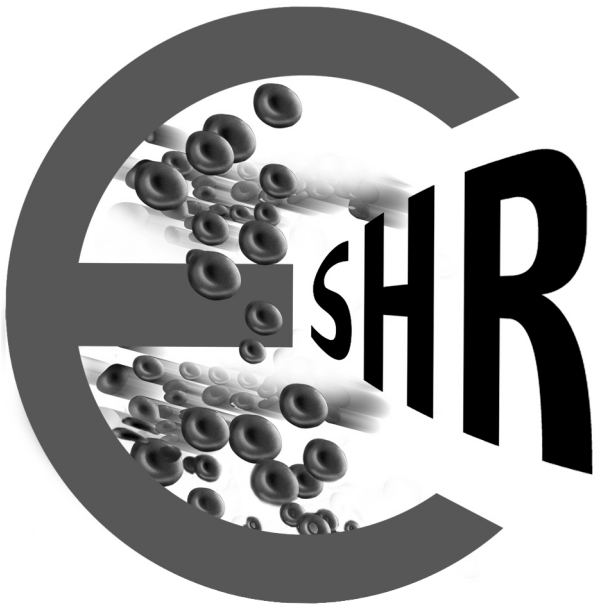
The Journal of the Egyptian Society of Haematology & Research



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ZAP-70 and CD38 Expression in Egyptian CLL

MAGDA ASSEM, M.D.*; SEHAM BAHGAT, M.D.**; MAGDA EL-MAHDEY, M.D.**; SONYA ARSANYOS, M.D.*, THORAYA ABD EL-HAMID, M.D.*** and SAMAH KOHLA, M.Sc.**

The Department of Clinical Pathology, National Cance Institute, Cairo University and Al Azhar University**, Cairo and the Department of Medical Oncology, National Cancer Institute, Cairo University***.*

ABSTRACT

Introduction: Following gene expression profiling which compared the two well established prognostic subsets (ZAP-70 and CD38), with unmutated and mutated IgV_H, ZAP-70 has emerged as the most promising surrogate marker for the IgV_H mutation status. CD38 expression has been also suggested as a surrogate marker for the IgV_H mutation status. However, in subsequent studies, researchers could clearly demonstrate the clinical value of CD38 as a prognostic marker with some degree of correlation to IgV_H mutation.

Aim of the Work: The aim of this study was to investigate the role of ZAP-70 and CD38 as predictors of disease progression in chronic lymphocytic leukaemia and their relations to the other prognostic markers.

Patients and Methods: This study included 50 CLL patients as well as 10 age and sex matched normal volunteers as a control group. All patients were subjected to Immunophenotyping using flowcytometer, Partec III to confirm the diagnosis of CLL with a wide panel of monoclonal antibodies. After appropriate lymphocyte gating, cytoplasmic ZAP-70 and surface CD38 expression were determined in (CD19+, CD5+) B cells. Results were correlated to other known prognosting markers of CLL.

Resuts: No significant association was found between ZAP-70% or CD38% expression and Rai staging at diagnosis; however, a significant positive association was found between CD38 measured as mean fluorescent index (MFI) and Rai staging at diagnosis (p=0.019).

A significant positive association was found between ZAP-70% expression and the non-responders group (those with stable or progressive disease after receiving 3-6 cycles of chemotherapy) and the bone marrow diffuse pattern of infiltration (p<0.001, 0.002 respectively). Also, a significant positive relation was found between ZAP-70% expression and P53% expression (p=0.005).

A significant increase in serum levels of LDH and $ß_2$M in ZAP-70 positive groups as compared to ZAP-70 negative group was detected (p=0.049 and 0.007 respectively).

CD38, either expressed as a percentage or as MFI, showed a significant positive association with the non-responders group (p=0.034, 0.006 respectively) and no significant association with BMB infiltration pattern.

A significant increase in serum levels of B2M in CD38% positive group as compared to CD38 negative group was encountered (p=0.045). A non-significant difference was found with LDH and P53 in CD38 positive as compared to CD38 negative groups. However CD38-MFI showed a significant positive relation to both LDH and B_2M (p=0.03 and 0.05 respectively).

By using the combined expressions of both markers, there was a significant association in concordant positive group (ZAP-70+/CD38+) with poor initial response to chemotherapy (p<0.001) and diffuse BMB infiltration pattern (p=0.015). Also, there was a significant positive relation with the increase in P53% expression (p<0.05), LDH (p=0.029) and $ß_2$-M (p<0.001).

A significant positive association was found between ZAP-70% expression as well as the combined expression of both ZAP-70 and CD38 and time to disease progression (TDP) (p=0.025 and <0.001) respectively.

A significant negative association was found between overall survival (O.S) and ZAP-70% expression as well as the combined expression of both ZAP-70 and CD38 (p=0.029 and 0.0312 respectively).

Conclusion: ZAP-70 is one of the most important prognostic markers in CLL, it appeared to be more predictive of disease progression and poor outcome than CD38 expression. Semi quantification of the CD38 antigen by flowcytometry greatly improves the prognostic value of the percentage expression. The combination of ZAP-70 and CD38 increases the prognostic power of either of the two factors.

Key Words: *ZAP-70 – CD38 – CLL.*

INTRODUCTION

B-cell chronic lymphocytic leukemia (B-CLL) is a heterogeneous disorder characterized by a variable clinical course **[1]**. Some patients have an aggressive disease requiring early therapy, whereas other patients exhibit a more stable, indolent disease with no benefit from palliative chemotherapy **[2]**. In a continual effort to identify

patients with poor prognosis and to facilitate the clinical management of B-CLL, several prognostic markers have been identified during the last two decades **[3]**.

At first, clinical staging systems based on leukemia cell burden were developed. Those systems have delineated the clinical presentation and natural history of B-CLL and have allowed predicting survival and treatment requirements **[4,5]**. However, the Rai and Binet staging systems lack the ability to distinguish prospectively patients with early stage B-CLL that will rapidly progress to aggressive disease from patients destined to remain in early stage for a long time **[1]**. Therefore, other parameters related to the genetics and biology of B-CLL, such as genomic aberrations and immunoglobulin variable heavy chain (IgV_H) mutation status, are increasingly used for prediction of disease prognosis **[3]**.

In landmark studies, it has been shown that survival probability in B-CLL is associated with mutational status of IgV_H genes **[6,7]**. Currently, IgV_H gene mutation status is considered as one of the most powerful prognostic factors, where B-CLL cases with unmutated IgV_H genes are characterized by an unfavorable clinical outcome **[1,3]**.

However, IgV_H mutation analysis is based on DNA sequencing, which is technically demanding and not widely available for routine clinical use. Thus, easily determined surrogate markers for IgV_H mutations are required. DNA microarray studies have shown that B-CLL cells with unmutated (IgV_H) genes can be distinguished from those with mutated IgV_H genes by the differential expression of a small number of genes, one of which encodes the 70-kDa zeta associated protein (ZAP-70) **[8,9]**.

ZAP-70, a member of the Syk-ZAP-70 protein tyrosine kinase family, is a key signaling molecule for T lymphocytes and natural killer cells. While ZAP-70 is not expressed in normal B lymphocytes, it is associated with increased intracellular signaling via the immunoglobulin receptor in B-CLL cells **[10,11]**.

Considering two published studies, **[12,13]**, ZAP-70 is the most promising surrogate marker for the IgV_H mutation status. In contrast to the technically demanding IgV_H analysis, ZAP-70 protein expression is conveniently measured by flowcytometry **[14,15]**.

Initially, CD38 expression also has been conveniently considered as a surrogate marker for the two important IgV_H mutated and unmutated subgroups of B-CLL **[16]**. CD38 is a type II transmembrane glycoprotein that acts as a complex ecto-enzyme and receptor molecule with signaling functions in B-CLL cells **[17]**.

However, while a plethora of subsequent studies could clearly demonstrate the clinical value of CD38 as a prognostic marker with some degree of correlation to IgV_H mutation status, both ZAP-70 and CD 38 are regarded as independent prognostic variables in B-CLL **[18]**.

PATIENTS AND METHODS

(A) Patients:

The present study was carried out in the Clinical Pathology Department of the National Cancer Institute (NCI), Cairo University during the period between November 2004 and November 2006.

The patients were selected from the outpatient clinic of the Medical Oncology Department. Fifty patients with chronic lymphocytic leukemia (CLL) were included in this study, forty males (80%) and ten females (20%) and their ages ranged from 37 to 80 years. Ten age and sex-matched normal volunteers were used as a control group.

The diagnosis of CLL was based on the criteria established by the International Work Shop on CLL and the National Cancer Institute-Sponsored Working Group Guidelines for CLL (NCI-WG) **[19]**. All cases were staged according to modified Rai stage system **[20]**. Twenty-six patients (52%) were at low risk Rai stages (0, I, II), 13 patients (26%) were at intermediate Rai stage (III) and 11 patients (22%) were at high-risk Rai stage (IV).

Comprehensive clinical information including treatment histories was available. Standard clinical criteria were used for the initiation of chemotherapy for all patients. The patients were followed up for a period ranging from 2-120 months.

Patients were subjected to:

1- Thorough history taking, full clinical examination, radiological examination including chest X ray, abdominal ultrasound and/or CT

scan were done whenever needed for proper clinical staging of the disease.

Patients were treated by one of the following lines of chemotherapy depending on age, performance status and stage of the disease:

- Chlorambucil (Clb) and prednisone: Clb was given orally at dose of 0.2 mg/kg/day and prednisone 20 mg/m^2/day.
- Cyclophosphmide, Vincristine, prednisone (CVP): Cyclophosphmide: 400 mg/m^2/IV on days 1-3, Vincristine: 1.4 mg/m^2 IV on day 1 and oral prednisone 100 mg/m^2 on days 1-5.
- Fludarabine and cyclophosphamide (FC): Fludarabine 25 mg/m^2 IV on days 1-3 and cyclophosphamide 250 mg/m^2 IV on days 1-3.

Response to induction chemotherapy was assessed according to the criteria proposed by the National Cancer Institute (NCI)-sponsored working group prior to study SWOG-9108 **[19]**.

Evaluation of response to chemotherapy had been made according to the following criteria:

Complete remission (CR):

Asymptomatic patients with no orgnomegaly or lymphdenopathy. Lymphocyte count < 4x 10^3/µl, neutrophils >1.5x10^3/µl, Hemoglobin (HB) >11 gm/dl, platelets count >100x10^6/µl and bone marrow lymphocytes < 30%.

Partial remission (PR):

More than 50% decrease in organomegaly or lymphadenopathy plus one of the following: neutrophils >1.5x10^3/µL, Hemoglobin >11 gm/dl and platelets >100x10^6/µl.

Progressive disease (PD):

New lesion or >50% increase in organomegaly or lymphadenopathy, circulating lympocytes revealing >50% increase.

Stable disease: Patients who do not fit the criteria for CR, PR, or PD **[19]**.

