Negative	Inadequate
Morphology	8/41 (19.5%)	11/41 (26.8%)	16/41 (39%)	6/41 (14.6%)
Immunohistochemistry	3/41 (7.3%)	–	8/41 (19.5%)	–
Total	11/41 (26.8%)	11/41 (26.8%)	24/41 (58.5%)	6/41 (14.6%)

Table (3): Frequencies of amplified IgH framework regions in 41 DLBCL cases.

FR	BM/PB	PB only	Single	Total
FRI	10	1	11	21
FRI, III	7		7	
FRI, II, III	2		2	
FRII	2		2	6
FRIII	2		2	12
FRI, FRII	0	1	1	
FRII, FRIII	1		1	

BMA : Bone marrow aspirate.
FR : Framework regions.
BM : And/or.
PB : Same FR patterns were detectable in peripheral blood and bone marrow of the cases.
(PB) : Rearrangements were detectable in peripheral blood only.

Table (4): Clinical staging vs. Staging by bone marrow biopsy morphology and IgH gene rearrangement.

Clinical stage	No	Morphology				Molecular		Confirmed clinical stage: Negative by all methods
		Inad	+ve	–ve	?	+ve	%	
I	12	2	2	5	3	7	58.3	True stage I 4/19 (21%)
IE	7	2	2	2	1	5	71.4	
Total Stage I	19	4	4	7	4	12	63.2	
II	9		2	4	3	5	55.6	True stage II 3/10 (30%)
IIE	1				1	0	0	
Total stage II	10		2	4	4	5	50	
III	12	2	5	4	1	9	75	True stage III 3/12 (25%)

Inad: Inadequate sample ?: Suspicious

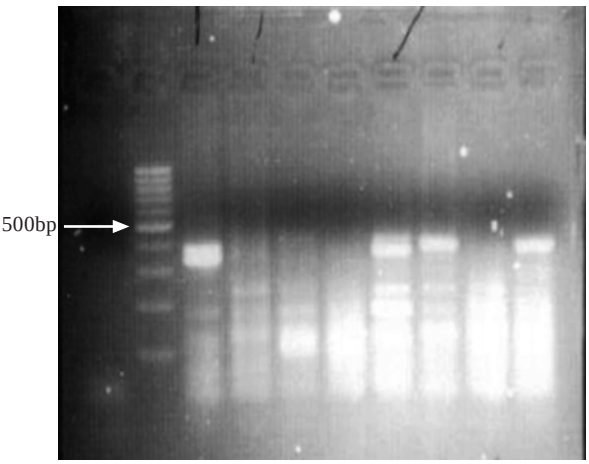

Fig. (1A): FRIc: Lane 1: Blank. Lane 2: 100 bp MWM, Lane 3: Positive control. Lane 4,5,6 and 9: Negative samples. Lane 7,8, and 10: One rearranged band 330-350 bp.

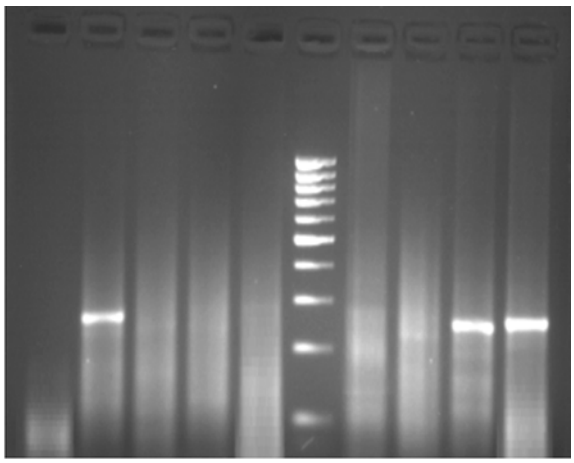

Fig. (1B): FRIIa: Lane 1: Blank. Lane 2: Positive control. Lane 3,4,5,7,8: Negative samples. Lane 6: 100 bp MWM. Lanes 9 and 10: One rearranged band 230-270 bp.

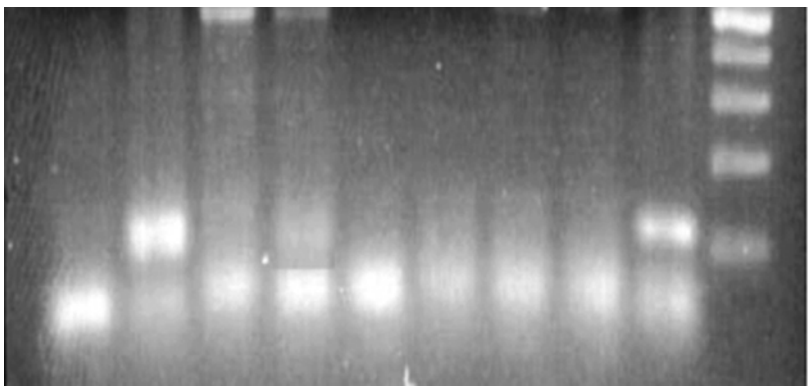

Fig. (1C): FRIIIa: Lane 1: Blank. Lane 2: Positive control. Lane 3,4,5,6,7,8: Negative samples. Lane 9: One rearranged band 70-110 bp. Lane 10: 100 bp MWM.

Fig. (1): Examples of PCR analysis of IgH gene rearrangements in BMA indicating the presence of clonally-rearranged cell population.

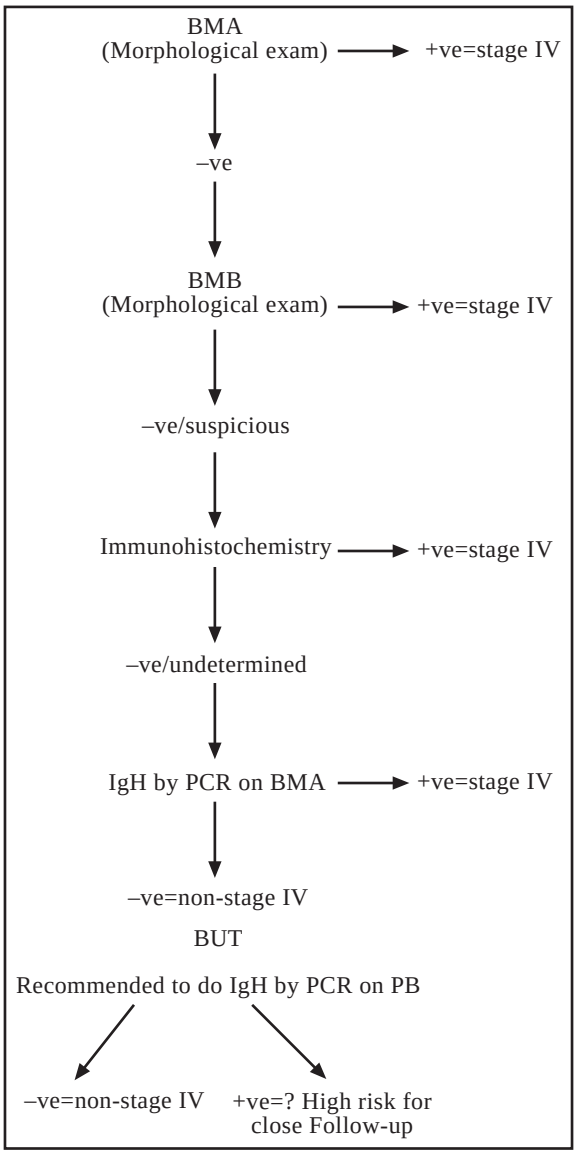

Fig. (2): Algorithm for detection of BM involvement in diffuse large B-cell lymphoma (DLBCL).

DISCUSSION

Evaluation of bone marrow biopsies is part of the standard work-up for diagnosis, classification, and staging of non- Hodgkin's lymphoma (NHL). In addition, it is of major importance in monitoring the course of disease and in assessing the response to treatment. The appraisal of bone marrow involvement is traditionally based on morphological examination and has been shown to be prognostically relevant **[30]**. There have been several previous studies on the clinical role of ancillary investigations such as IHC and flow cytometry **[16]**, in NHL. For a long time, Southern blot analysis has been the gold standard technique for molecular clonality studies **[31]**. However, despite the high reliability of Southern blot analysis, it is increasingly replaced by PCR techniques. The immunoglobulin heavy chain (IgH) gene represents the most useful for detecting B-cell clonality since it rearranges early during B lymphoid development and demonstrates extensive junctional diversity. The incidence of informativity of IgH PCR in B-cell lymphomas varies with the IgH PCR strategy and the pathological subtype **[15,16,32]**.

A major goal of the current study was to define an algorithm for the diagnosis of bone marrow involvement in NHL patients with a high detection rate and the least possible cost and laboratory work-up.

By exclusion of the morphologically positive BMA cases, our detection rate by morphological evaluation of BMB was 19.5% of the BMA negative cases. This was lower than that observed in 2 studies (28.6% and 35% respectively **[10,33]** but higher than a third study, where morphologic evaluation of BMB was positive for involvement in 10.6% of the specimens **[34]**.

Combining the BMB with IHC, the detection rate increased to 26.8% and hence 11/41 cases were upstaged which is higher than other studies **[35,36]**. Also a study that used immunohistochemistry and immunophenotyping by flow cytometry in staging bone marrow biopsies reported an upstage of 20-22% of their patients with DLBCL **[16]**.

There are several previous studies addressing the role of gene rearrangement (IgH/IgL) in NHL with widely varying detection rates **[15,16,30,33,37,38]**. Using PCR amplification of IgH gene on DNA extracted from BMB, high detection rates ranging between 54.5% and 75% were reported **[30,33,38]** especially when using FRIII **[30]** or FRIII and FRII in a seminested PCR method **[33]**.

To increase the detection rate of the IgH PCR, 3 sets of consensus primers were used in this study including the commonly used FRIII. The consensus primers used were FRII-VH and, FRIII-VH specific primers. In addition the consensus FRIc primer directed against a highly conserved region in the FRI region shared by

the 7 VH families was employed for the FRI-JH amplification **[39]**. The higher detection rate observed with FRI in our study signified that FRI might be the primer with the highest sensitivity of detection of monoclonal IgH gene rearrangement in DLBCL cases. This result is supported by the finding of others **[23,40]** who used FRI primer in a predetermined algorithmic format. It has been reported that primers from FRI compared to the primers from FRIII, were more sensitive (73% vs 58%) in detection of monoclonality **[41]**.

PCR amplification of IgH gene detected clonality in 7/11, (63.6%) of our morphologically suspicious cases which was higher than a study **[30]** that reported clonality in 50% of morphologically suspicious DLCL cases. However, another study detected clonality in 3/3 of morphologically suspicious DLCL cases **[33]**. The number in this latter study is too few to make any conclusions. On the other hand, using FRIII and FRII in a single step PCR method did not detect any clonality among the 4 morphologically suspicious DLCLs **[42]**.

In this study, we detected clonality in 41.7% of the morphologically/IHC negative cases. Lower detection rates were previously reported. A lower detection rate 6.5% was reported **[42]** which might be explained by the use of FRIII and FRII in a single step PCR method. However, another study, using the biomed 2 protocol showed an even lower detection rate of only ~5% **[16]**.

One study reported a rate as high as 60% but the study involved a very small number (3/5 cases) **[33]**.

It is to be noted that, although our study was performed on BMA; it revealed a sensitivity of detection of monoclonality of IgH gene which was comparable to studies performed on BMB. This could be attributed to the contribution of the FRI primer which was successfully amplified from DNA extracted from BMA. Another contributing factor was the use of semi-nested PCR method which increases sensitivity besides decreasing the frequency of false negative results compared to the single step PCR method **[43]**.

In 2 cases clonality could be detected in PB but not in the corresponding BMA i.e testing PB for monoclonality of IgH improved the detection from 24/41 (58.5%) to 26/41 (63.4%). This finding of positive PB and negative BMA was previously reported **[15,44]**.

The 2 cases with PB positive for IgH clonality without corresponding positivity of BMA were morphologically suspicious (interstitial in one case and mixed paratrabecular interstitial in the other). One of them revealed B cell clonality by IHC analysis. A possible explanation for a false-negative result is that the lymphoma is present in the BMB but not in the BMA from which the DNA for our assay originated. Lymphomatous involvement of the BM tends to be focal, and it is entirely possible that the tumor was fortuitously present in the location where the biopsy needle was inserted, but was not present in the place from which the aspirate was taken. Moreover, the area of lymphomatous involvement, particularly paratrabecular sites, may be fibrotic and thus, not amenable to aspiration by vacuum suction **[45]**. This denotes that including PB in the test will overcome the limitation of using BMA instead of BMB for DNA preparation.

In this study, of the 41 stages I-III cases, only 10 (24.4%) were negative by both methods. Of the remaining 31, 26 (83.9%) showed IgH rearrangement and hence were actually stage IV. The remaining 5 cases were either suspicious or inadequate by morphology and were found negative by PCR.

Although there are reports that demonstrated a significant difference in overall survival at 5 years amongst patients with positive histology and molecular studies, negative histology but positive molecular studies, and negative histology and molecular studies **[15,46]**, others were unable to demonstrate such a difference or a change in the predictive value of the international prognostic index (IPI) by inclusion of molecular staging **[16]**. Similarly we could not draw any conclusions about the response to treatment amongst patients with positive or negative histology and molecular studies.

In Conclusion, our results confirm that PCR based IgH gene rearrangement analysis is a sensitive and specific method for demonstrating B cell clonality in BMA and PB. It should be considered as an additional tool to properly stage DLBCL cases according to the suggested algorithm.

REFERENCES

1- Harris NL, Jaffe ES, Diebold J, et al. World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: Report of the Clinical Advisory Committee meeting-Airlie House, Virginia, November 1997. J Clin Oncol. 1999; 17 (12): 3835-49.