Time to disease progression (TDP): Duration of response was measured from the date of initial response until disease relapse or progression; or death from any cause, with observation censored at the date of last contact for patients last known to be alive without report of relapse **[21]**.

Overall survival (OS): Was measured from the date of presentation to the Medical Oncology Department until death from any cause, with observation censored at the date patients were last known to be alive for those not known to have died **[21]**.

2- Routine laboratory tests were also done including, liver and kidney function tests, uric acid and Coombs' test, LDH and $ß_2$ microglobulin ($ß_2$M).

3- Complete blood picture, bone marrow aspiration and biopsy.

4- Immunophenotyping using flowcytometer, Partec III to confirm the diagnosis of CLL with a wide panel of monoclonal antibodies including:

Fluorescein isothiocyanate (FITC) conjugated: CD45, CD3, CD4, CD20, FMC7, HLA-DR, and Kappa light chains.

Phycoerythin (PE) conjugated: CD5, CD23, CD10, CD22, CD56, CD79b, CD8 and lambda light chains.

Phycoerythin-Cyanine5 (PECya5) conjugated: CD19.

5- Immunophenotyping for the expression of our studied markers:

FITC conjugated: P53.

FITC conjugated: ZAP-70.

PE conjugated: CD38.

All monoclonal antibodies used were tested as surface expression except for, ZAP-70 and P53 in which we measured their cytoplasmic expression.

All monoclonal antibodies used were purchased from Dako except, ZAP-70 was (BD Bioscience). Results were expressed as a percentage of cells showing positive expression.

Intra-cytoplasmic staining of ZAP-70 and P53 proteins was done using the intrastain kit (purchased from Dako). As the antibody is directed against intracytoplsmic antigen, permeabilization of the cell membrane is necessary before incubation with antibody. Therefore, the kits contain two solutions: solution A is the (fixing) agent based on a paraformaldhyde solution and solution B is the (permeabilizing) agent based on a combination of lysing solution and detergent.

N.B the tube labeled for ZAP-70 contained ZAP-70/CD5/CD19, as anti-ZAP-70-FITC, anti-

CD5-PE and anti-CD19-PEcya5. This combination was done to quantify the cytoplasmic expression of ZAP-70 in B-CLL cells (CD5+, CD19+). As ZAP-70 is present in normal T (CD3+) and Natural killer (NK) cells (CD56+), so to exclude the ZAP-70 of normal T-lymphocytes (CD3+) and NK (CD56+) cells contamination. We can detect the level of ZAP-70 in CLL cells by gating on (CD19+, CD5+) B-CLL cells.

Interpretation of results:

For most studied markers, positive expression was considered when the marker is identified in more than or equal to 20%. ZAP-70 was defined as positive when identified in more than or equal to 20% of the gated CD19/CD5 positive cells **[14]** (Fig. 1). CD38 was considered positive when at least 30% of the gated (CD19+, CD5+) B cells expressed it **[18]**.

To establish the proper cut off value of P53% expression a Roc curve was done. Using this curve, a threshold of 4.95% was found to be appropriate for P53, above which the results were considered positive with a sensitivity of 60% and a specificity of 100%.

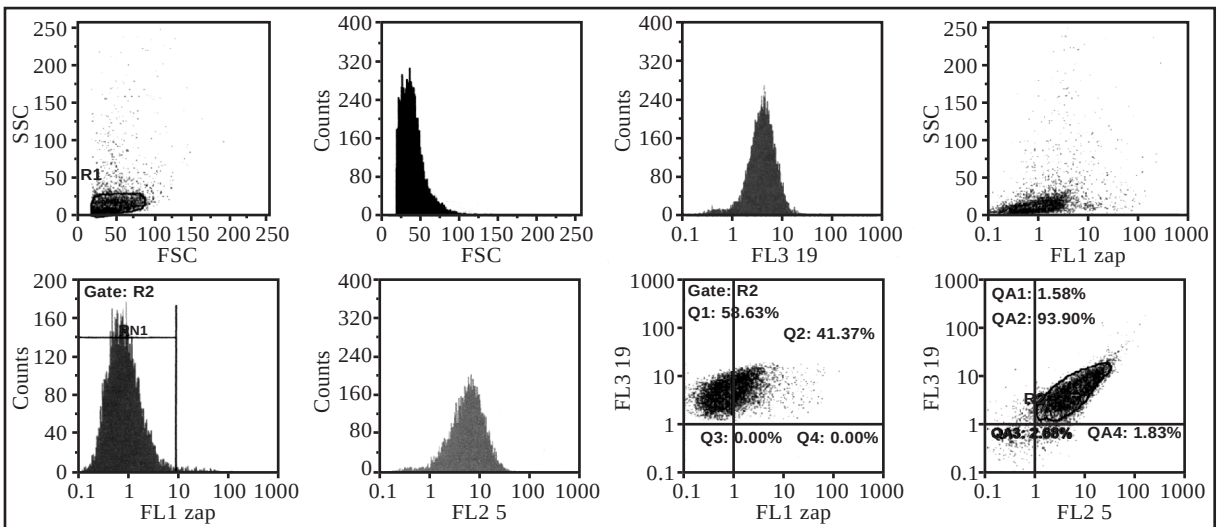
For CD38, results were also expressed as mean fluorescence index (MFI). This was done by dividing the mean fluorescence intensity of the test over that of the negative control **[22]**. Using ROC curve, a cut off value of 2.25 (with a sensitivity of 68% and a specificity of 70%) were found to be appropriate for CD38 MFI.

Statistical analysis:

Data was analyzed using SPSS win statistical package version 12. Numerical data were expressed as mean ± Standard deviation (SD), median, minimum and maximum. Qualitative data was 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 one corresponding to the student *t*-test) for variables not normally distributed. While, comparison between 3 groups was done using one way ANOVA on rank of variables followed by Post-Hoc test (Scheffe test) for multiple comparisons.

Receiver Operating Characteristic (ROC) curve was plotted for determination of the cut off values of MFI-ZAP, MFI-CD38 variables. Survival analysis was done using Kaplan-Meier Method. Comparison between two survival curves was done using log-rank test. Probability (*p*-value) ≤ than 0.05 was considered significant and less than 0.001 considered as highly significant.



Region	Gate	Ungated	Count	Count/ml	% Gated	GMn-x	Mean-x	CV-x%	GMn-y	Mean-y	CV-y%
R1	(None)	8858	8858	–	91.68	39.51	41.46	31.93	7.41	8.71	59.84
Q1	R2	5300	4918	–	58.63	0.54	0.59	38.39	3.80	4.17	44.38
Q2	R2	3907	3470	–	41.37	2.07	3.37	231.15	5.99	6.71	47.05
Q3	R2	303	0	–	0.00	–	–	–	–	–	–
Q4	R2	152	0	–	0.00	–	–	–	–	–	–
R2	(None)	8388	8388	–	86.81	6.43	7.96	66.85	4.59	5.22	53.13
QA1	(None)	153	153	–	1.58	0.55	0.61	38.95	1.77	1.95	61.98

Fig. (1): Flowcytometric analysis of a case of CLL, ZAP-70 Positive.

RESULTS

The present study included 50 patients with chronic lymphocytic leukemia (CLL). They were 40 males (80%) and 10 females (20%) with a male to female ratio of 4/1. Their ages ranged from 37 to 80 years with a mean of 58.6±9.31 SD, (median = 57) years. In addition, 10 age and sex-matched normal volunteers were taken as a control group.

All patients were followed up for their initial response after 3-6 cycles of the initial chemotherapy and then have been followed up for a period of 2-120 months.

Clinical data of the studied group:

Rai staging system of CLL patients at the time of diagnosis showed, seven patients (14%) in stage I, 19 patients (38%) in stage II, 13 patients (26%) in stage III and 11 patients (22%) in stage IV. According to the modified Rai staging system, 26 patients (52%) were at low and intermediate risk (0, I, II), while 24 patients (48%) were at high risk (III, IV).

After the initial chemotherapy, 32/50 patients (64%) showed response to the initial treatment (CR 14 or PR 18), while 18/50 patients (36%) showed no response (SD 11 or PD 7).

During the follow-up period, 6/50 (12%) had died due to causes related to CLL while, 44/50 (48%) were alive at the end of the study.

Laboratory findings:

**ZAP-70 results:*

Our studied CLL group had a mean ZAP-70% expression of 20.98±18.3 SD. We found that 22/50 patients (44%) were positive while 28/50 patients (56%) were negative for ZAP-70.

Correlation of ZAP-70 expression to other known poor prognostic criteria of CLL (Table 1):

No significant relation was found when comparing ZAP-70% expression with age, sex or initial modified Rai staging at the time of diagnosis (p=0.802, 0669 and 0.164 respectively).

On the other hand there was a significant negative relation of ZAP-70% expression with initial response to chemotherapy (p<0.001).

A significant positive relation was also found between ZAP-70% expression and the diffuse infiltration pattern of bone marrow biopsy p=0.002.

A significant increase in serum levels of LDH and $ß_2$M in ZAP-70 positive groups as compared to ZAP-70 negative groups was detected (p=0.049 and 0.007 respectively). There were non significant differences in TLC, HB and PLT values between ZAP-70 positive and ZAP-70 negative groups (p=0.356, 0.325 and 0.599 respectively).

A significant positive relation was found between ZAP-70% expression and P53% expression (p=0.005).

There was no significant relation between ZAP-70% expression and Bcl2% expression, (p=0.641).

Regarding survival status, six CLL related deaths occurred during the observation period and 5 patients of this group were ZAP-70 positive (83.3%). In contrast to only 1/6 in the ZAP-70 negative group (16.7%). In the ZAP-70 positive group 5/22 (22.7%) patients had died in contrast, only 1/28 (3.5%) patient in the ZAP-70 negative group.

The relation between the two groups showed statistically near significance (p=0.075).

** CD38 results:*

The mean CD38% expression of the studied group was 13.2%±14.3 SD (positive cases were 9/50 cases). The mean CD38 MFI expression of the studied group was 12.4±11 SD (positive cases were 22/50 cases).

Correlation of CD38% expression and the other bad prognostic criteria of CLL (Table 1):

There was no significant relation between CD38 expression on one hand and either age, sex, bone marrow infiltration pattern or modified Rai stage at diagnosis on the other hand (p= 1.00, 0.09, 0.428 and 0.721 respectively).

A significant negative relation was found between CD38% expression and the initial response to chemotherapy (p=0.034).

When measuring CD38 as percentage of cell expression, a significant increase in serum levels of B_2M in CD38 positive group as compared

to CD38 negative group was encountered (p= 0.045). A non significant differences was found for TLC, HB, PLT, LDH, P53 and Bcl2 in CD38 positive as compared to CD38 negative groups (p=0.960, 0.419, 0.733, 0.105, 0.802 and 0.305 respectively).

No significant relation was found between CD38% expression and ZAP-70% or p53% expression (p=0.441, 0.802 respectively).