2- Coiffier B. State-of-the-art therapeutics: Diffuse large B-cell lymphoma. J Clin Oncol. 2005; 23 (26): 6387-93.

3- Mokhtar N. Cancer pathology registry (1998-2000). National Cancer Institute, Cairo University.

4- Campbell J, Seymour JF, Matthews J, et al. The prognostic impact of bone marrow involvement in patients with diffuse large cell lymphoma varies according to the degree of infiltration and presence of discordant marrow involvement. Eur J Haematol. 2006; 76: 473-480.

5- Chung R, Lai R, Wei P, et al. Concordant but not discordant bone marrow involvement in diffuse large B-cell lymphoma predicts a poor clinical outcome independent of the International Prognostic Index. Blood. 2007; 110: 1278-1282.

6- Arber DA, George TI. Bone marrow biopsy involvement by non-Hodgkin's lymphoma: Frequency of lymphoma types, patterns, blood involvement, and discordance with other sites in 450 specimens. Am J Surg Pathol. 2005; 29: 1549-1557.

7- Sehn LH, Scott DW, Chhanabhai M, et al. Impact of concordant and discordant bone marrow involvement on outcome in diffuse large B-cell lymphoma treated with R-CHOP. J Clin Oncol. 2011; Apr, 10: 29 (11): 1452-7.

8- Abdelhamid T, Samra M, Ramadan H, et al. Clinical prognostic factors of diffuse large B cell non-Hodgkin lymphoma: A retrospective study. Journal of the Egyptian National Cancer Institute. 2011; 23: 17-24.

9- Palacio C, Acebedo G, Navarrete M, et al. Flow cytometry in the bone marrow evaluation of follicular and diffuse large B-cell lymphomas. Haematologica. 2001; 86 (9): 934-40.

10- Sah SP, Matutes E, Wotherspoon AC, et al. A comparison of flow cytometry, bone marrow biopsy, and bone marrow aspirates in the detection of lymphoid infiltration in B cell disorders. J Clin Pathol. 2003; 56 (2): 129-32.

11- Perea G, Altes A, Bellido M, et al. Clinical utility of bone marrow flow cytometry in B-cell non-Hodgkin lymphomas (B-NHL) Histopathology. 2004; 45 (3): 268-74.

12- Kremer M, Quintanilla-Martinez L, Nahrig J, et al. Immunohistochemistry in bone marrow pathology: A useful adjunct for morphologic diagnosis. Virchows Arch. 2005; 447: 920-937.

13- Talaulikar D, Dahlstrom JE, Shadbolt B, et al. Role of Immunohistochemistry in staging diffuse large B-cell lymphoma. J Histochem Cytochem. 2008; 56 (10): 893-900.

14- Kang YH, Park CJ, Seo EJ, et al. Polymerase chain reaction-based diagnosis of bone marrow involvement in 170 cases of non-Hodgkin lymphoma. Cancer. 2002; 94 (12): 3073-82.

15- Mitterbauer-Hohendanner G, Mannhalter C, Winkler K, et al. Prognostic significance of molecular staging by PCR-amplification of immunoglobulin gene rearrangements in diffuse large B-cell lymphoma (DLBCL) Leukemia. 2004; 18 (6): 1102-7.

16- Talaulikar D, Shadbolt B, Dahlstrom JE, et al. Routine use of ancillary investigations in staging diffuse large B-cell lymphoma improves the International Prognostic Index (IPI). J Hematol Oncol. 2009; 2: 49.

17- Talaulikar D, Dahlstrom JE. Staging bone marrow in diffuse large B-cell lymphoma: the role of ancillary investigations. Pathology. 2009; 41 (3): 214-22.

18- Baivee D, Warnke R, Natkunam Y. Lack of utility of CD20 immunohistochemistry in staging bone marrow biopsies for diffuse large B-cell lymphoma. Appl Immunohistochem Mol Morphol. 2009; 17 (2): 93-95.

19- Talaulikar D, Gray JX, Shadbolt B, et al. A comparative study of the quality of DNA obtained from fresh frozen and formalin-fixed decalcified paraffin-embedded bone marrow trephine biopsy specimens using two different methods. J Clin Pathol. 2008; 61 (1): 119-23.

20- Bishop PW, McNally K, Harris M. Audit of bone marrow trephines. J Clin Pathol. 1992; 45: 1105-8 .

21- Cheson BD, Horning SJ, Coiffier B, et al. Report of an international workshop to standardize response criteria for non-Hodgkin's lymphomas. NCI Sponsored International Working Group. J Clin Oncol. 1999; 17 (4): 1244.

22- Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Research. 1988; 16 (3): 1215.

23- Aubin J, Davi F, Nguyen-Salomon F, et al. Description of a novel FR1 IgH PCR strategy and its comparison with three other strategies of clonality in B cell malignancies. Leukemia. 1995; 9: 471-9.

24- Trainor KJ, Brisco MJ, Wan JH, et al. Gene rearrangement in B- and T-lymphoproliferative disease detected by the polymerase chain reaction. Blood. 1991; 78: 192-6.

25- Ramasamy I, Brisco M, Morley AA. Improved PCR method for detecting monoclonal immunoglobulin heavy chain rearrangement in B cell neoplasms. J Clin Pathol. 1992; 45: 770-5.

26- Mayer SP, Giamelli J, Sandoval C, et al. Quantitation of leukemia clone-specific antigen gene rearrangements by a single-step PCR and fluorescence-based detection method. Leukemia. 1999; 13: 1843-1852.

27- Fisher RI, Gaynor ER, Dahlberg S, et al. Comparison of a standard regimen (CHOP) with three intensive chemotherapy regimens for advanced non-Hodgkin's lymphoma. N Engl J Med. 1993; 328 (14): 1002-6.

28- Sonneveld P, de Ridder M, Lelie H, et al. Comparison of doxorubicin and mitoxantrone in the treatment of elderly patients with advanced diffuse non-Hodgkin's lymphoma using CHOP versus CNOP chemotherapy. J Clin Oncol. 1995; 13 (10): 2530-9.

29- Lambertenghi Deliliers G, Butti C, Baldini L, et al. A cooperative study of epirubicin with cyclophosphamide, vincristine and prednisone (CEOP) in non-Hodgkin's lymphoma. Haematologica. 1995; 80 (4): 318-24.

30- Maes B, Achten R, Demunter A, et al. Evaluation of B cell lymphoid infiltrates in bone marrow biopsies by morphology, immunohistochemistry, and molecular analysis. J Clin Pathol. 2000; 53: 835-840.

31- Langerak AW, Wolvers-Tettero ILM, Van Dongen JJM. Detection of T cell receptor ß (TCR ß) gene rearrangement patterns in T cell malignancies by southern blot analysis. Leukemia. 1999; 13: 965-974.

32- Van Dongen JJM, Langerak AW, Ggemann M, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: Report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia. 2003; 17: 2257-2317.

33- Rizk SH, El-Masry MW, Mansour IM, et al. Clonal rearrangement of immunoglobulin heavy chain gene (IgH) and detection of bone marrow involvement in B-cell Non-Hodgkin lymphoma. Journal of the Egyptian Society of Hematology and Research. 2005; 1 (1): 7-15.

34- Gomyo H, Shimoyama M, Minagawa K, et al. Morphologic, flow cytometric and cytogenetic evaluation of bone marrow involvement in B-cell lymphoma. Haematologica. 2003; 88: 1358-1365.

35- Chetty R, Echezarreta G, Comley M, et al. Immunohistochemistry in apparently normal bone marrow trephine specimens from patients with nodal follicular lymphoma. J Clin Pathol. 1995; 48 (11): 1035-8.

36- Raga M, Brousset P, Schlaifer D, et al. Bone marrow involvement in anaplastic large cell lymphoma. Immunohistochemical detection of minimal disease and its prognostic significance. Am J Clin Pathol. 1995; 103 (1): 82-9.

37- Kang YH, Park CJ, Seo EJ, et al. Polymerase chain reaction-based diagnosis of bone marrow involvement in 170 cases of non-Hodgkin lymphoma. Cancer. 2002; 94 (12): 3073-82.

38- Krober SM, Horny HP, Greschniok A, et al. Reactive and neoplastic lymphocytes in human bone marrow: Morphological, immunohistological, and molecular biological investigations on biopsy specimens. J Clin Pathol. 1999; 52: 521-526.

39- Thériault C, Galoin S, Valmary S, et al. PCR Analysis of Immunoglobulin Heavy Chain (IgH) and TcR-[gamma] Chain Gene Rearrangements in the Diagnosis of Lymphoproliferative Disorders: Results of a Study of 525 Cases. Moc Pathol. 2000; 13 (12): 1269-1279.

40- Lombardo JF, Hwang TS, Maiese RL, et al. Optimal Primer Selection for Clonality Assessment by Polymerase Chain Reaction Analysis. III. Intermediate and High-Grade B-Cell Neoplasms. Human Pathology. 1996; 27 (4): 273-280.

41- Magic Z, Novkovic T, Cikota B, et al. Genetic alterations in B-cell non-Hodgkin lymphoma. Vojnosanit Pregl. 2005; 62 (2): 87-96.

42- Coad JE, Olson DJ, Christensen DR, et al. Correlation of PCR-Detected Clonal Gene Rearrangements with Bone Marrow Morphology in Patients with B-Lineage Lymphomas. Am J Surg Pathol. 1997; 21 (9): 1047-1056.

43- Bagg A, Braziel RM, Arber DA, et al. Immunoglobulin Heavy Chain Gene Analysis in Lymphomas A Multi-Center Study Demonstrating the Heterogeneity of Performance of Polymerase Chain Reaction Assays. Journal of Molecular Diagnostics. 2002; 4 (2): 80-89.

44- Rockman SP. Determination of clonality in patients who present with diagnostic dilemmas: A laboratory experience and review of the literature. Leukemia. 1997; 11: 852-862.

45- Ben-Ezra J, Hazelgrove K, Ferreira-Gonzalez A, et al. Can Polymerase Chain Reaction Help Distinguish Benign From Malignant Lymphoid Aggregates in Bone Marrow Aspirates? Arch Pathol Lab Med. 2000; 124: 511-515.

46- Lassmann S, Gerlach UV, Technau-Ihling K, et al. Application of BIOMED-2 primers in fixed and decalcified bone marrow biopsies: analysis of immunoglobulin H receptor rearrangements in B-cell non-Hodgkin's lymphomas. J Mol Diagn. 2005; 7 (5): 582-91.

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Three Novel δ-Chain Variants Identified by DNA Sequencing: δcd 52 [GAT→CAT], δcd 81 [CTC→TTC] and δcd 2 [CAT→AAT]

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ABSTRACT

Background: The mutations in the δ-gene are clinically silent, thus of no clinical significance, yet δ-thalassaemia can mask the presence of ß-thalassaemia trait. A total of 67 mutations have been found in the δ-globin gene of which the majority are structural variants, but the number and different types in the UK population has not been investigated thoroughly at the molecular level.

Patients and Methods: One hundred twenty (120) cases with a suspected HbA_2 variant or δ-thalassaemia were collected for analysis by a genomic sequencing and ARMS-PCR strategy developed for the identification of novel δ-chain variants and δ-thalassaemia mutations. Cases were selected by HPLC analysis showing a shifted HbA_2 peak or a reduced amount of HbA_2.

Results: Twenty eight cases were selected for this publication. Ninty two cases (76.7%) diagnosed as HbA_2' (HbB_2) were published before and excluded from this publication. Ten different variants were identified, including three novel ones. The novel mutations were HBD: c.157G>C, HBD: c.244C>T and HBD: c.7C>A.

The other results were: One case carried HbA_2-Indonesia codon 69 GGT→CGT; 2 cases carried the HbA_2-Coburg mutation, cd 116 (CGC→CAC); 4 patients carried the HbA_2-Babinga, cd 136 (GGT→GAT); 2 cases carried the δ-thalassaemic mutation δ cd 4 (ACT→ATT) $delta^+$; 10 cases carried the HbA_2-Yialousa mutation, cd 27 (GCC→TCC); 1 case carried the Hb A_2-Troodos mutation, cd 116 (CGC→TGC) and 1 case was homozygous for the thalassaemic mutation -68 (C→T). Three of the variants (HbA_2-Indonesia, HBD: c.157G>C and HBD: c.244C>T) had a characteristic shifted retention time on HPLC that can be used for a probable diagnosis.

Conclusions: We are reporting 3 novel δ-globin gene mutations with their genotype/phenotype characterization which can be added to the list of δ-thalassaemia/variants as a database.

INTRODUCTION

At present over 1536 mutations have been found in the human haemoglobin genes the majority in the α- and ß-globin genes. A total of 67 mutations have been found in the δ-globin gene of which the majority are structural variants (HbVar Database). The mutations in the δ-gene are clinically silent as the gene is expressed at low levels, approximately 2-3% of the total haemoglobin **[1]**. Although the disorders of δ-globin are of no clinical significance, δ–thalassaemia can mask the presence of ß-thalassaemia trait **[2]**. A diagnosis of ß-thalassaemia trait is usually based on the presence of a raised HbA_2 (above 3.5%) in combination with reduced red cell indices, the coinheritance of δ-thalassaemia can reduce the HbA_2 level to that found in normal individuals, thus ß-thalassaemia carriers may be missed. Seventeen δ-thalassaemia mutations have been identified to date. δ–thalassaemia mutations can be divided into those which reduce δ-globin synthesis and those in which no δ-globin is produced, designated as δ^+ and δ^0 thalassaemia respectively **[3]**.

The δ-globin structural variants can also complicate the diagnoses of ß-thalassaemia because they may split the HbA_2 peak on HPLC into two equal amounts, and thus may lead to a misdiagnosis for ß-thalassaemia trait if the variant peak is not identifiable, for example due to running with HbA. Thirty two structural variants have been identified in the δ-globin gene, but the number and different types in the UK population has not been investigated thoroughly at the molecular level.

The approach described here applies forward and reverse DNA sequence analysis of the δ-globin gene using a set of primers designed to amplify the whole gene. The method enables both δ-thalassaemia mutations and mutations in the coding sequence that result in δ-chain variants to be identified.

PATIENTS AND METHODS

Blood samples:

Samples were referred to the National Haemoglobinopathy Reference Laboratory in Oxford during a 24-month period in 2007-2009 from the following UK haematology departments for haemoglobinopathy investigations: Walsgrave Hospital, Coventry; St George's Hospital, London; Royal Victoria Infirmary, Newcastle; City Hospital, Nottingham; Royal London Hospital, London; New Cross Hospital, Wolverhampton; Queens Medical Centre, Nottingham; Hope Hospital, Salford and Broomfield Hospital, Essex. All blood samples were sent as whole blood in EDTA for investigation of a possible HbA_2 variant or an abnormally low HbA_2 level. One hundred and twenty cases were identified as carriers of δ-globin gene mutations by cation exchange high performance liquid chromatography (HPLC) analysis. Cases were selected because they had abnormal HbA_2 peak by HPLC, indicating that the patient carried a δ-chain variant or exhibited a reduced HbA_2 level below the normal range or low HbA_2 for *ß*-thalassaemia. In this work, 28 cases out of 120 were DNA sequenced for δ-globin gene mutation; 92 cases out of 120 (76.7%) diagnosed as HbA_2' (HbB_2) were excluded and published before **[4]**.

Haemoglobin analysis:

Full blood counts were performed on all blood samples using the Micros 60 automated cell counter (ABX Diagnostics, Montpellier, France). HbA_2 values were measured by HPLC with the *ß*-thalassaemia short programme on the BioRad Variant-II testing system (BioRad laboratories, Hercules, California, USA). HPLC also identifies δ-chain variants as a separate peak if the variant HbA_2 has a different retention time to normal HbA_2. The HPLC retention time for the normal HbA_2 peak is 3.59-3.68 minutes.

Sequence analysis:

DNA was extracted from whole blood using the standard phenol/chloroform method **[5]**. Amplification of the δ-gene was performed by polymerase chain reaction (PCR) using primers designed to amplify the entire δ-gene (Gene Bank Accession Number: U01317). The forward primer (5' Delta Amp) (5` CAGGGCAAGTTAAGGGAATAGTGG 3`) was designed in the 5` URR sequence and the reverse primer (3' Delta Amp) (5` CAGGCAAAGGAAGGAGGAAGAA 3`) was designed in the 3` URR. The PCR was carried out in a 25 μL reaction containing 12.5 μL Qiagen Mastermix from Qiagen (http://www1.qiagen.com), 2.5 μL Qiagen Q Solution, 1 μL of each primer (10 pmol/μL), 7 μL Sigma molecular grade water from Sigma (www.sigmaaldrich.com) and 1μL genomic DNA. The PCR conditions were 15 minutes at 97°C for the initial step, then 30 cycles of denaturation for 30 seconds at 97°C, annealing for one minute at 65°C, and extension for 2 minute at 72°C then 5 cycles of denaturation for 30 seconds at 97°C, annealing for one minute at 65°C, and extension for 3 minute at 72°C, a final extension period of 10 minutes and a pause at 15°C. PCR amplification was performed in on a Biometra® Uno II machine.

Forward and reverse cycle sequencing was then performed using Beckman CEQ™ DTCS -Quick Start Kit (Beckman, High Wycombe, UK). Three nested forward sequencing primers were designed, one in the 5` UTR (5` GGGCAAGTTAAGGGAAT 3`), one in Intron I (5` ACTGCTGTCAATGCCCTGTG 3`) and one in Intron II (5` ATGCTGATGGGAATAACCTG 3`). Two nested reverse primes were used one in the 3' UTR 5` (ATCTGTAGAGCCTCAGGAAC 3`) and one in Intron II (5` GGAGAAGAGCAGGTAGGT 3`). The products were then processed according to the Beckman Coulter CEQ DNA analysis system user manual (http://www.beckmancoulter. com) and the sequence analysed on a Beckman Coulter CE 8000 automated sequencer according to the manufacturer's instructions.

RESULTS

DNA sequencing of 28 cases of low HbA_2 (other than HbA_2') revealed a δ-globin gene mutation coding for a δ-globin chain variant in 27 patients plus a δ-thalassaemic point mutation in the promoter region in one patient (Tables 1-3). Twenty one (21) cases out of 28 were found to have a δ-globin gene mutation reported previously (http://globin.cse. psu. edu / hbvar/

menu.html) in the Syllabus of Human Haemoglobin Variants **[6]**. Seven cases were found to have novel mutations.

Known mutations:

One case of Asian origin was identified with the δ-variant HbA_2-Indonesia (δ cd 69 GGT→CGT; p.Gly70Arg; HBD: c.208G>C) converting Glycine to Arginine (Fig. 1a). He had normal red cell indices and a split HbA_2 peak totalling 2.7% of the total haemoglobin. The abnormal HbA_2 peak (1.2%) had a retention time of 4.45 minutes (Table 1, Case 1).

Three cases were observed with different mutations at codon 116. Two of them were identified as being heterozygous for HbA_2-Coburg (δ cd 116 CGC→CAC) (p.Arg117His; HBD: c.350G>A) changing the amino acid arginine to histidine (Fig. 1b). These individuals had a low HbA_2 of 1.2% and 1.7% on HPLC. No abnormal HbA_2 variant peak was observed by HPLC (Table 1, Cases 2,3). The third one was identified with the δ-variant HbA_2-Troodos (δ cd 116 (CGC→TGC)) and converts the amino acid arginine to cysteine (p.Arg117Cys; HBD: c.349C>T) and was classified as a δ^+ thalassaemia mutation. No abnormal HbA_2 peak was observed by HPLC. This patient had reduced red cell indices due to iron deficiency, with a low HbA_2 of 1.2% (Table 2, Case 13).

Four cases were found heterozygous for the codon 136 mutation that converts the amino acid from glycine to aspartic acid. This results in the δ-chain variant named HbA_2-Babinga (δ cd 136 (GGT→GAT); p.Gly137Asp; HBD: c.410G>A) (Fig. 1c). None of these cases had an abnormal HbA_2 peak on HPLC analysis (Table 1, Cases 4-7). The mutation for HbA_2-Babinga is not reported to be a thalassaemic mutation and thus normal expression of the variant is expected. The fact that no abnormal peak was observed indicates it is running with one of the other Hb peaks normally observed on HPLC (P2, P3 and Hb A). Three of these four cases were found to have a P3 peak with a slightly shifted retention time of 1.77 minutes instead of the normal P3 retention time of approx 1.65 minutes. One case showed a P3 peak with a shoulder. Thus it is proposed that the HbA_2-Babinga peak is combined with the P3 peak and has a retention time of approx 1.75 minutes. One of these four cases was also a carrier for the ß-chain variant HbS. Individuals with HbS trait have a higher normal range for Hb A_2 (3.5-4.5%) than normal individuals. The case with HbS trait and HbA_2-Babinga had an HbA_2 level of 2.9% (Table 1, Case 6). This correlated with an approximately 50% reduction of the value expected in HbS trait individuals. This case also had a mild reduction in their red cell indices, which was confirmed, by the low % HbS measured by HPLC, to be α^+-thalassaemia trait. The case had 32% HbS, which is in the range of 29-34% observed in individuals with α^+-thalassaemia trait. The other cases with this mutation had normal red cell indices and a reduced level of HbA_2.

Two cases with low HbA_2 (1.7% and 0.6%) were found to have the δ-thalassaemic mutation (δ cd 4 (ACT→ATT) delta$^+$; p.Thr5Ile; HBD: c.14C>T) converting threonine to isoleucine (Fig. 1d). They were also found to be compound heterozygous for δ-thalassaemic mutation (δ cd 27 GCC→TCC; HbA_2-Yialousa) and a silent C→ T at codon 97 (Table 2, Cases 1,2). Both had reduced red cell indices consistent with α-thalassaemia. Gap-PCR revealed that both were homozygous for the common -$\alpha^{3.7}$ mutation. One of them was more anaemic than the other (Hb% were 11.9 and 10.4 respectively) which may account for the too low HbA_2 in the more anaemic one.

Ten cases were found to have codon 27 mutation (GCC→TCC) resulting in the conversion of alanine to serine and has been named HbA_2-Yialousa (p.Ala28Ser; HBD:c.82G>T) and is designated as a δ^+-thalassaemia mutation (Table 2, Cases 3-12) (Fig. 1e). These cases had a low HbA_2 consistent with the δ-gene mutation having a thalassaemic phenotype. Three out of these ten cases were homozygous for the mutation with Hb A_2 between 0.7 and 1% (Table 2, Cases 4,5,7). No normal HbA_2 can be synthesised. The variant HbA_2 peaks had a retention time of 3.62 minutes, which were in the position observed for the normal HbA_2 peak. Thus HbA_2-Yialousa does not have a characteristic retention time. One among these three homozygous also had reduced red cell indices, resulting from α-thalassaemic mutations, as it was shown by DNA analysis (Table 2, Case 7). The other seven cases were heterozygous for the mutation; four among them had also ß-thalassaemic mutations (two were heterozygous for the $ß^0$-thalassaemia mutation IVS1-1 G→A and the other two were heterozygous for the severe $ß^+$-

thalassaemia IVS1-110 G→A); they had reduced red cell indices consistent with this type of thalassaemia which explain their normal HbA_2 (2.8%, 3.3%, 3.1% and 3.4%) (Table 2, Cases 3, 6, 11,12). The other three heterozygous for the mutation, two among them showed HbA_2 of 1.9% and 1.5%; the third one had HbA_2 of 0.6% which was too low for this type of mutation which was confirmed by α sequencing to be due to α-thalassaemia trait (α2 cd 104 TGC→TAC; Hb Sallanches) (Table 2, Cases 8, 9,10).

One case that had low HbA_2 (1.5%) with no abnormal peak on the HPLC profile and normal red cell indices was found homozygous for the δ-thalassaemic mutation (δ -68 C→T; HBD: c.-118C>T) (Table 2, Case 14) (Fig. 1f).

Novel mutations:

Three novel mutations were identified in seven cases. All mutations show characteristic features of δ chain structural variants. The three novel mutations were identified by both forward and reverse sequencing.

One case was a 41-year old male of Indian origin, with reduced red cell indices and a split HbA_2 peak constituting 3.4% of the total haemoglobin. The abnormal HbA_2 peak (1.7%) had a retention time of 4.72 minutes. DNA sequence analysis demonstrated a mutation (HBD: c.157G>C) at codon 52 of the δ-globin gene (GAT→CAT) converting aspartic acid to histidine (Table 3, Case 1) (Fig. 1g).

Three cases had the mutation (p.Leu82Phe; HBD: c.244C>T) at codon 81 of the δ-gene (CTC→TTC), converting leucine to phenylalanine (Table 3, Cases 2,3,4) (Fig. 1h). Among them two cases presented with normal red cell indices and a split HbA_2 peak on HPLC, constituting 2.7% and 2.9% of the total haemoglobin. The abnormal HbA_2 peak (1.3% and 1.5% respectively) had a retention time of 3.82 and 2.9 minutes respectively (Table 3, Case 2,3). The third one showed low HbA_2 (0.5%) and no abnormal peak on the HPLC; it was found heterozygous for the same novel mutation. This case had normal red cell indices and normal α-globin gene screening which could not explain the too low HbA_2 (Table 3, Case 4).

The other three cases with novel mutations had either no or very low HbA_2. DNA sequence analysis demonstrated a mutation (p.His3Asn; HBD: c.7C>A) at codon 2 [δ cd 2 (CAT → AAT)] converting histidine to asparagine (Fig. 1i). One of them was homozygous for the mutation and had no HbA_2 with mild anaemia (Hb: 10 g/dl) with normal red cell indices (Table 3, Case 6). The other two were compound heterozygous for the mutation and the δ^+– thalassaemic mutation (δ cd 27, HbA_2-Yialousa). One of them had no HbA2 with mild anaemia (Hb: 10.6 g/dL) and reduced red cell indices which was diagnosed as iron deficiency [ferritin: 19 µg/L (N: 30-185)] (Table 3, Case 5). The other one had HbA_2 of 0.7% with normal red cell indices (Table 3, Case 7).

Table (1): Known δ-globin gene variants detected in 120 cases of low HbA_2 (other than HBA_2').

Case No	Variant name	HPLC observation	Hb A (%)	δ variant % and rt in min	Hb (g/dl)	MCV (fl)	MCH (pg)	Hb F (%)	Other findings	Ethnic origin
1	HbA_2-Indonesia	Split HbA_2	1.5	1.2 at 4.45	11.8	94	28.9	0.7	–	Far East Asian
2	HbA_2-Coburg	Low A_2	1.2	No abnormal peak	11.5	87	27.9	0.6	–	Dutch
3	HbA_2-Coburg	Low A_2	1.7	No abnormal peak	7.1	66.7	23.3	0.3	–	Cypriot
4	HbA_2-Babinga	Low HbA_2	1.6	No abnormal peak	11.3	93.8	29.7	0	–	Afro-Carribean
5	HbA_2-Babinga	Low HbA_2+ P3 with a shoulder	1.1	No abnormal peak	–	–	–	–	–	Western European
6	HbA_2-Babinga	Low HbA_2	2.9	No abnormal peak	10	–	25	–	32% HbS; α^+ thalassaemia trait	African
7	HbA_2-Babinga	Low HbA_2+ P3 with a shoulder	1.5	No abnormal peak	12.7	99	29.4	1.5	–	African

rt = Retention time.