Correlation of CD38 MFI to the other poor prognostic markers in CLL patient group: A significant positive relation was found with advanced modified Rai staging at diagnosis and with disease progression (DP) (p=0.019 and 0.033 respectively) and a negative significant relation with good initial response to chemotherapy (p=0.006).

A significant increase in TLC, LDH and $ß_2$M levels was found in CD38 MFI positive group as compared to CD38 MFI negative groups (p=0.020, 0.033 and 0.05 respectively). There was a significant decrease in HB level between CD38 MFI positive and negative groups (p=0.015). No significant differences was found in PLT, P53% and Bcl2% expressions in CD38 MFI between the two groups (p=0.178, 0.358 and 0.679 respectively) (Table 1).

Table (1): Correlation of ZAP-70%, CD38% and CD38 MFI with clinical and laboratory data in the studied 50 B-CLL cases.

	ZAP-70% Cut off 20% Positive cases: 22/50	CD38% Cut off 30% Positive cases: 9/50	CD38 MFI Cut off 2.25 Positive cases: 22/50
Rai staging at diagnosis:			
• 0, I, II	N.S.	N.S.	p=0.019
• III, IV			
Initial response to chemotherapy:			
• CR, PR	p<0.001	p=0.034	p=0.006
• SD, PD			
Bone marrow infiltration pattern:			
• Diffuse	p=0.002	N.S.	N.S.
• *Non-diffuse:*			
TLC	N.S.	N.S.	p=0.02
Hb	N.S.	N.S.	p=0.015
Platelet count:	N.S.	N.S.	N.S.
LDH	p=0.049	N.S.	p=0.03
B_2M	p=0.007	p=0.045	p=0.05
P53%	p=0.005	N.S.	N.S.

N.S.: Non significant. p value: Significant ≤0.05.

Results of the combined analysis of (ZAP-70 and CD38):

Our CLL patients were classified according to the combined expression of ZAP-70% cut off 20% and CD38% cut off 30% into 3 main groups:

The first group with concordant ZAP-70+, CD38+), which included 5/50 patients (10%).

The second group with discordant (ZAP-70+, CD38-) and (ZAP-70-, CD38+), which included 21/50 patients (42%).

The third group with concordant (ZAP-70–, CD38-), which included 24/50 patients (48%).

Table (2): Comparison of clinical and laboratory data between ZAP-70/CD38 concordant and discordant group in the studied 50 B-CLL patients.

Parameter	ZAP-70 and CD38 Total (n) 50			p value
	Concordant both positive	Discordant one positive, one negative	Concordant Both negative	
Number of patients	5 (10%)	21 (42%)	24 (48%)	
Rai stage diagnosis:	a	a	b	
(0.I, II)%	2/5 (40%)	9/21 (42.8%)	15/24 (62.5%)	0. 396
(III, IV)%	3/5 (60%)	12/21 (57.1%)	9/24 (37.5%)	
Initial response:	a	b	c	
(CR, PR)%	0/5 (0%)	11/21 (52%)	21/24 (87.5%)	<0.001
(SD, PD)%	5/5 (100%)	10/21 (48%)	3/24 (12.5%)	
Bone marrow biopsy:	a	b	c	
Diffuse%	4/5 (80%)	12/21 (57.1)	5/22 (22.7%)	0.015
Non-diffuse%	1/5 (20%)	9/21 (42.8%)	17/22 (77.2%)	
LDH (IU/L):	a	b	c	
Mean (n)	914 (n=4)	716 (n=19)	543 (n=20)	0.029
B_2M (mg/L):	a	a	b	
Mean (n)	3.7 (n=4)	3.5 (n=18)	2.2 (n=16)	<0.001
P53%:	a	b	c	
Mean (n)	25 (n=3)	10 (n=13)	3 (n=15)	0.050

*The p-value for comparison among the three subgroups, was calculated using the one way ANOVA on rank of variables followed by Post-Hoc test.

The concordant both positive group showed decreased response to chemotherapy, more diffuse pattern of bone marrow infiltration and increased

LDH, B_2M levels and P53%. The concordant both negative group showed increased response to chemotherapy, non diffuse pattern of bone marrow infiltration and lower LDH, B_2M levels and P53%. The discordant group showed intermediate response to chemotherapy, less diffuse pattern of bone marrow biopsy than the first group and LDH, B_2M and P53% levels intermediate between the other two groups.

Relation of time to disease progression (TDP) with different parameters in the study:

In our studied CLL patients 32/50 responded to the initial chemotherapy while, 18/50 patients showed no response. The follow-up period of TDP of CLL patients ranged between 2-120 months.

The cumulative survival (CS) at 1 year was 79% (for ZAP-70 negative patients, compared to 42% in ZAP-70 positive patients (p=0.020) (Fig. 2).

In CD38 negative patients with CS at one year was 88%, which was significantly higher when compared to CD38 positive patients, the CS at 1 year was 33% (p=0.033) (Fig. 3).

No patients in the concordant both positive markers responded to the initial chemotherapy. In concordant both (ZAP-70 and CD38) negative group, the CS was 86%, the median was 29 months. In the discordant (ZAP-70+ and CD38– or ZAP-70– and CD38+) group the CS was 41%, the median was only 12 months. There was a significant relation between the combined CD38 and ZAP-70% expression groups and TDP (p<0.001) (Fig. 4).

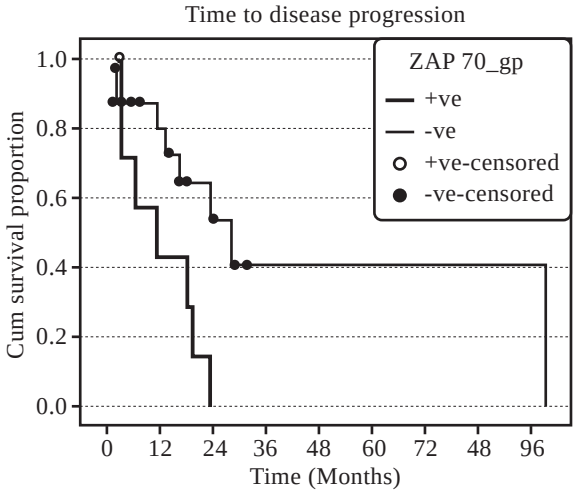


Fig. (2): Time to disease progression in relation to ZAP-70 groups (p=0.020).

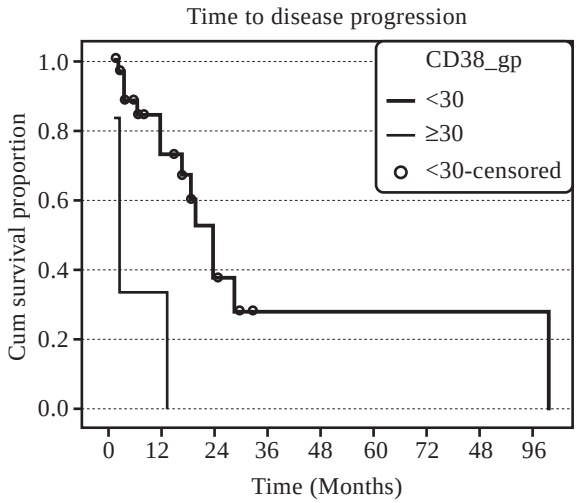


Fig. (3): Time to disease progression in relation to CD38 groups (p=0.033).

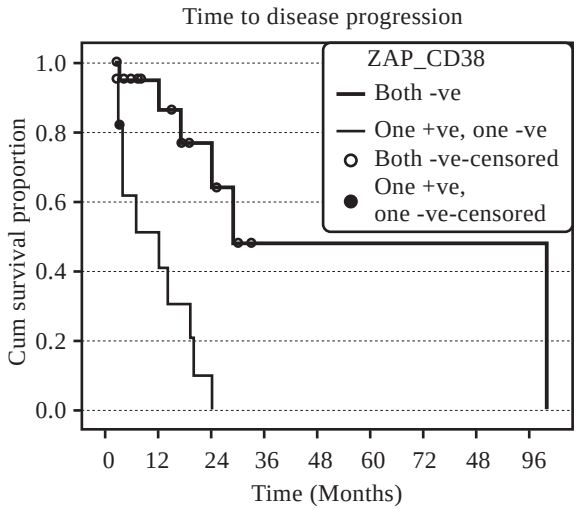


Fig. (4): Time to disease progression in relation to ZAP-70/CD38 groups (p<0.001).

Survival study:

The follow-up period of CLL patients ranged between (2-120) months with a median follow-up of 18 months. The cumulative overall survival (CS) was 89% with a mean of 95.3±11.75 SD, with (95% confidence interval (CI) 72-118%). The median survival was not reached as >50% of cases (88%) was still alive at the end of the study.

Survival analysis regarding the expression of the studied markers was as follows:

The cumulative overall survival (CS) at 1.5 year was 96% for ZAP-70 negative patients, but only 81% for ZAP-70 positive patients. This difference was statistically significant (p=0.029) (Fig. 5).

At 1.5 years the CD38+ CLL patients had a cumulative overall survival (CS) 76% compared to CD38 -ve patients with CS 92%. However, this difference was not statically significant (p=0.1970) (Fig. 6).

In concordant both ZAP-70 and CD38 positive group, the CS was 80%. In the discordant (ZAP-70+ and CD38– or ZAP-70– and CD38+) group the CS was 81%. In the concordant both ZAP-70 and CD38 negative group the cumulative survival was 100% since all patients in this group were alive at the end of the study. This relations between the combined (CD38 and ZAP-70) expression groups and overall survival were statistically significant (p=0.031) (Fig. 7).

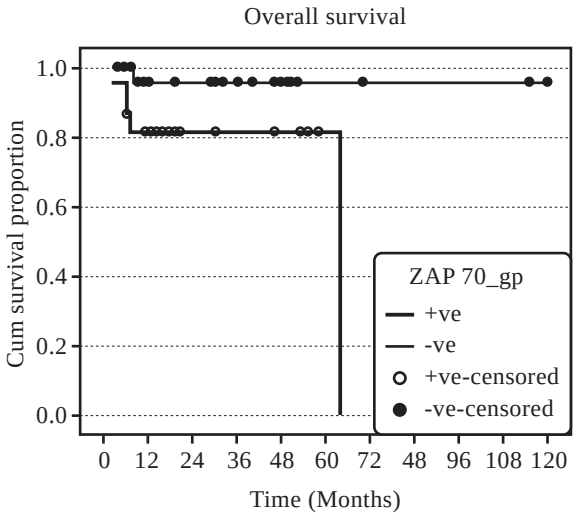


Fig. (5): Overall survival in relation to ZAP-70 percentage expression (p=0.029).