Table (2): Known δ-globin gene thalassaemic mutations and thalassaemic variants detected in 120 cases of low HbA_2.

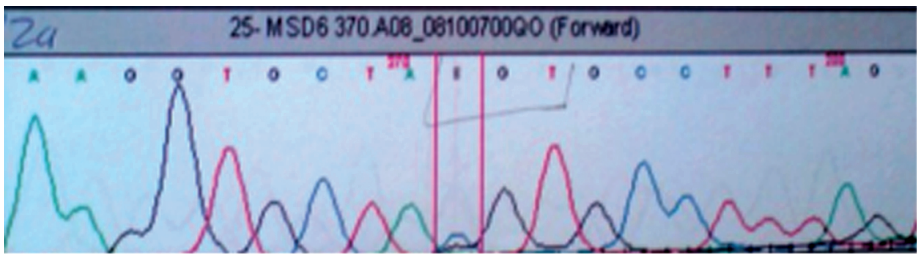
Case No	Variant name	HPLC observation	Hb A_2 (%)	δ variant % and rt in min	Hb (g/dl)	MCV (fl)	MCH (pg)	Hb F (%)	Other findings	Ethnic origin
1	cd 4 (ACT>ATT) delta+	Low HbA_2	1.7	No abnormal peak	11.9	78	22.8	0	$-\alpha^{3.7}$ thalassaemia homozygous; HbA_2 Yialousa;97 polymorphism	Cypriot
2	cd 4 (ACT>ATT) delta+	Low HbA_2	0.6	No abnormal peak	10.4	73	18.3	0.9	$-\alpha^{3.7}$ thalassaemia homozygous; HbA_2 Yialousa; 97 polymorphism	Guyanese
3	HbA_2-Yialousa	Normal HbA_2 for ß-Th	2.8	No abnormal peak		59	19.7		$ß^0$-Thalassaemia trait IVS1-1 G>A by ARMS	Turkish
4	HbA_2-Yialousa	Low HbA_2	0.9	No abnormal peak	11.2	84	28.1	0.6	-	Asian/Malaysian
5	HbA_2-Yialousa	Low HbA_2	1	No abnormal peak	18.1	91	29.3	0.2	-	Egyptian
6	HbA_2-Yialousa	Low HbA_2	3.3	No abnormal peak	10.2	80	23.9	9.1	$ß^0$-Thalassaemia trait IVS1-1 G>A by ARMS	Italian
7	HbA_2-Yialousa	Low HbA_2	0.7	No abnormal peak	11.6	69.7	22.2	0.5	$-\alpha^{3.7}$ thalassaemia homozygous	Pakistani
8	HbA_2-Yialousa	Low HbA_2	1.9	No abnormal peak	13	102	32.8	2.2	-	Polish
9	HbA_2-Yialousa	Low HbA_2	1.5	No abnormal peak	12.3	87	28.3	0.4	-	Iranian
10	HbA_2-Yialousa	Low HbA_2	0.6	No abnormal peak	12.4	78	25.5	0.6	α_2-Thalassaemia cd104 TGC>TAC (Hb Sallanches)	Indian
11	HbA_2-Yialousa	Normal HbA_2 for ß	3.1	No abnormal peak	11.8	64.2	21.5	1.5	B^+-Th (IVSI-110 G>A)	European
12	HbA_2-Yialousa	Normal HbA_2 for ß	3.4	No abnormal peak	12.7	66	21.9	<0.5	B^+-Th (IVSI-110 G>A)	Greek
13	HbA_2 Troodos	Low HbA_2	1.2	No abnormal peak	11.6	72.2	21.3	0	iron def	British
14	- 68 C>T	Low HbA_2	1.5	No abnormal peak	16.3	84	27	1.2		Asian Indian

Table (3): Three novel δ-globin gene mutations.

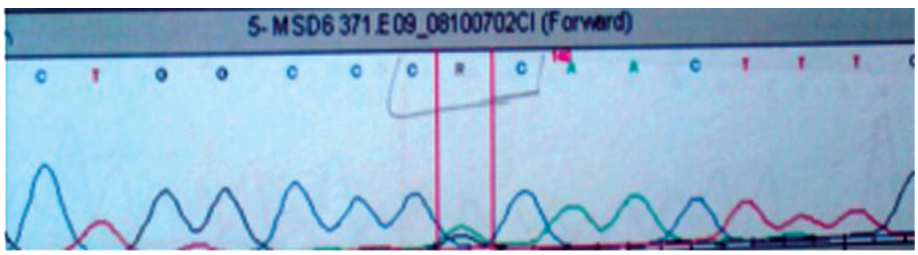
Case No	Variant name*	HPLC observation	Hb A_2 (%)	δ variant % and rt in min	RBC ($x10^{12}$/L)	Hb (g/dl)	MCV (fl)	MCH (pg)	Hb F (%)	Other findings	Ethnic origin
1	HBD:c. 157G>C	Split HbA_2	1.7	1.7 at 4.72	5.02	12	74	23.9	0.3	–	Indian
2	HBD:c. 244C>T	Split HbA_2	1.4	1.3 at 3.82	3.98	12.5	98	31.5	0.5	–	South Korean
3	HBD:c. 244C>T	Split HbA_2	1.4	1.5 at 2.9	4.46	13.7	95	30.6	0.9	–	Japanese
4	HBD:c. 244C>T	Low HbA_2	0.5	No abnormal peak	3.78	12.2	94	32.3	0	Normal α-globin gene screen	Irish
5	HBD:c. 7C>A	No HbA_2	-	No abnormal peak	4.44	10.6	83	23.9	1.6	Ferritin=19 (N: 30-85 ug/L). HbA_2 Yialousa	Asian Indian
6	HBD:c. 7C>A	No HbA_2	-	No abnormal peak	3.53	10	87	28.4	0.6	–	Unknown
7	HBD:c. 7C>A	Low HbA_2	0.7	No abnormal peak	4.01	12.7	100	31.7	3.4	HbA_2 Yialousa	Pakistani/ Indian

* HGVS nomenclature.

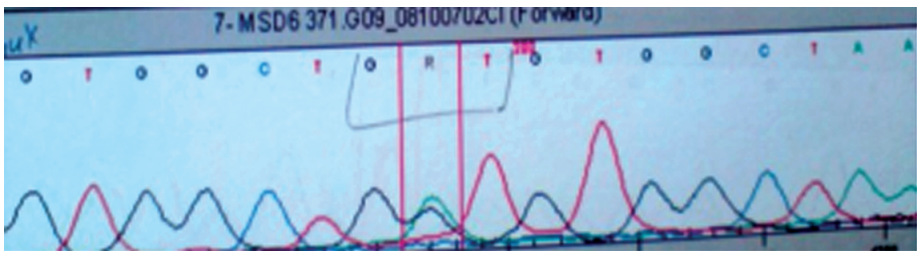
Fig. (1): DNA sequencing chromatograms of the mutation detected (except HbA_2 Troodos) show the depression of the normal wave by the half and the presence of the abnormal wave. f is homozygous, so there is complete replacement of the wave.



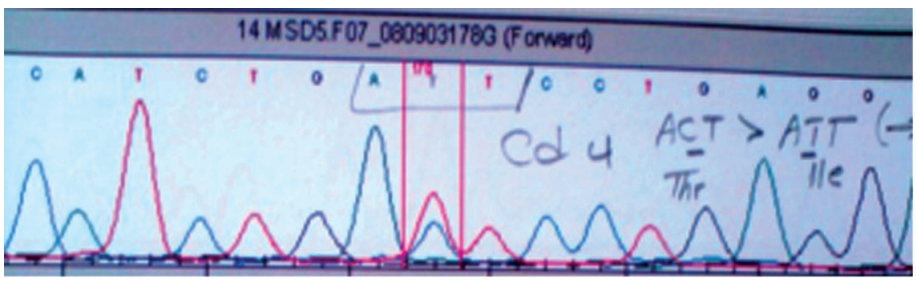
(A): Cd 69 mutation (GGT>CGT), Hb A2 indonesia (HBD:c.208G>C).



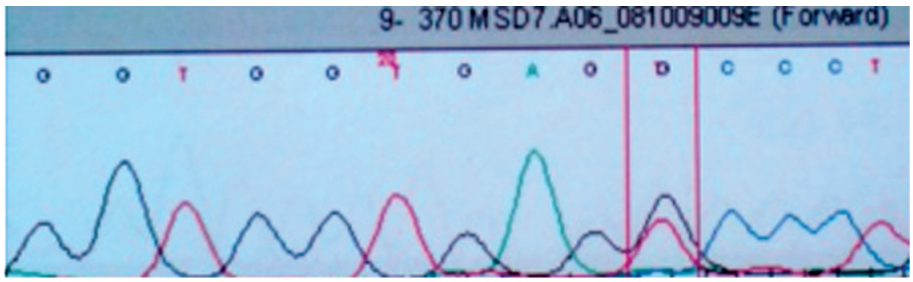
(B): Cd 116 mutation (CGC>CAC), Hb A2-Coburg (HBD:c.350G>A).



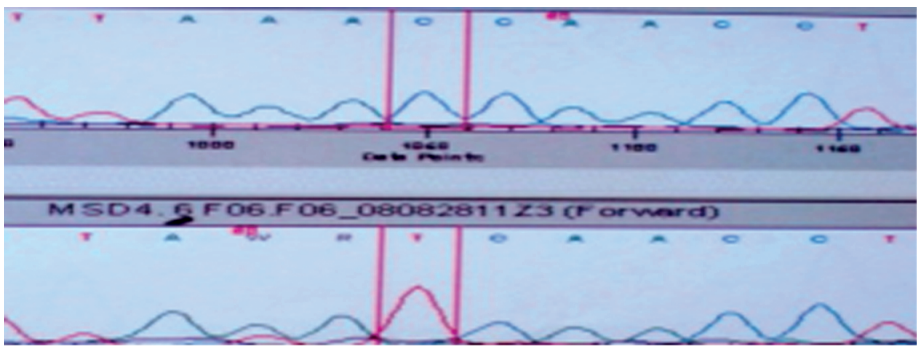
(C): Cd 136 mutation (GGT>GAT) Hb A2–Babinga (HBD:c.410G>A).



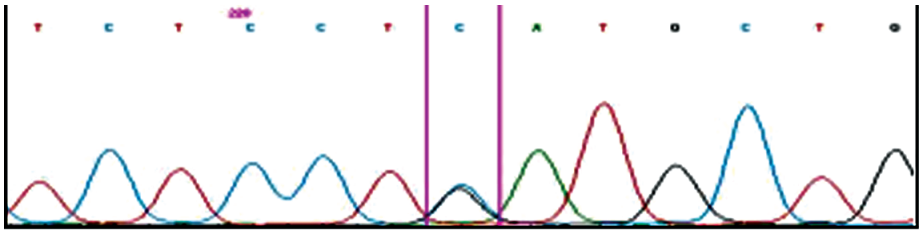
(D): Cd 4 mutation (ACT>ATT) (HBD:c.14C >T).



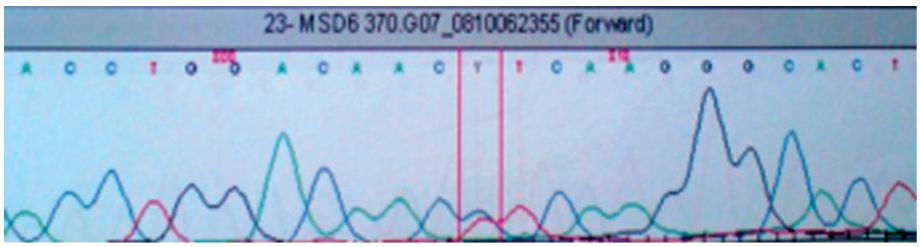
(E): Cd 27 mutation (GCC>TCC) Hb A2–Yialousa (HBD: c.82G>T).



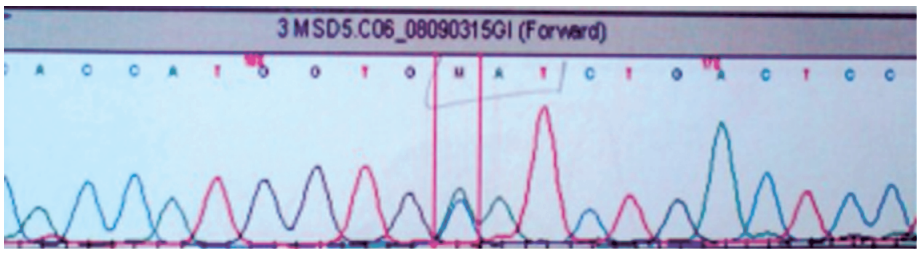
(F): -68 mutation (C> T) (HBD c.-118C>T).



(G): Novel cd 52 mutation (GAT-CAT) (HBD: c.157G>C).



(H): Novel cd 81 mutation (CTC>TTC) (HBD: c.244C>T).



(I): Novel cd 2 mutation (CAT>AAT) (HBD: c.7C >A).

DISCUSSION

In this study 120 case of low HbA_2 were examined by DNA analysis; 92 cases out of 120 (76.7%) diagnosed as HbA_2' (HbB2) were excluded and published before **[4]**. Twenty eight cases out of 120 were DNA sequenced for δ-globin gene mutation.

One case out of 120 (0.8%) of Far East Asian origin was identified with the δ-variant HbA_2-Indonesia. It had been described in the Indonesian and Malay population at low frequency and in association with HbE and with ß–thalassaemia **[7,8]**. This case had normal red cell indices and a split HbA_2 peak constituting 2.7% of the total haemoglobin. The abnormal HbA_2

peak (1.2%) had a retention time of 4.45 minutes which can be useful in identification on the HPLC.