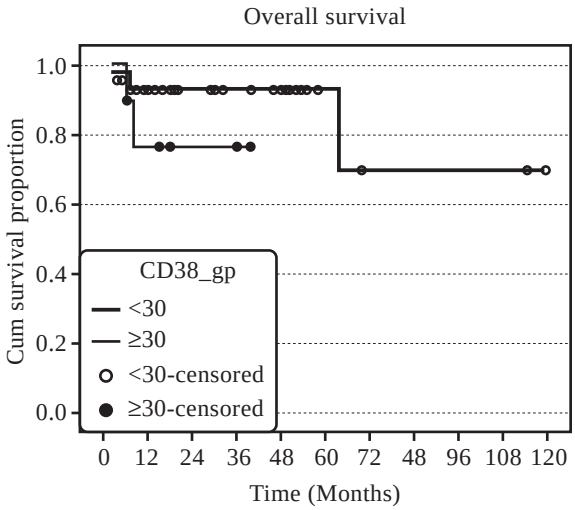


Fig. (6): Overall Survival in relation to CD38 percentage expression (p=0.1970).

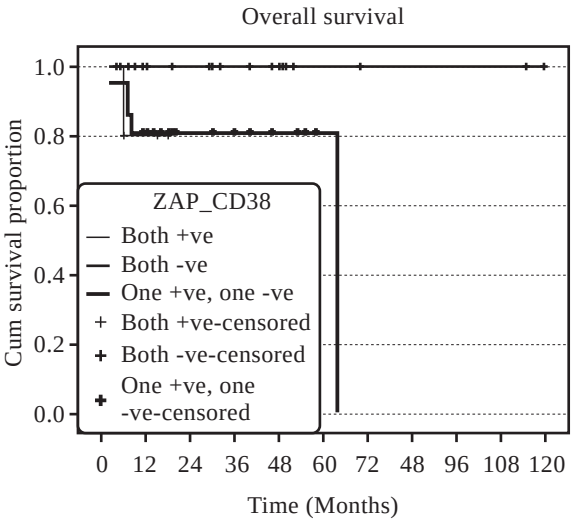


Fig. (7): Overall survival in relation to ZAP-70/CD38 groups (p=0.031).

DISCUSSION

Chronic lymphocytic leukemia (CLL) is defined as a proliferation of B lymphocytes that express surface CD19 or CD20, CD5, CD23, and low levels of immunoglobulin (Ig), CD79b, and CD22 **[23]**.

In Egyptian National Cancer Institute (NCI), CLL constituted 0.4% out of 556 cancer cases and 7.4% out of 621 leukemic cases registered during the year of 2001 **[24]**. In 2005, the male CLL/leukemia was 20/311 (6.4%) and constituted 0.5% out of all cancer cases. In female CLL/leukemia was 26/414 (6.3%) and constituted 0.4% out of all cancer cases **[25]**.

Heterogeneity in the clinical behavior of CLL makes it difficult to identify, which patients will benefit most from earlier or more aggressive treatment and those who should be treated with more conservative and less toxic approaches. Clinical researchers have long thought to identify a marker (or markers) for use as a prognostic tool **[3]**.

ZAP-70, a member of the syk family of tyrosine kinases, has demonstrated an equivalent clinical utility to IgH mutational status in correlation with disease progression and survival **[26]**.

CD38 has been associated with disease progression in many different types of leukaemias, including B-CLL **[22]**.

The present study included 50 patients with chronic lymphocytic leukemia (CLL). They were 40 males (80%) and 10 females (20%) with a male to female ratio of 4/1. Their ages ranged from 37 to 80 years. In addition, 10 age and sex-matched normal volunteers were taken as a control group.

ZAP-70 expression:

In this study, when using the percentage expression for interpretation, the leukemic cells were ZAP-70 positive in 22/50 (44%) and ZAP-70 negative in 28/50 (56%). These results were similar to recently reported researches **[27,28]**. However, a higher percentage of positivity was detected when using MFI, suggesting a higher sensitivity for this method.

A significant differences were found between ZAP-70 positive and ZAP-70 negative patients with some of B-CLL poor prognostic parameters (LDH and B_2M p=0.049 and 0.007 respectively). This is in accordance with what was reported earlier **[27,29,30]**.

Our results showed a positive significant relation between ZAP-70% expression and the diffuse pattern of bone marrow biopsy (p= 0.002). This is in agreement with recent studies which reported that the expression of ZAP-70 was related to the infiltration type **[27,28]**.

A significant negative relation was found between ZAP-70% and the initial response to chemotherapy (p<0.001). These results are in accordance with what was previously reported **[1,32,33,34]**. In addition, our results are in agreement with the earlier results, which found that ZAP-70 positive patients required more intensive chemotherapy over longer periods of time than ZAP-70 negative patients **[14]**.

This study showed a positive relation between ZAP-70 and P53% expression (p=0.005) with no significant relation with Bcl2 and this relation was in accordance with previous studies **[31,33,35]**.

Six CLL-related deaths had occurred during the observation period. Five out of those six patients were ZAP-70+ (83.6%). In the ZAP-70 positive group 5/22 (22.7%) patients had died during the follow up period In contrast only 1/28 (3.5%) patients was in the ZAP-70 negative group. The difference between the two groups was statistically of near significance (p= 0.075). Our results are in agreement with that of other researchers, who reported that, 15/156 CLL-related deaths occurred during their observation period and 12/15 patients of this group were ZAP-70+ (80%). In contrast only 3/15 patients deceased in the ZAP-70– group (20%) **[30]**.

ZAP-70 was proven by many authors to be a sensitive and specific surrogate marker for IgV_H mutational status though, the mechanisms accounting for the relation between both factors remain unknown **[26,36]**. Moreover, the prognostic value of ZAP-70 expression is even more significant than IgV_H mutational status **[13]**.

CD38% expression:

In B-CLL cells, CD38 was considered of prognostic value when ≥30% of (CD19+, CD5+) B CLL cells expressed this membrane antigen **[37]**. Based on the current study using this cut off value, 9/50 (15%) were positive while 41/50 (85%) were negative. The low percentage of CD38 positivity may be attributed to the fact that most of our patients (80%) had received many cycles of chemotherapy before the time of testing. Previous studies have reported that CD38, expression can change with time and under different conditions and chemotherapy selectively eliminate the CD38 clone **[18]**. It was therefore suggested for the accurate assessment of the prognostic significance of CD38 positivity is to ensure that only samples close to or at the time of presentation are tested **[22]**.

Our results were more or less similar to previously reported ratios **[29,38]**. This can be explained as most of their patients had received chemotherapy at the time of analysis. In contrast, a different ratio was obtained in a more recent study, where CD38 positivity was observed in 89% of the studied CLL cases. However, none of his patients had received chemotherapy at the time of sampling **[27]**.

In this study, no significant relation was found between CD38% expression and age, sex, the infiltration pattern of bone marrow biopsy or modified Rai staging at diagnosis. Also, no significant relation was found between CD38% expression and P53% or Bcl2% expressions and these results are in accordance with other studies **[22]**.

A significant negative relation was found between CD38% expression and the initial response to chemotherapy (p=0.034). Only 3/9 (33.3%) of CD38% positive cases responded to chemotherapy either by CR or PR; in contrast 6/9 (66.7%) did not respond either by SD or PD. These results are in agreement with recent studies **[30,33,38]**.

Our results showed a significant increase in serum level of $ß_2$M in CD38 positive as compared to CD38 negative (p=0.045). These results were in accordance with previous results **[22,29]**.

In the present study, no significant differences were found between CD38% positive and CD38% negative cases regarding TLC. This is in accordance with what was reported earlier **[39]**.

By using the mean fluorescent intensity for measuring CD38, a positive significant relation was found between advanced modified Rai Staging at diagnosis and CD38 MFI (p=0.019).

A negative significant relation was found between initial response to chemotherapy and CD38 MFI (p=0.006).

No significant relation was found between CD38 MFI and the other bad prognostic markers and this was in accordance with previous studies **[22]**.

On comparing CD38 MFI positive and negative cases of the studied CLL patients, a significant difference was found regarding TLC, HB, LDH and $ß_2$M. Patients with CD38 MFI positive cases showed increased TLC, decreased HB, increased LDH and B_2M (p=0.02, 0.015, 0.033 and 0.05 respectively) as compared to CD38 MFI negative cases.

As a result, we can conclude that, semiquantification of the CD38 antigen by flowcytometry greatly improves the prognostic value of the percentage expression and this is in accordance with previous reports **[22,40]**.

Combined ZAP-70 and CD38 analysis:

Combined analysis of (ZAP-70 and CD38) allowed separation of our patient cohort into 3 subgroups, that were, concordant ZAP-70–, CD38– (42%), concordant ZAP-70+ CD38+ (10%) and patients with discordant results either (ZAP-70+, CD38– or ZAP-70–, CD38+) (48%). Importantly, these three subgroups differed in their clinical characteristics and laboratory findings. The combination of ZAP-70 and CD38 increased the prognostic power of both factors.

A significant relation was found between the three subgroups with the initial response to chemotherapy (p<0.001), pattern of bone marrow biopsy (p=0.015), serum level of LDH (p= 0.029) and $ß_2$M (p<0.001) and P53% expression (p=0.05).

There was no relation between the three subgroups and the advanced modified Rai staging at the time of diagnosis.

No significant relation was found between the three subgroups and sex or age. All these results are in accordance with other reports **[14, 29,30,41]**.

A negative significant relation was found between the OS and the combined expression of the both markers (p=0.031). The worse OS was found in the concordant both positive groups with CS 80%. Intermediate survival was found in the discordant both markers with CS 81% and the best survival was found in the concordant both negative groups with CS 100%. These results are in accordance with previous researches **[14,27,29,30]**.

Also a negative significant relation was found between TDP and the combined expression of both markers (p<0.001). The longest TDP was found in the concordant both negative group (median 29 months and CS 86%), intermediate TDP was found in the discordant both markers (median 12 months and CS 41%) and the shortest TDP was found in the concordant both positive group. These results are in agreement with recent studies **[27,30]**.

Conclusion:

ZAP-70 positive B-CLL group has a decreased response rate, a worse stage of the disease, a decrease in overall survival and less TDP than that of ZAP-70 negative patients. This suggests that ZAP-70 assay is a powerful predictor of outcome in CLL.

On comparing the two methods used for evaluating CD38 expression, (percentage expression and MFI), it revealed that the use of both MFI in association with percentage positivity can be a better predictor of disease pro-

gression and outcome of the disease than the percentage alone.

Our data showed that the prognostic information given by ZAP-70 and CD38 expression is complementary. Combined analysis of these two markers allows for the separation of three B-CLL patient subgroups with good, intermediate and poor prognosis and therefore could be used to guide treatment decisions especially in early clinical stages of the disease. Since flowcytometry can be used reliably to assess CLL samples for ZAP-70 and CD38, it should be more suitable for application in clinical laboratories than IgV_H mutation analysis, which is a technically more demanding and expensive assay to perform.

Recommendations:

The expression of ZAP-70 is a good prognostic marker and should be used in the routine work up of CLL.

CD38 can change with time and under different conditions, as chemotherapy selectively eliminates the CD38 clone. It is important therefore, for the accurate assessment of the prognostic significance of CD38 positivity to ensure that only samples close to or at the time of presentation are tested before taking any type of chemotherapy.