Two cases, from Holland and Cyprus, out of 120 (1.7%) have been identified as being a carrier for variant HbA_2-Coburg **[6]**. This mutation has been previously described in a Sicilian family in association with ß-thalassaemia trait **[9]**. Hb A_2 is low at 1.2-1.7% and there was no identifiable abnormal HbA_2 peak. HbA_2 Coburg is a δ-chain variant without any reported reduction in expression and thus does not appear to have a thalassaemia phenotype. This means that the variant δ-chain peak representing HbA_2-Coburg may run in a hidden position on the HPLC i.e. masked by HbA, P2 or P3 peaks, thus this variant is not identifiable by a characteristic HPLC retention time.

The codon 136 mutation found in four cases out of 120 (3.3%) produces the δ-chain variant Hb A_2-Babinga, previously reported in Babinga Pygmies (1-2%) and a small number of Black families in the United States **[10]**. Two of the four cases were known to be of African origin, one of these being a carrier for HbS, which is commonly found in African communities. Our studies show that Hb A_2-Babinga does not have a characteristic retention time that can be used for its identification by HPLC. Our HPLC results suggest it runs with the P3 peak on HPLC, causing a slight shift in retention time of the P3 peak, which could possibly be of use in identifying the presence of this variant.

Fourteen of the cases were found to have mutations known to cause δ^+-thalassaemia trait. Two cases (Cypriot and Guyanese) out of 120 (1.7%) were found to have the δ^+-thalassaemic mutation (Codon 4 (ACT->ATT) $delta^+$; HBD: c.14C>T). They were also found to be compound heterozygous for Hb A_2-Yialousa and a silent C>T at codon 97. This thalassaemic variant had been described in Greek Cypriots associated with a C>T at - 199, a silent C>T at codon 97, and an AT deletion at position 722 in IVS-II **[11]**. Both had reduced red cell indices and were found homozygous for the common $-\alpha^{3.7}$ mutation.

Ten cases out of 120 (8.3%) had the δ-globin gene mutation HbA_2-Yialousa indicating that HbA_2-Yialousa is the common δ-thalassaemic mutation and the second common HbA_2 mutation in general. HbA_2-Yialousa has been previously described in a Cypriot family **[12]**, however our cases were of different ethnic origin which means it could be ubiquitous. Three cases were homozygous for this mutation and consequently had an extremely low HbA_2 peak of 0.7-1%, which ran in the normal HbA_2 position. Homozygosity for this mutation means that the variant must have the same retention time as HbA_2 and thus cannot be identified by a characteristic HPLC retention time. Four cases were found compound heterozygous for the mutation and ß-thalassaemia mutations, suggesting that HbA_2-Yialousa is the common δ-thalassaemic mutation associated with ß-thalassaemia in our population. Their HbA_2 level were within normal and low for ß-thalassaemia (2.8-3.4%), indicating that HbA_2-Yialousa could compromise the diagnosis of ß-thalassaemia heterozygosity when this is based on the HbA_2 level only.

One British case out of 120 (0.8%) had the δ-thalassaemic variant HbA_2-Troodos previously described in a Cypriot family **[12]**. No abnormal HbA_2 peak was observed for this patient, but it is not clear if the reduced amount of variant HbA_2 runs with normal HbA_2 or is masked by other Hb peaks.

One case of Asian Indian origin out of 120 (0.8%) had the δ-thalassaemic mutation δ-68 C→T (HBD: c.-118C→T). This mutation had been described by Bouva et al. **[13]**, and was localized on the AACCAAC sequence [HBD from c.-120 to -114 (δ -70 to -64)]. The sequence is considered to be a regulatory element and can compromise the diagnosis of ß-thalassaemia carrier.

One case out of 120 (0.8%) with a novel mutation at codon 52 (HBD: c.157G>C) was observed in a patient of Indian origin with a split HbA_2 on HPLC. The variant peak expression (1.7%) was the same as the normal HbA_2 peak expression (1.7%) and thus the variant mutation does not have a thalassaemic phenotype. The new variant has a characteristic retention time of 4.72 minutes which can be used for identification by HPLC.

A second novel mutation was discovered in three cases (South Korean, Japanese and Irich) out of 120 (2.5%). The mutation, at codon 81

(HBD: c.244C>T), also appears to be a δ-chain variant without a thalassaemia phenotype as the HPLC profiles indicate the presence of a split HbA_2 with peaks of equal size. The new variant has a characteristic retention time of 2.9-3.82 minutes, which can be used for identification by HPLC. One case among these three could not be explained for its result (Table 3, Case 4).

The third novel mutation was at codon 2 (HBD: c.7C>A). Three cases (Asian Indian and Pakistani) out of 120 (2.5%) were found to have the mutation. One of them was homozygous for the mutation with no HbA_2 on the HPLC which means that it is δ^0-thalassaemia mutation. The other two were compound heterozygous for the mutation and the common δ^+-thalassaemic mutation (HbA_2-Yialousa); one of them had no HbA_2 which can be explained by mutations in-trans i.e. the two δ-genes are mutated. The other one had Hb-A_2 of 0.7% which can be explained by mutations in-cis i.e. one δ-gene has the two mutations leaving one δ-gene intact.

Although our data do not reflect the real epidemiology of δ-molecular defects in the UK due to the selection criteria used for detection, they do suggest that δ-globin gene defects are very common, raising the possibility of misleading the diagnosis of ß-thalassaemia carriers. In cases with HbA_2 variants, it is important to consider the HPLC chromatogram carefully to avoid an incorrect diagnosis concerning ß-thalassaemia.

REFERENCES

1- Steinberg MH, Adams JG. Haemoglobin A_2; Origin, evolution and aftermath. Blood. 1991; 78: 2165-2177.

2- Li HW, Codrington JF, Schiler UG, Wadsworth LD, Bertie P, Adekile A, Huisman TH. The usefulness of sequence analysis of amplified DNA for the identification of δ-chain variants. Hemoglobin. 1991; 15 (1-2): 77-84.

3- Weatherall DJ, Clegg JB. The thalassaemia syndromes. Fourth edition Blackwell Science. UK. 2001.

4- Khalil MSM, Old J: HPLC and DNA diagnosis of HBA_2': The commenst δ-globin gene variant. Egyptian J. Haematol. Sep. 2010; 35 (4): 377-381.

5- Old JM, Higgs DR. The thalassaemias, Methods in haematology (ed by D.J. Weatherall) 1983; 6: 74-102. Churchill Livingstone, Edinburgh.

6- Huisman TH, Carver M, Efremov G. A syllabus of human haemoglobin variants. The sickle cell Foundation (USA) 1998.

7- Lie-Injo LE, Poey-Oey HG, Mossberger RJ. Haptoglobins, transferrins, and hemoglobin A_2 in Indonesians. Am J Hum Genet. Sep. 1968; 20 (5): 470-3.

8- Eng LI, Pribadi W, Westendorp Boerma F, Efremov GD, Wilson JB, Reynolds CA, Huisman TH. Hemoglobin A_2-Indonesia or $\alpha_2\delta_2$ 69 (E13) Gly → Arg. Biochim Biophys Acta. Feb. 1971; 16: 229 (2): 335-42.

9- Sharma RS, Williams L, Wilson JB, Huisman TH. Hemoglobin-A_2-Coburg or $\alpha_2\delta_2$116Arg lead to His (G18). Biochim Biophys Acta. Jun. 1975; 26: 393 (2): 379-82.

10- De Jong WW, Bernini LF. Haemoglobin Babinga (delta 136 glycine-aspartic acid): New delta chain variant. Nature. Sep. 1968; 28: 219 (5161): 1360-2.

11- Trifillis P, Kyrri A, Kalogirou E, Kokkofitou A, Loannou P, Schwartz E, Surrey S. Analysis of δ-globin gene mutations in Greek Cypriots. Blood. Sep. 1993; 1: 82 (5): 1647-51.

12- Trifillis P, Ioannou P, Schwartz E, Surrey S. Identification of four novel delta-globin gene mutations in Greek Cypriots using the polymerase chain reaction and automated florescence-based DNA sequence analysis. Blood. Dec. 1991; 15: 78 (12): 3298-305.

13- Bouva MJ, Harteveld CL, Peter van Delft, Giordano PC. Known and new δ globin gene mutations and their diagnostic significance. Haematologica. Jan. 2006; 91 (1): 129-32.

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Circulating Micro-Particles as Potential Hemostatic Biomarkers for Cerebrovascular Ischemic Infarction

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ABSTRACT

Background: Cellular micro-particles (MPs) are submicron plasma membrane derived vesicles shed into the circulation by a variety of blood cells and vascular cells during cellular activation and apoptosis. Currently no practical, rapid and sensitive test is available for the diagnosis of acute ischemic stroke. Current knowledge from earlier studies on MPs suggests that they represent reliable biomarkers as they are cell specific and released early in the pathophysiological cascade of the disease.

Patients and Methods: This study included 20 patients with acute cerebrovascular ischemic infarction confirmed by neuroimaging as well as 20 matched healthy controls. Full evaluation of clinical, radiological and laboratory data was done. Peripheral blood endothelial, platelet, erythrocyte and monocyte micro-particles were measured by flowcytometry using their corresponding monoclonal antibodies (anti CD 62 E, anti CD 61 P, anti CD235 and anti CD 14) respectively.

Results: A significantly higher CD 235 and highly significantly elevated CD 61P and CD 14 were observed in stroke patients compared to controls where platelet derived micro-particles PMPs were the most commonly occurring (28.8%). Co-expression of CD61P and CD62E was a common feature in stroke patients (39.5%) which was found to be highly significant when compared to controls. A cutoff value for the co-expression of CD 61P and CD 62 E as a marker of thrombotic stroke was suggested to be 13.5% using ROC curve statistical method. This co-expression was higher among stroke patients with diabetes mellitus and cardiac disease while CD61P expression was significantly higher in diabetic patients when compared to non-diabetics. A significant higher expression of CD 61P and CD 235 was found in patients not receiving anticoagulation at the time of sampling when compared to controls.

Conclusions: The higher levels of CD 61P, CD 62 E, CD 14 and co-expression of CD 61P and 62E suggests that the systemic endothelial, platelet and inflammatory cell activation increases the risk for cerebrovascular morbidities especially in patients with diabetes mellitus and history of cardiac disease. MPs co-expressing CD62E and CD 61P can be used as a test for the early diagnosis of thrombotic stroke with high sensitivity and specificity. Establishing a cutoff value for co-expression of CD62E and CD61P in stroke patients can contribute to the clinical applications as using MP assay in diagnosis of thrombotic propensity, monitoring of anticoagulant therapy, and detection of risk of stroke and ischemic heart disease in high risk patients.

Key Words: *Stroke – Micro-particles – Biomarker – Flowcytometry.*

INTRODUCTION

Cellular micro-particles are small submicron plasma membrane derived vesicles which are shed into the circulation by a variety of blood cells and vascular cells during cellular activation or apoptosis **[1]**. Micro-particles are present in low concentrations in normal plasma and possess some specific cell surface proteins that indicate their cell of origin, as well as other cell surface molecules that regulate their physiological and pathologic interactions as coagulation, cell signaling and cellular interactions **[2]**.

Their procoagulant properties are mediated through their phospholipid rich surfaces as well as cell surface molecules reflecting their cell of origin: Tissue factor (TF), large multimers of vWF and P-selectin **[3]**. In the recent years circulating MPs of endothelial, platelet and leucocyte origin have been implicated in contributing to the pathogenesis of thrombosis in different thrombotic disorders **[4]**. Their newly diagnosed role in vascular accidents is an area of immense interest that promises to yield important advances into diagnosis and therapy **[5]**.

Increasing studies are exploring the role of circulating MPs in thrombotic stroke. Currently, no practical, rapid and sensitive test is available for the diagnosis of acute ischemic stroke. A number of soluble molecules have been identified that are merely associated to these cerebrovascular accidents. Current knowledge suggests that these membrane derived micro-particles may represent reliable biomarkers as they are cell specific and released early in the pathopysiological cascade of a disease. MPs can be found not only in the cerebrospinal fluid but also in tears and circulating blood in case of blood brain barrier dysfunction. They represent a new challenge in stroke diagnosis and management **[6]**.

The aim of this work was to study circulating platelet, endothelial, monocyte, and erythrocytes micro-particles in patients with ischemic stroke in comparison with healthy controls to assess their clinical application as sensitive biomarkers for diagnosis, prediction and management of the disease.

SUBJECTS AND METHODS

An informed written consent was obtained from all patients or their legally authorized relatives as well as the healthy controls prior to their enrolment. Approval of the Institutional Ethical Review Board was obtained.

This is a case control cross-sectional study which included 20 patients and 20 age matched controls. Patients enrolled were those who have had experienced focal neurological symptoms and signs lasting 24 hours or longer with a relevant ischemic lesion within the brain assessed by neuroimaging. Patients above 50 years of both genders were recruited from the stroke unit at Kasr Al Aini Hospital. Patients were excluded if they presented with stroke of other determined etiology as cardioembolic, hemorrhagic, non-atherosclerotic vasculopathy, hypercoagulable states or borderzone infarction secondary to cerebral infarction.

Patients were subjected to full medical history taking and clinical assessment with emphasis on previous thrombotic or bleeding disorders, previous angina, myocardial infarction (MI), transient ischemic attacks (TIA), previous cerebrovascular accidents, presence of cerevascular risk factors as hypertension, diabetes mellitus, smoking, hyperlipidemia and history of medications as antihypertensives and anticoagulants.

Both patients and controls were subjected to neuroimaging studies (CT brain or MRI), echocardiography and duplex, ECG, routine laboratory investigations (complete blood count, renal functions, liver functions, lipid profile and hemostatic profile) and flowcytometric measurement of circulating micro-particles.

Citrated whole blood (2ml) was collected and processed for platelet poor plasma (PPP). Micro-particles were obtained by centrifugation in two steps. The initial centrifugation to obtain platelet poor plasma was done at 3000 rpm for 25 minutes at room temperature (20ºC-24ºC). The supernatant was removed and transferred to another test tube leaving 200µl above the cell pellet. This was followed by another centrifugation of the supernatant at 5000 rpm for 5 minutes at room temperature. One ml supernatant was collected for micro-particle measurement leaving a 100µl pellet which was discarded. MPs were stained for 30 minutes in the dark at room temperature, with periodic vortexing, by mixing 100µl of PPP with 20µl of each of the anti-human fluorochrome- conjugated antibodies (fluorescein isothiocynate-labeled anti-CD 61P with phycoerythrin-labeled anti-CD 62E in one tube and allophycocyanin-labeled anti-CD 14 with phycoerythrin-labeled anti-CD 235 in a second tube). All antibodies were obtained from Beckman Coulter except CD 235-PE which was obtained from Dako, Denmark. Then 100µl of red cell lysing buffer was mixed with the sample, followed by vortexing and 10 minutes incubation in the dark. The sample was diluted with 200µl phosphate buffered saline (PBS) analyzed on a Beckman Coulter MCL-XL2 setting the stop condition for both patients and controls at 3000 events. An initial micro-particle-size gate was set at 1.0µ based on the forward scatter results.

Statistical method:

In this case-control study, sample size calculation was not possible due to insufficient literature that suggests the expected mean difference of micro-particle expression in stroke patients compared to control subjects.

Clinical and laboratory data were analyzed using SPSS software (SPSS 17.0, SPSS Inc., Chicago, Il). Continuous data was calculated

as mean and standard deviation (SD). Clinical findings were reported in frequency tables. Micro-particles phenotypic expression comparison was performed using the Student *t*-test when data was of normal distribution. For non-parametric data, Mann-Whitney U test was used for comparison of mean values. Receiver operator characteristic (ROC) curve was used for estimation of cutoff value for expression of CD61P/CD62E in stroke patients, to contribute to the future test evaluation. Data were found significant when *p*-value was less than 0.05.

RESULTS

The patient population comprised 20 cases including 17 males and 3 females with an age range of 50-73 with a mean of 63.65±6.43 and a median of 64 years.

As regards risk factors, hypertension was encountered in 18 (90%), hyperlipidemia and diabetes, each in 15 (75%), thrombophilia and family history of stroke, each in 2 (10%) and 12 (60%) of patients were smokers.

Other pathological conditions included associated cardiac disease and renal affection, each in 5 (25%) as well as respiratory disease and hepatic affection, each in 3 (15%) of patients. Presenting complaint and neurological symptoms and signs encountered in the patients' cohort are presented in Table (1). Table (2) presents the medications received by the patients at the time of diagnosis.

Various circulating micro-particles levels are presented in Table (3). Table (4) represents the findings in various patients' subgroups.

No association was encountered between any of the circulating micro-particle types on one hand and any of the presenting complaints, symptoms or signs on the other hand except for an association between erythroid micro-particles (CD235) and blurred vision. Patients who presented with blurred vision showed a level of 31.0500±29.37667 versus 9.7700±8.44552 for those who did not present with blurred vision (p=0.043).

Studying the difference between micro-particles levels in patients and controls as regards the variable risk factors, no statistically significant association was found with either hyperlipidemia, hypertension or smoking. Neither was there any statistically significant association with the various neurological symptoms and signs (headache, neuropathy, vertigo, muscle weakness or memory affection) or with receiving medication.

Receiver operating characteristic (ROC) curves were used to determine a cutoff value for CD61P/CD62E co-expression as a marker of thrombotic stroke. Suggested cutoff value is 13.5%. Other micro-particles showed an overlap between patients and controls and calculation of cutoff values were not possible.

Table (1): Complaint and neurological symptoms/signs in 20 stroke patients.

Presentation	No. (%)	Neurological symptoms/signs	No. (%)
Coma	8 (40.0)	Fainting attack	1 (5.0)
Drowsiness	3 (15.0)	Fits	3 (15.0)
Rt side hemiplegia	3 (15.0)	Headache	11 (55.0)
Lt side hemiplegia	3 (15.0)	Neuropathy	10 (50.0)
Muscle paresis	3 (15.0)	Memory affection	2 (10)
Quadriplegia	2 (10.0)	Muscle weakness	9 (45)
		Vertigo	2 (10)
		Blurred vision	8 (40)

Table (2): Type of medications received by 20 stroke patients at time of sampling.

Medication	Frequency	Percentage
Oral anticoagulant	5	25.0
Antiplatelet therapy	2	10.0
Aspirin + oral anticoagulant	4	20.0
Antihypertensive drugs	18	90.0
Insulin	11	55.0
Oral hypoglycemic	2	10.0

Table (3): Type of medications received by 20 stroke patients at time of sampling.

Cell specific micro-particle	Patients	Controls	*p*-value
Platelet (CD 61P)	28.81±22.67*	5.31±5.79	0.001
Endothelial (CD 62E)	2.02±3.16	3.58±4.81	0.233
Erythrocyte (CD 235)	22.67±22.62	7.72±3.71	0.031
Monocyte (CD 14)	15.05±19.31	2.45±3.43	0.007
Co-expression CD 61P/CD 62E	39.52±24.79	1.95±1.90	0.023*

Mean ± SD

Table (4): Circulating micro-particles level in 20 stroke patients' subgroups.

Patients' subgroups	Circulating Micro-particle Type									
	Platelet (CD 61P)		Endothelial (CD 62E)		Erythrocyte (CD 235)		Monocyte (CD 14)		CD 61P/ CD 62E	
Cardiac:										
Yes	20.82±21.54*	0.37**	1.98±1.55	0.97	17.80±15.96	0.89	15.76±21.29	0.92	66.73±26.97	0.006
No	31.47±23.12		2.03±3.58		21.54±24.68		14.79±19.40		25.91±5.04	
Diabetes:										
Yes	23.08±23.08	0.047	2.12±3.35	0.802	19.11±20.44	0.97	12.54±18.64	0.286	44.67±26.07	0.05
No	46.00±17.78		1.70±2.81		19.62±32.96		24.45±21.58		21.50±0.707	

* Mean ± SD ** *p*- value

DISCUSSION

Although micro-particles are present in low concentration in normal plasma, increased levels are generated in response to platelet activation, direct vascular endothelial damage and thrombin activity on the cell surface. Indeed their altered numbers and characteristics that are exhibited in many vascular diseases with increased thrombotic predilection (both arterial and venous) has urged for the need to achieve further advances towards their use in diagnosis and therapy in vascular accidents.

Since few reports are available on the role of MPs in thrombotic cerebral stroke, the current study has aimed to assess the clinical application of circulating MPs in the diagnosis, prediction and management of thrombotic cerebrovascular accidents through the measurement of levels of platelet (CD61P), endothelial (CD 62E), erythrocyte (CD 235) and monocyte (CD 14) microparticles by flowcytometry in a cross sectional sample of 20 patients and 20 healthy controls.

In the current study, the most common type of micro-particles was PMP (28,8%), MPs co-expressing both platelet and endothelial markers (39.5%) followed by MP of erythrocyte origin (19.2%), then those of monocyte origin (15%) and finally endothelial derived MP (2%). Comparing MP levels in stroke patients versus controls revealed significantly higher CD 235 expression and highly significant greater expression of both CD 61P and CD 14 in stroke patients compared to control but not endothelial MPs. This finding is in accordance with other studies. A study of cerebro-occlusive events, reported high PMP levels in cerebrovascular accidents in small and large vessels and multifactorial dementia with no apparent effect of antiplatelet therapy on PMP levels **[7]**. Again other studies reported increased levels of PMP in both acute and chronic phase of cerebral infarction **[8,9]** and another study reported that this significantly correlated with intima media thickness and intracranial stenosis of carotid arteries **[10]**. In contrast to this study, other studies detected elevation of endothelial derived MP levels in acute ischemic stroke **[11,12]**. Also a recent study on endothelial micro-particles reported that high levels of CD 62E was associated with cardiovascular events in patients with stroke. This study did not simultaneously assay PMP **[13]**.

In the current study, CD 61P/CD62E co-expression was a common feature in stroke patients. Only a single report on co-expression of platelet and endothelial markers was found. This "remarkable" finding, as stated by the authors, was suggested to be a result of an interaction between platelets (or platelet fragments) and endothelial cells resulting in cellular activation and generation of micro-particles of bi-lineage origin **[14]**.

In healthy control subjects of the present study erythroid MPs exhibited the highest level of expression, followed by platelet then endothelial and the least was monocyte derived MPs. This is not in agreement with other reports, in which the majority of micro-particles in healthy controls were of platelet origin, followed by endothelial, red blood cells and monocytes.

MPs level was compared according to presence or absence of other clinical data of stroke patients. MPs of erythroid origin were significantly higher in patients complaining of blurred vision. However this finding was not supported by other studies on stroke patients. In the current

study, both CD 61P and CD61P/CD 62E co-expression were significantly higher in diabetic patients when compared to non-diabetics. In partial agreement with our results, endothelial derived MPs were found to be elevated in patients with DM with no reports on co-expression of endothelial and platelet MPs **[15]**. Other studies have shown that elevated MPs in diabetic patients especially of endothelial, platelet and monocyte origin correlate with diabetic complications **[16]**. Another study related platelet and endothelial MPs levels in diabetic patients to vascular complications **[17]**.

No significant difference in MPs levels was found between hypertensive and non–hypertensive stroke patients in the present study. This is in contrast to other studies which reported the association between elevated levels of endothelial derived MPs and hypertension **[18,19]**.

The current study showed no significant difference when comparing MPs levels in patients with renal dysfunction with disease free patients. This is in disagreement with a study done on patients with chronic renal failure that reported that renal disease is accompanied by endothelial activation and their results in all their patient subgroups showed an increase mainly of platelet-derived MPs, with minor populations of endothelial and TF bearing MPs **[20]**.

A recent review on micro-particles as reliable markers of thrombosis highlighted that the difficulty in identification, standardization and quantification methods of MPs, hinders its use as a practical and clinical diagnostic tool. Further prospective studies and more evaluation of diagnostic value of MP assays in different diseases are worth doing to promote the utility of this technique in clinical practice **[21]**. Improved decision making in diagnosis and patient management is only one route by which tests affect patient health, and empirical evaluations are needed to compare the effect of test strategies on patient health. To establish whether a new diagnostic test will change health outcomes, it must be examined as part of a broader management strategy **[22]**.

In conclusion, increased circulating microparticles (CD 61P, CD 62 E, CD 14 and co-expression of CD 61P and 62E) suggests that the systemic endothelial, platelet and inflammatory cell activation increases the risk for cerebrovascular morbidities especially in patients with diabetes mellitus and history of cardiac disease. Establishing a cutoff value for co-expression of platelet and endothelial circulating MPs and significant elevation of its level in stroke patients especially those with history of DM and cardiac ischemia can contribute to the clinical application. Evaluation of its plasma level may be used in diagnosis of thrombotic propensity, monitoring of anticoagulant therapy, and detection of risk of stroke and ischemic heart disease in diabetic patients.

REFERENCES

1- Piccin A, Murphy WG, Smith OP. Circulating microparticles: Pathophysiology and clinical implications. Blood Rev. 2007; 21: 157-171.

2- Morel O, Toti B, Hugel B, Bakouboula B, Camoin-Jau L, Dignat-George F, Freyssinet JM. Pro-coagulant microparticles: Disrupting the vascular homeostasis equation? Arterioscler Thromb Vas Biol. 2006; 26: 2594-2604.

3- Van der Meijden PE, Van Schlifgaarde M, Van Oerle R, Renne T, Ten Cate H, Spronk HM. Platelet and erythrocyte-derived microparticles trigger thrombus generation via factor XIIa. J Thromb Haemost. 2012; 10: 1335-1362.

4- Mackman N. Role of tissue factor in hemostasis and thrombosis. Blood Cells Mol Dis. 2006; 36: 104-107.

5- Mallat Z, Benamer H, Huger B, Benessiana J, Steg PG, Freyssinet JM. Elevated levels of shed membrane microparticles with procoagulant potential in the peripheral circulating blood of patients with acute coronary syndromes. Circulation. 2000; 101: 841-843.

6- Morel O, Hugel B, Jesel L, Lanza F, Douchet MP, Zupan M. Chauvin M, Cazenave JP, Freyssinet JM, Toti F. Sustained elevated amounts of procoagulant membrane microparticles and soluble GPV after acute myocardial infarction in diabetes. Thromb Haemost. 2004; 91: 345-353.

7- Lee JM, Zipfel GJ, Choi DW. The changing landscape of ischaemic brain injury mechanisms. Nature. 1999; 399 (6738 Suppl): A7-14.

8- Cherian P, Hankey GJ, Eikelboom JW, Thom J, Baker RI, McQuillan A, Staton J, Yi Q. Endothelial and platelet activation in acute ischemic stroke and its etiological subtypes. Stroke. 2003; 34: 2132-2137.

9- Pawelczyk M, Baj Z, Chmielewski H, Kaczorowska B, Klimek A. The influence of hyperlipidemia on platelet activity markers in patients after ischemic stroke. Cerebrovasc Dis. 2009; 27: 131-7.