We recommend the adoption of MFI. Wherever, CD38 positivity is currently measured as part of the clinical assessment of CLL patients. Trials should be performed in different centers to evaluate the prognostic significance of quantification of CD38.

However, further studies are required to develop a standardized flowcytometry protocol that will allow comparison of ZAP-70 and CD38 measurements between different laboratories.

Finally, the prognostic value of combined ZAP-70/CD38 analysis should be tested in the setting of a controlled prospective trial. Performing this combined analysis could serve for either precise definition of prognostic subgroups or might be an option to confirm the prognosis if the value of one predictive factor is borderline.

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Evaluation of the Role of NPM/ALK as a Marker in Lymphoid Malignancies

MAGDA EL-SAYED, M.D.*; SONYA ARSANYOS, M.D.* and IMAN SEDHOM, M.D.***

The Departments of Pathology, Clinical Pathology** and Pediatric Oncology***, National Cance Institute, Cairo University.*

ABSTRACT

Introduction: Conventional methods of NHL diagnosis are based principally on morphology and immunophenotyping, which have a significant shortage in diagnosis of large cell lymphoma. The discovery of anaplastic lymphoma kinase gene has permitted the definition of a distinct molecular genetic subtype of NHL.

Aim of the Work: Was to determine the fraction of ALK +ve lymphoma among lymphoid malignancy cases. Also to detect the relation of ALK expression to age, sex, pathologic types and staging. Correlation of ALK positivity to the overall survival was studied, to determine its significance as a prognostic marker.

Patients and Methods: This study included 114 cases of lymphoid malignancies (44 ALL and 70 NHL cases), divided according to the sampling technique: 44 cases of bone marrow aspiration samples, 20 cases of bone marrow biopsy and 50 cases of lymph node biopsy samples.

Patients of the study group were subjected to full clinical examination, complete blood count, bone marrow morphological examination (for group I and II only), cytochemistry and immunophenotyping by flowcytometer. All cases were subjected to immunostaining using the monoclonal antibody ALK-1. It recognizes an epitope in both the 80-KD NPM-ALK chimeric and 200-KD wild type ALK protein. Cytoplasmic and nuclear labeling was seen in the t (2;5) and also in other ALK variant proteins.

Results: ALK positivity was detected in 25 cases out of the 114 studied lymphoma and leukaemia cases.

ALK positivity showed a wide morphological spectrum. Positivity was more encountered in large cell lymphoma (10/47), immunoblastic (1/3), peripheral T cell (1/1), Lennert's (1/1) and lymphoblastic lymphoma (1/3).

ALK expression was positively correlated with disease stage. This correlation was statistically significant (p=0.02).

A clear finding of a significantly better survival in ALK +ve cases was found, compared to ALK -ve cases (p<0.02).

Conclusion: The definition of ALK lymphoma on the basis of ALK protein expression has the great advantage that it is more conclusive than morphological examination. The diagnosis of ALK +ve lymphoma is of great importance because of their apparent improved prognosis compared with ALK -ve cases. Immunostaining provides a mean of detecting ALK +ve cells even if few tumor cells are present, so important in detecting minimal residual disease. So, ALK protein immunohistochemistry is recommended to be included in the diagnostic workup to identify this distinct lymphoma entity.

Key Words: *NPM/ALK – Lymphoid malignancies.*

INTRODUCTION

Lymphomas are a heterogeneous group of lymphoproliferative malignancies. Their classification has evolved from a scheme utilizing purely morphologic assessment (the working formulation) to a system that attempted to integrate morphologic, immunophenotypic and genotypic data (the REAL classification) and finally to a classification that integrate all these features into clinically relevant categories of equal use to pathologists and clinicians (the WHO classification).

Anaplastic large cell lymphoma is an example, initially described as a neoplasm characterized by large pleomorphic lymphoid cells that expressed the lymphoid activation antigen, CD30. However, variants composed of smaller cells are also recognized **[1]**. Anaplastic large cell lymphoma (ALCL) harbors the reciprocal chromosomal translocation t (2;5) (p23;q35) in approximately 80% of the cases.

With the discovery of t (2;5) as a cytogenetic marker of ALCL and characterization of the implicated genes (NPM and ALK), there is now convincing literature that ALCL expressing the anaplastic lymphoma kinase (ALK) protein is considered as a distinctive group **[2]**. This group

can be called anaplastic large cell lymphoma, primary systemic form, ALK+ or given the acronym (ALKoma) **[3]**. It is a homogenous entity with the following characteristics:

- Young age, bimodal age distribution with one peak in adolescents and young adults and other in eldery patients.
- Patients with primary systemic disease, frequent expression of epithelial membrane antigen (EMA) and no association with Ebestien Barr virus (EBV) and a highly favourable prognosis **[4]**.

The genes involved which are nucleophosmin (NPM) and anaplastic lymphoma kinase (ALK) and the resulting chimeric NPM-ALK protein is thought to play a key role in the pathogenesis of t (2;5) positive ALCL **[5]**. The anaplastic lymphoma kinase (ALK) is the oncogene of most anaplastic large cell lymphomas (ALCL), driving transformation through many molecular mechanisms **[6]**.

The hybrid gene, NPM-ALK encodes the formation of 80-KD ALK protein in which 40% of N terminal portion of NPM is fused to the complete intracytoplasmic and nuclear compartment of the cell. Heterodiamers form between NPM-ALK and normal NPM, which are translocated to the nucleus **[7]**. A novel function of NPM-ALK was suggested recently. It acts through phosphorylation and activation of JNK and cJun, which may contribute to uncontrolled cell-cycle progression and oncogenesis.

The frequency of marrow involvement of ALCL is 10-17% on morphologic assessment but increases to 36% if immunostaining is performed on the marrow biopsies to highlighten the dispersed neoplastic cells among the haemopoietic cells **[8]**.

Immunostaining with the monoclonal antibody ALK-1 is a quick and efficient method as a marker for the diagnosis of ALK +ve lymphoma. Because ALK protein is normally absent in all normal tissues, ALK-1 staining represents significant advances in the detection of NPM-ALK anomaly **[9]**.

MATERIAL AND METHODS

This study included 114 cases of lymphoid malignancies, divided according to the sampling technique: 44 cases of bone marrow aspiration samples, 20 cases of bone marrow biopsy and 50 cases of lymph node biopsy samples.

Patients of the study group were subjected to full clinical examination, complete blood count, bone marrow morphological examination (for group I and II only), cytochemistry and immunophenotyping by flowcytometer. All cases were subjected to immunostaining using the monoclonal antibody ALK-1.

**Sample preparation:*

1- Bone marrow aspiration samples were subjected to staining by leishman stain for morphological examination.

2- One ml of bone marrow aspirate was collected on a preservative free heparin (50 I.U./ml blood) and processed to cytopreparation for immunostaining.

To purify leukemic cells, BM samples were mixed with an equal volume of RPMI-1640 medium and centrifuged on Ficoll Hypaque. Also mononuclear cells from the control group were separated using Ficoll Hypaque. Drops of MNCs from patients and the control group were blotted on slides.

3- Bone marrow biopsy samples were subjected to:

• *Fixation:* The obtained core biopsies were immediately preserved in 10% buffered formol-saline for at least 24 hours.

• *Decalcification:* Specimen were placed in large quantities of formic acid-sodium citrate for 48 hours, Washed in running water for 4-8 hours then transferred directly to 70% alcohol to continue dehydration, clearing and impregnation.

• *Processing:* The decalcified core was transported into a wire-mesh basket where it was dehydrated in an ascending concentration of alcohol (70%, 80%, 90%), cleared in xylene and embedded in paraffin wax.

• *Sectioning:* By using the rotating microtom (Shandon-SA325) sections (4-5 micron thick) were obtained and transerfered on adhesive coated glass-slides using a floating warm water bath. The sections were then dried in a hot air oven (60ºC for 1 hour).

4- Lymph node biopsy samples were subjected to paraffin impregnation and paraffin embedding. Sectioned tissue (5µ thick) from paraffin embedded blocks were collected on clean glass slides. For increased adhesion of tissue sections during immunohistochemistry (IHC) staining procedures, poly-L-Lysine coated slides were used.

**Immunoperoxidase:* Staining with monoclonal antibody against ALK-1 (DAKO Envision TM system, Peroxidase (DAB) Mouse).

Before immunostaining, both bone marrow and lymph node biopsy samples were subjected to:

a- Deparaffinization and rehydration:

- Slides were placed in xylene bath for 5 min.
- Excess liquid was removed and slides placed in absolute ethanol and incubated for 5 minutes. Bath is changed and this step was repeated for another 5 minutes.
- Excess liquid was removed and slides were then placed in 90% ethanol, incubated for 5 minutes. Bath was changed and this step was repeated for another 5 minutes.
- Excess liquid was removed and slides were then placed in distilled water for 1 minute.

b- Target retrieval:

- Tissue sections were placed in citrate buffer (DAKO Target retrival solution, 10X concentrate) and heated in an autoclave at 121ºC for 9 minutes (3 min x 3 times).
- Slides were left to cool for 15 minutes and then washed thoroughly with phosphate buffered saline. All samples were then subjected to immunoperoxidase staining **[10]**.

**Interpretation of results:*

Normal lymphoid cells do not contain detectable ALK protein and a positive immunostaining reaction usually indicates that a lymphoma expresses the NPM-ALK fusion protein as a result of the t (2;5) anomaly. Positive staining pattern is characterized by the brown-stained nucleus and cytoplasm (Fig. 1) **[11]**. However, a new pattern of ALK protein expression, mixed membranous and cytoplasmic was recently reported **[1]**.

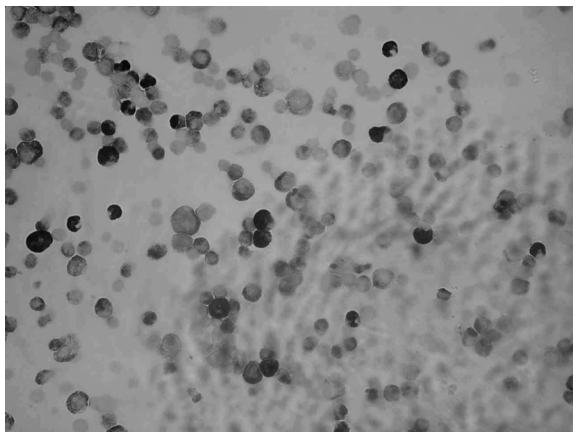

Fig. (1): A case of disseminated lymphoblastic lymphoma showing ALK positivity (by immunocytochemistry).

RESULTS

This study involved 3 groups of lymphoid malignancy cases, received at the pathology and Clinical Pathology Departments, National Cancer Institute, Cairo University. Groups were divided according to the type of sample examined:

- Group I, Bone marrow aspirate group (44 cases). Their age ranged from 1.5-65 years with a mean of 12 years. This group included 32 (72.7%) males and 12 (27.3%) females.
- Group II: Bone marrow biopsy group (20 cases). Their age ranged from 16-71 years with a mean of 49 years. This group included 16 (80%) males and 4 (20%) females.
- Group III: Lymph node biopsy group of 50 cases. Their age ranged from 15-86 years with a mean of 47 years. This group included 29 (58%) males and 21 (42%) females.
- Peripheral blood samples were taken from 10 normal individuals and used for preparation of mononuclear cells to be used as normal control.

Patients of the study group were subjected to full clinical examination, complete blood count, bone marrow morphological examination (for group I and II only), cytochemistry and immunophenotyping by flowcytometer. All lymphoma cases and the control group were subjected to immunostaining using the monoclonal antibody ALK-1. It recognizes an epitope

in both the 80-KD NPM-ALK chimeric and 200-KD wild type ALK protein. Cytoplasmic and nuclear labeling was seen in the t (2;5) and also in other ALK variant proteins.

Laboratory data of the three-studied groups are represented in Table (1).

Immunocytochemical and histochemical results:

ALK positivty was detected in 22.7% in group I (with a mean age of 8.22 years), 14.3% in group II (with a mean age of 25 years) and 24% of cases in group III (with a mean age of 39.5 years). There was no statistically significant difference between the 3 groups regarding ALK positivity (p=0.65). All the samples of the control group were negative for ALK-1.

The mean age of ALK positive cases was slightly lower than that of ALK negative cases (28.3 compared to 35.7 years respectively). However this difference was statistically insignificant (p=0.15).

ALK positivity showed a wide morphological spectrum. Positivity was encountered in large cell lymphoma (10/47), immunoblastic (1/3), peripheral T cell (1/1), Lennert's (1/1) and lymphoblastic lymphoma (1/3).

**Correlation studies of ALK positivity:*

It was correlated to:

1- Age:

Out of the 114 cases of the whole study group, the mean age of ALK positive cases was 28.3 years compared to 35.7 years in ALK negative cases with no statistically significant difference (p=0.15). ALK positivity showed age distribution from 5-20 years in the form of a plateau. Positivity was minimal between 26-44 years. In older age, ALK positivity peaked at 45 years with age distribution between 45-65 years. Positivity was decreasing between 65-80 years.

2- Haemogloin percent and platelet count:

For all groups together, the mean Hb level and platelet count were higher in ALK +ve cases compared to ALK -ve cases. However, this difference was statistically insignificant (p=0.32, 0.54 respectively).

3- Cell morphology:

ALK positivity was detected in 25 cases out of the total of 114 cases. The +ve NHL cases were non- anaplastic in morphology.

4- Bone marrow cellularity:

ALK positivty was detected in 16 out of 64 cases with normocellular marrow (25%) and in 8 out of 44 cases with hypercellular marrow (18.2%). It was detected in 1 out of 6 cases with hypocellular marrow (16.7%).

5- Immunophenotyping of the 1st group (bone marrow aspirate group):

ALK positive cases were 1/7 of C-ALL phenotype cases (14.3%), 1/7 of mature B cell phenotype cases (14.3%) and 8/30 of T cell phenotype cases (26.7%).

6- Pathologic subtypes in the 2nd group (bone marrow biopsy group):

The ALK positivity was high in large cell lymphoma (33.3%), peripheral T cell (30%) and lymphoblastic lymphoma (30%) types. This results is of borderline significance (p=0.08). No ALK positivity was detected in either small cell or mixed cell types.

7- Pathologic subtypes in the 3rd group (lymph node biopsy group):

Seventy five percent of ALK positive patients were of large cell lymphoma type (21 cases), compared to 8.3% positivity in Immunoblastic (3 cases), Lennert's lymphoma (1 case) and other undefined cases. Although highest expression in large cell lymphoma, this correlation was statistically insignificant (p=0.38).

8- Nodal and extranodal presentation in the 3rd group (lymph node biopsy group):

ALK +ve expression was detected in 25.6% of nodal cases and in 24% of the extranodal cases. This correlation was statistically insignificant (p=0.52).

9- Liver enlargement in the whole study group:

In the present study, hepatomegaly in ALK +ve lymphoma was minimal. In cases with either mild or moderate liver enlargement, ALK positivity was detected in 20% of cases. No positivity was detected in cases with massive hepatomegaly.

10- Lymphoma stage in the 3rd group (lymph node biopsy group):

Patients in stage I and II together showed ALK positivity in 12.9% of cases, while patients in stage III and IV, together showed positivity in 42.1% of cases. This higher positivity in stage III and IV was statistically significant (p=0.02).

11- Overall survival:

Out of the 114 studied cases, only 51 cases were followed up for 5 years to detect the overall survival. In positive cases, the overall survival was 91.9% (22 out of 24 positive cases) compared to 70.4% (19/27) in ALK negative cases. This difference was statistically significant (p<0.02) (Fig. 2).

Table (1): Laboratory data of the three-studied groups.

	Group I	Group II	Group III
	Immunophenotyping *C-ALL, 7 cases (15.9%) *Mature B cell, 7 cases (15.9%) *T cell 30 cases (68%)	Pathological types *Small cell, 4 cases (20%) *Large cell, 6 cases (30%) *Mixed cell, 6 cases (30%) *Peripheral T cell, 1 case (5%) *Lymphoblastic, 3 cases (15%)	Pathological types *Large cell, 41 cases (82%) *Immunoblastic, 3 cases (6%) *Mixed cell, 2 cases (4%) *Lennert's, 1 case (2%) *Other types, 3 cases (6%)
Hb gm/dl	8.29±2.3*	8.05±2.7*	10.98±1.8*
Platelet count x10^9/L	92.7±100.1*	130.6±124.5*	229.3±188.3*
BM cellularity:			
Hyper	27 (62.8%)	12 (60%)	5 (10%)
Normo	14 (32.6%)	6 (30%)	43 (86%)
Hypo	2 (4.6%)	2 (10%)	2 (4%)

*: Mean±SD.

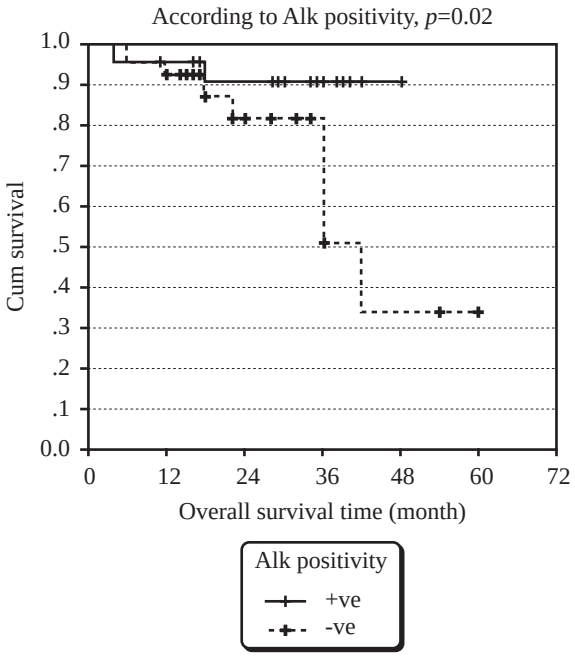

Fig. (2): The overall survival in the studied NHL patients in correlation to ALK positivity.

DISCUSSION

Acquired chromosomal anomalies (most commonly translocations) in leukemias and lymphomas usually result in either activation of a quiescent gene and expression of an intact protein product or creation of a fusion gene encoding a chimeric protein. Thus immunohistochemical detection of the products of many rearranged genes in leukemias and lymphomas can be clinically informative and provide information on cellular and subcellular protein expression that cannot be inferred from studies based on messenger RNA **[13]**.

Anaplastic large cell lymphomas (ALCL) represent a subset of lymphomas in which the anaplastic lymphoma kinase (ALK) gene is fused to several partners, most frequently to the NPM gene **[14]**. The discovery of anaplastic lymphoma kinase (ALK) gene has permitted the definition of a distinct molecular genetic subtype of NHL. Because ALK is not normally

expressed in haemopoietic cells, immunostaining of lymphoma cells with anti ALK antibodies can be used to detect ALK positive lesions. The monoclonal antibody, ALK1 specifically recognize an epitope that is present in both the NPM-ALK chimeric protein (produced by 2;5 chromosomal translocation) as well as by the full length wild type ALK protein **[15]**.

This study included 114 cases of lymphoid malignancies (44 ALL and 70 NHL cases). Cases were divided according to the sampling technique: 44 cases of bone marrow aspiration samples, 20 cases of bone marrow biopsy and 50 cases of lymph node biopsy samples. The number of the whole cases was 114 with age ranging from 1.5 to 86 years. ALL cases were classified according to the immunophenotyping and NHL cases according to REAL classification **[16]**.

In the present study, immunostaining with the monoclonal antibody, ALK1, was used to detect ALK expression in samples of the whole study group. ALK positive cases showed an age range from 2-63 years with a mean of 28.3 years which was slightly lower than that of ALK negative cases (a mean of 35.7 years). This difference was statistically insignificant. However, other researchers have stated that ALK expression occurs in younger populations when compared to lymphoma patients lacking ALK reactivity **[11,17]**.

Also ALK positivity showed age distribution from 5-20 years in the form of a plateau. Positivity was minimal between 26-44 years. In older age, ALK positivity peaked at 45 years with age distribution between 45-65 years. Positivity was decreasing between 65-80 years.

The ALK positive cases were 25 of the whole study group; none of those cases were anaplastic in morphology. These results agree with what was reported earlier that ALK expression is not only associated with large cell lymphomas that have anaplastic morphology **[17,18]**. In recent studies, it was reported that ALK-negative ALCL were found to have a high number of anaplastic cells compared with ALK-positive cases. It was suggested that the use of antibodies specific for ALK protein is a must, for the identification of the tumor entity ALK-positive from the poorly defined morphological category of anaplastic large cell lymphoma **[19,20]**. It was also suggested that immunohistochemistry is superior to molecular analysis in identification of this group, because this is a simpler test and is more sensitive: ALK immunoreactivity is seen even in cases showing variant translocations involving ALK fusion with a partner other than NPM **[4]**.

In the present study, the mean haemoglobin level in ALK +ve cases was higher than that of ALK -ve cases. However, this difference did not reach a statistically significant level. This goes with previous reports **[21]**.

In our study, ALK +ve cases showing hepatomegaly constituted 20% of ALK +ve cases. This is in contrast with previous reports which showed a lower incidence (8%) **[22]**. This increase in frequency of hepatomegaly in Egyptian cases could possibly be due to an already existing liver affection due to prevalent HCV or Bilharziasis.

The ALK positivity was more evident in cases with advanced stage of the disease (sage III & IV) when compared to those with stage I & II. This is in agreement with previous researches **[23]**.

A clear finding of a significantly better survival in ALK +ve cases was found, compared to ALK -ve cases ($p<0.02$). This prognostic difference was previously described documenting that ALK-positive ALCL has a superior outcome **[24,25]**. Recently, it was suggested that ALK-positive ALCL has a good prognosis compared to ALK-negative ALCL, possibly as a result of the immune recognition of the ALK proteins **[26]**.