10- Kuriyama N, Nagakane Y, Hosomi A, Ohara T, Kasai T, Harada S, Takeda K, Yamada K, Ozasa K, Tokuda T, Watanabe Y, Mizuno T, Nakagawa M. Evaluation of factors associated with elevated levels of platelet-

derived microparticles in the acute phase of cerebral infarction. Clin Appl Thromb Hemost. 2010; 16: 26-32.

11- Simak J, Gelderman MP, Yu H, Wright V, Baird AE. Circulating endothelial microparticles in acute ischemic stroke: A link to severity, lesion volume and outcome. J Thromb Haemost. 2006; 4: 1296-1302.

12- Jung KH, Chu K, Lee ST, Park HK, Bahn JJ, Kim DH, Kim JH, Kim M, Kun Lee S, Roh JK. Circulating endothelial microparticles as a marker of cerebrovascular disease. Ann Neurol. 2009; 66: 191-199.

13- Lee ST, Chu K, Jung KH, Kim JM, Moon HJ, Bahn JJ, Im WS, Sunwoo J, Moon J, Kim M, Lee SK, Roh JK. Circulating CD62E+ microparticles and cardiovascular outcomes. PLoS One. 2012; 7: e35713.

14- Trappenburg MC, van Schilfgaarde M, Marchetti M, Spronk HM, ten Cate H, Leyte A, Terpstra WE, Falanga A. Elevated procoagulant microparticles expressing endothelial and platelet markers in essential thrombocythemia. Haematologica. 2009; 94: 911-918.

15- Bernard S, Loffroy R, Sérusclat A, Boussel L, Bonnefoy E, Thévenon C, Rabilloud M, Revel D, Moulin P, Douek P. Increased levels of endothelial microparticles CD144 (VE-Cadherin) positives in type 2 diabetic patients with coronary noncalcified plaques evaluated by multidetector computed tomography (MDCT). Atherosclerosis. 2009; 203: 429-435.

16- Nomura S. Dynamic role of microparticles in type 2 diabetes mellitus. Curr Diabetes Rev. 2009; 5: 245-251.

17- Tsimerman G, Roguin A, Bachar A, Melamed E, Brenner B, Aharon A. Involvement of microparticles in diabetic vascular complications. Thromb Haemost. 2011; 106: 310-321.

18- Amabile N, Heiss C, Chang V, Angeli FS, Damon L, Rame EJ, McGlothlin D, Grossman W, De Marco T, Yeghiazarians Y. Increased CD62e (+) endothelial microparticle levels predict poor outcome in pulmonary hypertension patients. J Heart Lung Transplant. 2009; 28: 1081-1086.

19- Wang JM, Su C, Wang Y, Huang YJ, Yang Z, Chen L, Wu F, Xu SY, Tao J. Elevated circulating endothelial microparticles and brachial-ankle pulse wave velocity in well-controlled hypertensive patients. J Hum Hypertens. 2009; 23: 307-315.

20- Trappenburg MC, van Schilfgaarde M, Frerichs FC, Spronk HM, ten Cate H, de Fijter CW, Terpstra WE, Leyte A. Chronic renal failure is accompanied by endothelial activation and a large increase in microparticle numbers with reduced procoagulant capacity. Nephrol Dial Transplant. 2012; 27: 1446-1453.

21- Chopra S, Microparticles: Reliable Markers of Thrombosis," The Meducator: Vol. 1:Iss. 20, Article 7. Available at: 2011 http://digitalcommons.mcmaster.Ca /meducator/vol1/iss20/7

22- Ferrante di Ruffano L, Hyde CJ, McCaffery KJ, Bossuyt PM, Deeks JJ. Assessing the value of diagnostic tests: A framework for designing and evaluating trials. BMJ. 2012; 344: e686.

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MDR Gene and LRP As Prognostic Indicators in Adult AML Patients

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ABSTRACT

Background: Multidrug resistance (MDR) genes, multidrug resistance 1 (MDR1) protein and lung associated resistance protein (LRP), are correlated with the outcome of treatment of acute myeloid leukemia (AML).

Objective: Our aim in this study was to evaluate the frequencies of occurrence of MDR1, and LRP in Egyptian adult AML patients, and to correlate between their expression and disease prognosis.

Patients and Methods: In this study, the expression of MDR1 protein (P-gp) & LRP were measured using flowcytometry on bone marrow samples of 46 de-novo adult AML patients. Expressions were correlated to clinical & laboratory variables, response to treatment and overall survival.

Results: MDR1 protein was found positive or over expressed in 14 patients (30.4%), 4 (28.6%) of them achieved CR where as 10 (71.4%) were refractory (p=0.034). LRP was found positive in 12 patients (26.1%), 6 of them (50%) achieved CR and 6 (50%) were refractory (p=0.861). MDR1 showed significant correlation with hemoglobin level (p=0.034), and response to therapy (p=0.034).

None of the 2 parameters had any correlation with age, gender, WBC count, organomegally, BM blasts, FAB classification, BM cellularity or overall survival.

Conclusion: Only positive expression of MDR1 represents a significant prognostic indicator in adult AML cases.

Key Words: *Adult acute myeloid leukemia – MDR – LRP.*

INTRODUCTION

Drug resistance is a major obstacle in the successful treatment and an important cause of death in acute leukemia. Such resistance may be present before beginning treatment or may develop during chemotherapy. Drug resistance that extends to structurally and functionally unrelated drugs is termed multidrug resistance (MDR) **[1,2]**.

Several molecular biological mechanisms have been identified as being associated with MDR **[3]**. P-glycoprotein (P-gp) is a product of the multidrug resistance1 gene (MDR1) and is an ATP-dependent pump capable of expelling drugs out of cancer cells **[4]**. P-gp is a transmembrane glycoprotein conferring cross-resistance to a variety of mechanistically and structurally unrelated cytotoxic drugs, such as anthracyclines, taxanes, vinca alkaloids and epipodophyllotoxins **[4]**. In addition, a 110 kDa protein has been identified in a P-gp negative MDR lung cancer cell line. This protein was termed the lung resistance protein (LRP). LRP is the human major vault protein frequently overexpressed in drug resistant cancer cells and its expression has been correlated with poor prognosis. Vault proteins are present in all eukaryotic cells, and they are highly conserved. Several clinical data have indicated that LRP expression can be of high clinical value to predict the response to chemotherapy in some tumor types such as non-small cell lung carcinoma, osteosarcoma, melanoma and neuroblastoma **[5]**.

Despite the identification of these proteins, the pathways that result in drug resistance in leukemic cells remain largely uncharacterized. While drug resistance gene expression has been studied in acute leukemia, the value of MDR1 and LRP gene expression as independent predictors of treatment success is still controversial **[4,6]**.

Our aim in this study is to evaluate the frequencies of occurrence of MDR proteins P-gp, and LRP in Egyptian adult AML patients, and to correlate them with disease prognosis and clinical and laboratory variables.

PATIENTS AND METHODS

Patients:

The present study was carried out on 46 adult patients with de novo AML, who presented to the National Cancer Institute, Cairo University, in the period between October 2009 and December 2010. After an informed consent all studied patients were subjected to thorough history taking and full clinical examination. In addition radiology examination in the form of chest X-ray, abdominal ultrasound and CT scan whenever needed were done.

Complete blood picture, bone marrow aspiration and morphological examination, liver and kidney function tests were also done.

Immunophenotyping was done by flow cytometry (Partec III from DAKO cytomation), on marrow blast cells with a panel of monoclonal antibodies, purchased from DAKO (Denmark), including FITC and PE conjugated CD13, CD33 and MPO. Specific isotype controls for FITC, PE conjugated monoclonal antibodies were used. Results were expressed as percentage of cells showing positive expression.

Diagnosis of AML was based on the presence of ≥20% blast cells in BM film according to WHO proposal **[7]**, together with MPO staining and immunophenotyping.

Of the 46 newly diagnosed AML patients enrolled in this study, forty-one patients received the standard AML induction chemotherapy protocols applied at the NCI, Cairo University which are differentiated according to age and the subtype of AML. Four patients with acute promyelocytic leukemia received adriamycin and vesanoid. Thirty-three patients younger than 55 years received ARA-C plus adriamycin (3&7) protocol. For the 4 patients aged above 55 years, three of them received ARA-C plus adriamycin (2&5) protocol while one patient received oral vepside capsule. Response to induction therapy was assessed between days 21 & 28 after induction therapy. Patients achieving complete remission (CR) received consolidation therapy. Patients who did not achieve CR are considered refractory cases.

Methods:

Detection of MDR1 and LRP expression by flow cytometry (Partec III from DAKO cytomation) was done on lysed whole blood using anti-human MDR1 and LRP (FITC) monoclonal antibodies, purchased from DAKO (Denemark). Irrelevant monoclonal antibodies of the same isotypes and protein concentration were used as negative controls.

For interpretation of the results, the mean fluorescence index ratio (MFIR) was used, which represents the ratio between the mean fluorescence intensity of cells stained with the specific antibody and that of cells stained with the isotype-matched control antibody, the case was considered over expressing or positive for P-gp at a ratio of ≥1.1 **[8]**, and was considered positive for LRP when the ratio exceeds ≥0.3.

Statistical methods:

Data was analyzed using SPSSwin statistical package version 17 (SPSS Inc., Chicago, IL). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test (Fisher's exact test) was used to examine the relation between qualitative variables. For quantitative data, comparison between two groups was done using Mann-Whitney test (non parametric *t*-test). Survival analysis was done using Kaplan-Meier method and comparison between two survival curves was done using log-rank test. A p-value<0.05 was considered significant.

RESULTS

Forty six consecutive patients with de novo acute myeloid leukemia, who presented to the National Cancer Institute, Cairo University, in the period between October 2009 and December 2010, were included in this study. They were 33 males and 13 females, their ages ranged from 18 to 65 with a median of 37.5 years.

The clinico-hematological findings, treatment status and number of deaths of the 46 adult AML patients included in this study are presented in (Table 1).

The expressions of LRP & MDR were studied in the 46 patients. In these patients, we found 9 (19.5%) patients with simultaneous activity of MDR & LRP, 29 (63%) without activity of both, 5 (11%) with MDR activity only, and 3 patients (6.5%) with LRP activity only. Therefore, 17 (37%) patients had functional activity of one or both proteins.

Table (2) represents the hematological parameters of 46 adult AML patients in relation to positive and negative expression of MDR and LRP. There was no statistically significant difference between patients with negative and those with positive MDR and LRP expression as regards the hematological parameters except for the Hb (p=0.034).

Table (3) sums the positive and negative LRP-MDR1 co-expression in relation to other hematological parameters with no significant differences (p>0.05).

Of the newly diagnosed 46 adult AML patients, 24 (52.2%) achieved complete response, 5 (10.9%) died early during the study, while 17 patients (37%) failed to achieve response.

Among those who have positive MDR expression, 4/14 (28.8%) achieved CR compared to 20/32 (62.5%) with MDR negative expression (p=0.034).

Regarding the complete response rate in relation to positive and negative LRP expressions, 6 out of 12 (50%) patients who have positive LRP expression achieved CR, compared to18/34 (52.9%) of those who have negative LRP expression (p=0.86).

Finally, we analyzed the coexpression of LRP and MDR status in relation to clinical outcome (Table 4). Response to induction chemotherapy was best (CR rate 62%) in patients lacking expression of both genes, intermediate (CR rate 44.4%) in patients expressing both genes and worst in those with expression of either of these two genes, (CR rate 25%). Although our results showed that MDR expression is significantly associated with CR rate, contribution of the combined activity of MDR and LRP in the CR rate did not achieve statistical significance (p=0.079).

Table (5) represents the impact of MDR1 expression on response to therapy showing that –ve MDR1 expression is associated with a higher CR rate (p=0.034).

Table (1): Clinical and Laboratory Characteristics of 46 adult AML patients.

Parameter	Median	Range
Age (years)	37.5	18-65
BM Blasts (%)	75	18-95
TLC (x10^9/L)	58	4.3-470
Plt. (x10^9/L)	36	5-34
Hb (g/dL)	7.3	3.1-11
Parameter	Number (No.)	Percent (%)
Gender:		
Female	13	28.26
Male	33	71.74
FAB classification:		
M0	2	4.3
M1	16	34.8
M2	18	39.1
M3	5	10.9
M4	3	6.5
M5	2	4.3
Treatment Arm:		
Ara-C+ADR (3&7)	33	71.73
Ara-C+ADR (2&5)	3	6.52
ADR+Vesanoid	4	8.69
VP16	1	2.17
Early death	5	10.86

BM : Bone Marrow.
TLC : Total leucocytic count.
Plt : Platelets.
Hb : Hemoglobin.
FAB : French-American-British classification of AML.

Table (2): Hematological parameters of 46 adult AML patients in relation to MDR and LRP expression.

Parameter	MDR		LRP	
	Negative	Positive	Negative	Positive
Age (years)	39±14	35.2±11.6	37.3±14	39.4±11.4
	(20–65)*	(18–58)	(18–65)	(24–58)
	42.5**	33	33	43
BM Blasts (%)	70.1±20	69.4±20.7	71.8±19.9	65±20
	(18–91)	(30–95)	(18 –95)	(30–90)
	74	76	75.5	74.5
TLC (x10^9/L)	79.6±92.7	51.2±33.3	79.8±91.4	48.1±31.5
	(4.3–470)	(5–116)	(4.3–470)	(5–97)
	65.5	52	64	49.5
Plt. (x10^9/L)	45.2±30.2	40.9±38.1	43±31.2	46.5±36.7
	(5–134)	(8.8–134)	(5–134)	(10–134)
	37.9	27	37	33
Hb (g/dL)	6.8±1.8	8.1±2	7.1±1.8	7.3±2.4
	(3.1–11)	(4.3–11)	(3.1–11)	(4.3–11)
	6.9	8.2	7.1	7.8

* Mean±SD (range). ** Median.
No significant p-value was detected except with Hb p-value (0.034).