Due to the selective over expression of ALK in tumour cells, its direct involvement in the process of malignant transformation and its frequent expression in ALCL patients, ALK was suggested as a suitable candidate for the development of molecularly targeted strategies for the therapeutic treatment of ALK-positive lymphomas **[6,27]**.

From this study we conclude that:

* ALK positive expression is not only associated with large cell lymphomas that have anaplastic morphology. So, ALK immunostaining should be tested in all types of lymphoma regardless the morphologic evaluation. ALK

positive lymphomas are suggested to be referred to as ALK lymphoma.

* Immunostaining provides a mean of detecting ALK +ve cells even if few tumor cells are present, so important in detecting minimal residual disease. As minimal BM disease monitoring could identify patients at risk of relapse.

* ALK expression is not detected in normal haemopoietic cells. This suggests that it should be used as diagnostic marker of malignancy as well as a good prognostic marker of different types of lymphomas.

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Rearrangement of the Mll Gene in Acute Myeloid Leukemia and Myelodysplastic Syndromes

FARIDA H. GADALLAH, M.D.*; MUHAMMAD R. KHALAF, M.D.**; SHERINE I. SALEM, M.D.*; ABEER M. DARWISH, M.Sc** and WALEED MAHANNA, M.D.***

The Department of Clinical Pathology, National Cance Institute, Assiut** and Ain Shams*** Universities.*

ABSTRACT

Background: The MLL gene on 11q23 locus is involved in common translocations in human acute leukemias including acute myelogenous leukemia, T-cell ALL, B lineage ALL, myelodysplastic syndrome, lymphoblastic lymphoma and Burkitt's lymphoma.

Aim of Work: This study was intended to examine the frequency of MLL gene rearrangments (translocations & deletions) by conventional cytogenetics and FISH in adult and pediatric newly diagnosed cases with AML and MDS.

Subject and Methods: The study included 56 newly diagnosed AML (40) and MDS (16) patients with age range 3 days- 77 years, as well as 10 matched controls. All cases were subjected to complete blood picture, bone marrow aspirate for morphologic examination, cytochemical stains and immunophenotyping, karyotyping, and fluorescence in situ hybridization (FISH) using MLL gene split signal probe.

Result: MLL gene rearrangement was detected by FISH in 3/40 cases of AML, while conventional cytogenetics could only reveal one of them with 11q23 abnormality. There was a strong correlation between AML M4/M5 and MLL gene rearrangements. In AML-M4, 2/7 cases showed MLL gene rearrangements, while 1/7 cases of AML-M5 carried rearrangements of MLL gene. All MDS cases included in this study did not have MLL abnormalities by FISH. In AML patients, t (8;21) (q22;q22) was found in 20% either as a sole abnormality (10%) or with additional chromosomal abnormalities, + 8 and +19 in two cases each, t (15;17), +21, 7q-, double Philadelphia, +10, t (8;15), t (8;15;21) and i6p, in one case each. In MDS group of patients, chromosome -7/7q- abnormalities was detected in 3/10; complete loss (2 cases), or partial loss (one case), add5q, +19, -22, +2 and del9q in one case each.

Conclusion: FISH detected MLL gene rearrangement in 3 cases of AML while conventional cytogenetics could only reveal one case. FISH being more sensitive and more specific than conventional cytogenetics should be routinely used to screen for MLL gene rearrangements in AML patients.

Key Words: *AML – MDS – MLL – Gene rearrangement – Cytogenetics – FISH.*

INTRODUCTION

The MLL gene, also known as ALL-1, HRX, and Htrx on 11q23 locus is involved in recurrent translocations in human acute leukemias including acute myelogenous leukemia (AML), T-cell ALL, B lineage ALL, myelodysplastic syndrome (MDS), lymphoblastic lymphoma and Burkitt's lymphoma **[1-3]**. The MLL has been labeled a "promiscuous" oncogene since over 60 partner genes or regions have been identified. Included among these is an internal reduplication and self-fusion of MLL in some patients with trisomy 11 **[4]**.

MLL gene translocations are identified in about 5-6% of AML patients with t (6;11) (q27; q23), t (9;11) (p22;q23) and t (11;19) (q23;p13) being the most common. In AML cases showing MLL gene rearrangement, mostly FAB M4 and M5, the blast cells may express lymphoid markers in addition to myeloid markers. They are identified also in up to 10% of ALL patients with t (4;11) (q21;q23) and t (11;19) (q23;p13). ALL cells typically show a very immature phenotype (pro-B) with aberrant expression of mature myeloid or monocytic membrane markers **[5]**. Duplication or amplification of chromosome band 11q23 including the unrearranged MLL gene is a recurrent abnormality in therapy related MDS and AML **[6]**.

In addition 11q23 translocations occur in up to 80% of leukemia in children under one year of age **[7]**. Finally drugs that target topoisomerase II are very effective therapeutic agents, however they are associated with rearrange-

ments of 11q23 as a consequence of treatment **[8]**. Because MLL translocations are much less frequent in de novo leukemias of older patients but frequent in leukemias following chemotherapeutic DNA topoisomerase II poisons, it has been proposed that leukemia in infants may have an etiology resembling treatment related cases **[7]**.

MLL Gene: The MLL gene consists of 36 exons distributed over 100 kb and produces a 12 kb mRNA that encodes a 3968 aa protein with an estimated molecular weight of 430 kD **[1]**. The MLL protein is widely expressed in the developing embryo and is expressed at lower levels in most adult tissues. The predicted amino acid sequence of MLL indicates that it is homologous to the trithorax gene of D. melanogaster. MLL-functions in a large super complex of at least 29 proteins that is involved in the remodeling, acetylation, deacetylation and methylation of nucleosomes and histones.

Several lines of evidence indicate that one important function of MLL is in the maintenance of HOX gene expression during embryonic development. HOX genes are a large family of genes which are important in mammalian developmental cell fate determination **[4]**. Loss of MLL function in flies leads to homeotic transformation and deletion of MLL in mice leads to embryonic lethality and homeotic transformation **[1]**. Biochemical analysis of MLL suggests that it normally functions as a transcription regulator **[1-3]**.

The known fusion partners of MLL are diverse. This would appear to suggest that the fusion partners per se are not functionally important; rather it is MLL disruption that is the critical molecular event. Arguing against this hypothesis, however, is the fact that simple MLL truncation mutations are not frequently observed in AML and some of the common MLL fusion partners themselves share functional motifs of nuclear transcription factors, proteins involved in transcriptional regulation or other nuclear proteins **[7]**.

All of the breakpoints within MLL occur within an 8.3-Kb genomic region **[9]**. By mapping the translocation breakpoints in de novo leukemia, the breaks appeared to be located preferentially in the 5' half of the MLL breakpoint cluster region (BCR), whereas treatment-related leukemias and infant leukemias break more frequently in the 3' half of the BCR. This has led to the suggestion that a similar mechanism of breakage may be involved in MLL translocations in secondary leukemia and in infant leukemia **[8-10]**.

It has been speculated that MLL rearrangements are the initiating event for infant leukemias as they have been shown to occur in utero. **[11]**.

Patients carrying such a MLL translocation respond badly to therapy and have a poor clinical outcome **[4]**. Although, t (9;11) (p22;q23) involving AF9 provides the best outcome compared to other MLL translocations in AML, survival is relatively short **[12]**. Whereas patients with B-lineage ALL and MLL translocations tend to have a poor prognosis. In contrast, although MLL translocations are relatively rarely associated with T-ALL, these patients tend to have a very good prognosis **[13]**.

The Aim of this study was to report the frequency of rearrangments of the MLL gene in newly diagnosed cases of AML and MDS in relation to specific FAB subtypes using both conventional Cytogenetics and FISH.

MATERIALS AND METHODS

This study included newly diagnosed 56 AML and MDS patients (37 adults, 18 children and one infant), aged from 3 days to 77 years admitted to the National Cancer Institute, Cairo University from January 2004 to August 2006. Patients were subjected to complete blood picture, bone marrow aspirate for morphologic examination, cytochemical stains and immunophenotyping on routine basis; karyotyping, and fluorescence in situ hybridization (FISH) using MLL gene probe (LSI MLL Dual color rearrangement probe, Vysis Inc, Downers Grove IL) were also performed. Ten normal controls [7 adults (4 males and 3 females) and 3 children], age ranged from 9 years to 60 years (Median 29 years) were also included in this study. For controls, cytogenetic analysis was performed on peripheral blood lymphocytes which were cultivated for 72 hours in RPMI supplemented with 20% fetal calf serum and antibiotics. The cells were stimulated with 2% phytohemaglutinin (PHA). FISH study using MLL gene probe was performed on metaphase and/or interphase nuclei.

Cytogenetic methods:

Bone marrow or blood samples preserved in sodium heparin were cultured for each subject as previously described **[14]**. Chromosomes were karyotyped using an image analysis system (Vysis Quips XL Genetic work station) according to the international system of hemo-cytogenetic nomenclature **[15]**.

Fluorescence in situ hybridization:

FISH studies were performed according to the manufacture's instructions. MLL Dual Color Probe (Vysis Inc, Downers Grove, IL) designed for detecting the 11q23 rearrangements, was applied in this study. The probe consists of a centromeric portion labeled in spectrum green and a 190 Kb telomeric portion labeled in spectrum orange. The centromeric probe begins between MLL exons 6 and 8 and extends 350 Kb toward the centromere on chromosome 11, and thus covers the centromeric region of the breakpoint cluster region. The telomeric probe begins between exons 4 and 6 and covers a region primarily telomeric of the breakpoint cluster region.

Interphase nuclei lacking the MLL rearrangement are expected to contain two green/orange fusion signals. In the interphase nucleus showing the MLL rearrangement, the telomeric orange signal would move to the partner chromosome and the centromeric green signal would remain on the long arm of chromosome 11. Consequently, separate green and orange signals represent the MLL rearrangement.

RESULTS

Control group:

Ten normal control subjects matched for age and sex were included in the study all of which showed normal karyotype and FISH results for MLL gene. Fig. (1) shows a control with normal MLL gene.

Patients were divided into 2 groups:

Group 1 (AML):

It comprised 40/56 patients (71.4%) including 25 adults, 14 children and one infant. Their age ranged between 3 days and 77 years (Median 24 yrs). Of the 40 patients, 39 were diagnosed as AML based on FAB criteria. When immunophenotyping was performed, 3 patients of the 39 fulfilled the criteria for biphenotypic leukemia diagnosis being Myeloid/T-ALL in one case and Myeloid/B-ALL in two cases. The 40th patient was diagnosed as MDS based on FAB criteria as the percentage of the blast cells in the BM was 16, but when karyotype was performed, t (8;21) was detected so the diagnosis was changed into AML according to the WHO classification **[16-17]**.

The age, sex, FAB subtype, percentage of blast cells in the BM, karyotype and FISH results using MLL gene probe of group 1 patients are shown in Table (1).