Table (3): LRP-MDR1 co-expression in relation to other hematological parameters in 46 adult AML patients.

Parameter	Positive co-expression	Negative co-expression
Age (years)	36.7±11.9 (24–58)* 39**	38.1±13.8 (18–65) 36
BM Blasts (%)	66.3±19.5 (30–84) 75	70.8±20.2 (18–95) 75
TLC ($x10^9$/L)	43.2±30.5 (5–97) 46	78.4±88 (4.3–470) 65.5
Plt. ($x10^9$/L)	50.4±42.8 (10–134) 34.5	42.3±29.9 (5–134) 36
Hb (g/dL)	8±2.3 (4.3–11) 8.2	7±1.8 (3.1–11) 7

* Mean±SD (range).
** Median. No significant value (p>0.05).

Table (4): LRP & MDR status in relation to response to therapy in 46 adult AML patients.

Status \ Response	CR	RF	Total
LRP^-/MDR^-	18 (62%)	11 (38%)	29
LRP^-/MDR^+ or LRP^+/MDR^-	2 (25%)	6 (75%)	8
LRP^+/MDR^+	4 (44.4%)	5 (55.6%)	9

CR: Complete remission.
RF: Refractory.

Table (5): Impact of MDR1 (P-gp) expression on response to therapy in 46 adult AML patients.

P-gp expression	CR	RF	Total	p
Positive >=1.1	4 28.6%	10 71.4%	14 100%	0.034
Negative <1.1	20 62.5%	12 37.5%	32 100%	

CR: Complete remission.
RF: Refractory.

Figs. (1,2,3) represent the impact of LRP, MDR separately and combined on overall survival. No significant differences was encountered with any (p=0.381, 0.190 and 0.714 respectively).

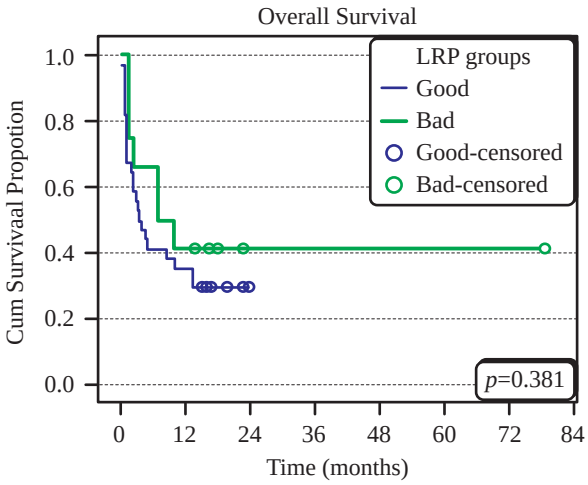

Fig. (1): Overall survival in relation to LRP expression in 46 AML patients.

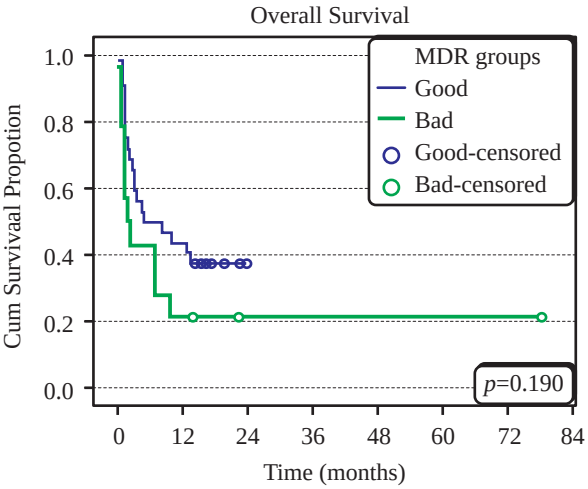

Fig. (2): Overall survival in relation to MDR expression in 46 AML patients.

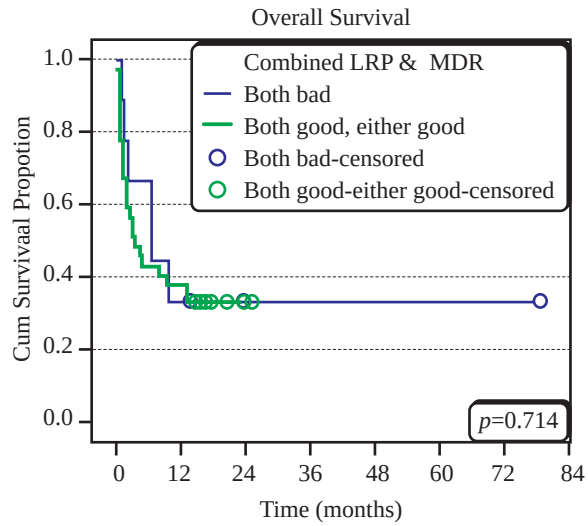

Fig. (3): Overall survival in relation to combined LRP and MDR expression in 46 AML patients.

DISCUSSION

Drug resistance is a multifactorial phenomenon and several mechanisms have been recognized for clinical resistance to chemotherapy in solid tumors as well as in hematologic malignancies. The two important mechanisms of drug resistance in leukemia are expression of drug resistance genes and activation of anti-apoptotic mechanism **[4]**.

With the advent of better chemotherapy and supportive therapy care in the past decade, clinical outcome has improved considerably for adult patients with both acute myeloid leukemia (AML) as well as acute lymphoblastic leukemia (ALL). However, leukemic cells from adults are intrinsically more resistant to drugs commonly used in induction chemotherapy as compared to those from pediatric patients **[6]**. Unfavorable karyotype, poor treatment tolerance and over expression of multi drug resistant genes in adults could account for this difference **[9]**.

Studies on the treatment of adult AML have shown only modest improvements over the last 2 decades, with the actual cure rate still ranging between 15% and 40%. The resistance of tumor cells to chemotherapeutic drugs is a major limitation in cancer treatment. MDR phenotype is the most frequently studied mechanism for intrinsic drug resistance, yet the prognostic role of P-gp and other MDR-associated proteins in adult AML is still largely unknown **[4]**.

Our aim in this study is to evaluate the frequencies of occurrence of MDR agents P-gp (MDR1), and LRP in Egyptian adult acute myeloid leukemia patients, and to correlate them with disease prognosis and clinical and laboratory variables.

P-gp expression:

In the present study, P-gp expression was found positive in 14/46 patients (30.4% of all AML cases), 4 of them (28.6%) achieved complete remission (CR), and was found negative in 32 patients 69.6%, 20 (83.3%) of them achieved CR. In agreement with our study Huh et al. **[5]** reported P-gp mRNA expression by RT-PCR in adult and childhood acute leukemia in 25% of cases at diagnosis. Our data are also in the range of that described before by Tafuri et al 21.7% **[10]**. The percentage of expression in most of studies is widely variable. This may be attributed to the use of different techniques and methods, (different cut-off values) and pooling of heterogenous groups of patients such as AML and ALL, initial and relapse samples, and adult and pediatric cases.

LRP expression:

In our study, we found LRP was expressed in 12 (26.1%) patients, 6 of them (50%) had CR. Negative expression was present in 34 patients (73.9%), 18 (75%) of them entered CR.

Similar to our study Tafuri et al. **[10]** found that 60.5% of cases were positive for LRP, and that LRP expression had no influence on CR. Another study **[13]** found that MRP1, LRP, BCRP and GSTP1 expressions showed no significant association with response to induction chemotherapy in AML patients. Recent literature on expression of MRP1, LRP and BCRP mRNA at diagnosis has also found no significant association of these genes with response rates **[12]**.

However Pradeep et al. **[11]** found that the clinical relevance of other drug resistance genes LRP, BCRP, GSTP1, DHFR and apoptosis related genes need to be elucidated.

Double expression P-gp and LRP:

In the current study dual expression of P-gp and LRP was detected in 9 (19.6%) cases, 4 of them (44.4%) had CR, and 5 (55.6%) were refractory. Although the result is suggestive of significant difference, the sample size does not support statistical confirmation. Huh et al. **[5]** reported coexpression of MDR1 and LRP in 21.1% of cases with no significant influence on CR rate.

Correlation of P-gp and LRP with rates of CR and refractory cases:

Our results showed significant correlation between CR group and refractory group in the expression of MDR1 with (p=0.034) but showed no significant correlation with LRP (p=0.861).

In agreement with our results, earlier study **[14]** stated that only MDR1/P-gp expression and cyclosporine-inhibited efflux were significantly associated with complete remission (CR) rate (p=0.012 and 0.039 respectively). Our data are also in accordance with recent different groups who stated that MDR1 expression in AML cases could be one of the mechanisms responsible

for induction failure in adult patients **[10,11,12]**. These results can concur with a report showing that P-gp expression does not correlate with CR rates **[1]**.

In contrast to our results, Huh et al. **[5]** found that LRP expression was associated with lower CR rate, while MDR1 appeared to have statistically no significant effect on CR.

Although our data reported that MDR expression is significantly associated with CR rate, contribution of the combined activity of MDR and LRP did not achieve significance. These contradictory results might be partially caused by the relatively small patient numbers in our study. Earlier studies reported that coexpression of LRP and MDR1 might result in worst prognosis **[1,10]**.

Correlation of P-gp and LRP with clinical and laboratory parameters:

In the current study a significant correlation was detected between Hb level & MDR1 expression (*p*-value 0.034). However no correlation was detected between P-gp or LRP and other known prognostic markers such as age and WBCs count, which represent tumor cell mass, as well as BM blasts. Our results are in accordance with other researchers who revealed no relation between either P-gp or LRP and either age or WBCs count **[3]**. Moreover, Ozlem and his colleagues **[3]** supported our results; they also found no significant relation between P-gp and LRP with other prognostic markers.

In conclusion, the present study demonstrates that only positive expression of MDR1 appears to represent a significant prognostic indicator in adult AML, whereas LRP expression has no significant impact on prognosis in adult AML cases.

The expression of the tested parameters does not correlate with other known prognostic factors such as age or WBCs count.

For better understanding of the factors involved in MDR, we recommend involving alternative drug-resistance mechanisms as MRP and BCRP with MDR1 and LRP in one study. Also we recommend conducting studies on large number of uniformly treated AML patients so that the statistical studies are more conclusive.

REFERENCES

1- Tsimberidou AM, Paterakis G, Androutsos G, et al. Evaluation of the clinical relevance of the expression and function of P-glycoprotein, multidrug resistance protein and lung resistance protein in patients with primary acute myelogenous leukemia, Leuk Res. 2002; 26: 143-154.

2- Plasschaert SL, De Bont ES, Boezen M, et al. Expression of multidrug resistance-associated proteins predicts prognosis in childhood and adult acute lymphoblastic leukemia, Clin Cancer Res. 2005; 11: 8661-8668.

3- Ozlem DI, Meltem DK, Pelin M, et al. Expression of Multidrug Resistance 1, Lung Resistance Protein and Breast Cancer Resistance Protein Genes in Chronic Leukemias. International Journal of Hematology and Oncology. 2011; 21: 92-100.

4- Bhushan B, Chauhan PS, Saluja S, et al. Aberrant phenotypes in childhood and adult acute leukemia and its association with adverse prognostic factors and clinical outcome, Clin Exp Med. 2010; 1: 33-40.

5- Huh HJ, Park CJ, Jang S, et al. Prognostic significance of multidrug resistance gene 1 (MDR1), multidrug resistance-related protein (MRP) and lung resistance protein (LRP) mRNA expression in acute leukemia. J Korean Med Sci. 2006; 2:253-258.

6- Pulte D, Gondos A, Brenner H. Improvements in survival of adults diagnosed with acute myeloblastic leukemia in the early 21st century. Haematologica. 2008; 93: 594-600.

7- Harris NL, Jaffe ES, Diebold J, et al. World Health Organization classification of neoplastic diseases of the haematopoitic and lymphoid tissues: Report of the Clinical Advisory Committee Meeting-Airline House, Virginia Histopathol. 2000; 2: 193-207.

8- Kamel AM, EL-Sharkawy N, Yassin D, et al. P-gp Expression and Rh 123 Efflux Assay Have no Impact on Survival in Egyptian Pediatric Acute Lymphoblastic Leukemia Patients. Journal of the Egyptian Nat, Cancer Inst. 2005; 3: 165-172.

9- Vitale A, Guarini A, Ariola C, et al. Absence of prognostic impact of CD13 and/or CD33 antigen expression in adult acute lymphoblastic leukemia, Haematologica. 2007; 92: 342-348.

10- Tafuri A, Gregorj C, Petrucci MT, et al. MDR1 protein expression is an independent predictor of complete remission in newly diagnosed adult acute lymphoblastic leukaemia, Blood. 2002; 100: 974-981.

11- Pradeep SC, Bharat B, Singh LC, et al. Expression of genes related to multiple drug resistance and apoptosis in acute leukemia. Experimental and Molecular Pathology. 2012; 92: 44-49.

12- Van den Heuvel-Eibrink MM, van der Holt B, Burnett AK, et al. CD34-related coexpression of MDR1 and BCRP indicates a clinically resistant phenotype in

patients with acute myeloid leukemia (AML) of older age, Ann Hematol. 2007; 86: 329-337.

13- Galimberti S, Rossi A, Palumbo GA, et al. FLT 3 mutations do not influence MDR-1 gene expression in acute myeloid leukemia. Anticancer Res. 2003; 23: 3419-3426.

14- Leith CP, Kopecky KJ, Chen IM, et al. Frequency and clinical significance of the expression of the multidrug resistance proteins MDR1/P-glycoprotein, MRP1, and LRP in acute myeloid leukemia. A Southwest Oncology Group Study. Blood. 1999; 94: 1086-1099.