In this study, conventional cytogenetics (CC) revealed 2 patients with 11q23 deletion, while FISH analysis for MLL gene proved the abnormality in one patient as a true 11q23 positive /MLL+. This patient (Table (1) case No. 30) was a male child aged 4.5 years (1/15=6.7%) diagnosed as M5a with 75% blast cells in his bone marrow and showed 11q- in the karyotype which was consistent with the FISH result.

FISH showed MLL gene rearrangements in another 2 adult patients (2/25=8%) in whom 11q23 band was normal by CC: One patient (Table (1) case No. 23) was a male aged 20 years diagnosed as M4 with 34% blast cells in the bone marrow and showed t (8;21) in his karyotype. By FISH, MLL gene deletion was detected. The other patient (Table (1) case No. 28) was a female aged 64 years diagnosed as M4 with 70% blast cells in the bone marrow and showed normal karyotype and MLL gene translocation by FISH.

Using FISH technique, collectively, 3/40 (7.5%) AML patients, showed gene rearrangements (Fig. 2).

Group 2 (MDS):

It comprised 16/56 patients (28.6%), including 12 adults and 4 children. Their age ranged between 4 and 68 years (Median 30 years). MDS diagnosis was based on FAB criteria. The age, sex, FAB subtype, percentage of blasts and normoblasts in the BM, karyotype and FISH results using MLL gene probe for these patients are shown in Table (2).

Using FISH technique, none of the MDS patients in this study showed MLL gene rearrangement.

Table (1): Karyotyping and MLL gene rearrangement in 40 de novo AML cases.

Case no.	Age	Sex	FAB	Blasts in BM %	Karyotype	FISH for MLL gene
1	38	Female	M1	90	46, XX	Neg
2	27	Male	M1	93	46, XY	Neg
3	18	Male	M1	73	46, XY	Neg
4	41	Male	M1	67	Not done	Neg
5	24	Female	M1	81	47, XX + 8 [14] 45X-X [6]	Neg
6	14	Female	M1	80	46, XX, add 4q, t(8;21)	Neg
7	14	Female	M1	82	48, XX, 7q-, +19, ++Ph	Neg
8	12	Male	M2	66	46, XY	Neg
9	10	Male	M2	35	47, XY, +21	Neg
10	31	Male	M2	20	46, XY	Neg
11	16	Female	M2	40	46, XX, t(8;21)	Neg
12	23	Male	M2	34	50, XY, +8, +10, +19, +21	Neg
13	12	Male	M2	44	46, XY	Neg
14	15	Male	M2	63	Not done	Neg
15	9	Male	M2	68	46, XY, add 4q t(8;21)	Neg
16	34	Male	M2	20	45, X, t(8.;21), -Y	Neg
17	29	Male	M2	44	Not done	Neg
18	25	Male	M2	71	48, XY, +i(6p), t(8;21) der 12 +mar	Neg
19	24	Female	M2	16	46, XX t(8;21)	Neg
20	47	Male	M3	9	46, XY t(15;17)	Neg
21	20	Female	M3	2	46, XX t(15;17)	Neg
22	24	Female	M3	15	46, XX t(15;17)	Neg
23	20	Male	M4	34	46, XY t(8;21)	MLL gene del
24	28	Male	M4	55	46, XY	Neg
25	37	Male	M4	77	46, XY	Neg
26	22	Male	M4	57	47, XY, +8	Neg
27	51	Female	M4	33	46, XX, der 7	Neg
28	64	Female	M4	70	46, XX	MLL translocation
29	36	Male	M4	60	46, XY	Neg
30	4.5	Male	M5a	75	46, XY, del 11q	MLL deletion
31	32	Female	M5a	85	47, XX, + der8	Neg
32	37	Male	M5a	70	46, XY, t(8;21)	Neg
33	2	Male	M5a	72	47, XY, +8	Neg
34	9	Male	M5a	82	46, XY	
35	77	Female	M5a	55	Not done	Neg
36	36	Female	M5b	35	46, XX	Neg
37	3 d	Male	M7	55	47, XY, +21	Neg
38	2	Male	Biphenotypic	48	46, XY, +4, t(16;17)	Neg
39	35	Male	Biphenotypic	24	46, XY, del 11q	Neg
40	3	Male	Biphenotypic	38	46, XY, add 1q, t(8;15) add 11p, -17, +mar [12] 46, XY [8]	Neg

N.B. MLL gene probe (LSI MLL Dual color rearrangement probe, Vysis Inc, Downers Grove IL).

Table (2): Karyotyping and MLL gene rearrangement in 40 MDS cases.

Case no.	Age	Sex	FAB	Blasts in BM %	Normoblasts in BM %	Karyotype	FISH for MLL gene
1	58	Male	RARS	4	58 >15% RS	46, XY	Neg
2	29	Male	RA	2	22	46, XY	Neg
3	5	Male	RAEB	16	30	46, XY	Neg
4	68	Female	RA	3	16	46, XX	Neg
5	41	Male	RA	4	15	46 XY	Neg
6	32	Male	RA	3	24	46, XY, +19, -22	Neg
7	4	Female	RAEB	18	30	45, XX, -7	Neg
8	29	Male	RA	4	23	46, XY	Neg
9	38	Male	RAEB	15	22	46, XY	Neg
10	28	Female	RA	2	28	47, XX, +2, 13q+	Neg
11	31	Male	RA	3	22	45, XY, -7	Neg
12	18	Male	RAEB	16	26	46, XY, del 7q	
13	33	Female	RA	1	47	46, XX, add 5q, der 5	Neg
14	25	Female	RA	5	28	46, XX	Neg
15	50	Female	RA	1	76	46, XX	Neg
16	10	Male	RA	3	58	46, XY, del 9q	Neg

RARS: Refractory anemia with ringedsiderblast, RA: Refractory anemia, RAEB: Refractory anemia with excess blasts.
N.B. MLL gene probe (LSI MLL Dual color rearrangement probe, Vysis Inc, Downers Grove IL).

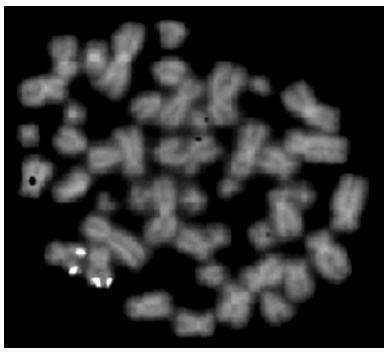

Fig. (1): Negative control for FISH using MLL break apart probe (Vysis).

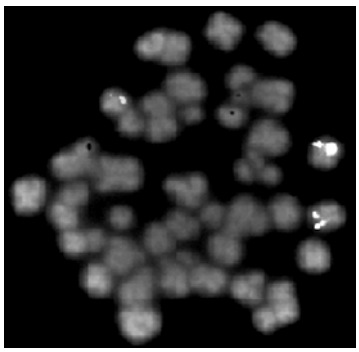

Fig. (2): FISH showing split of signals of the MLL gene probe denoting MLL gene translocation.

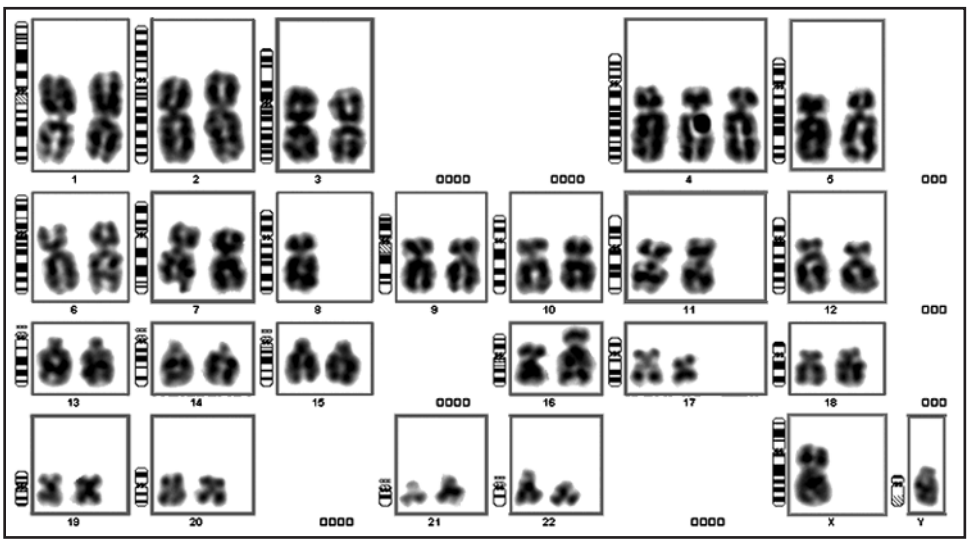

Fig. (3): Karyotype of Case No. 38 showing 47, XY, +4, t (16;17) (q22;11).

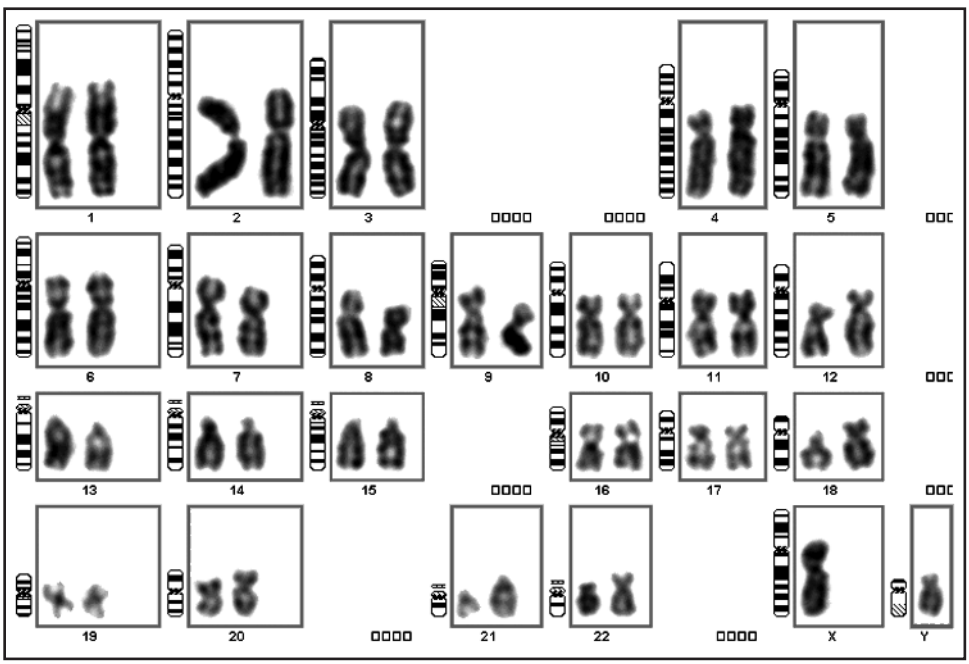

Fig. (4): Karyotype of case No. 11 showing t (8;21) (q22; q22).

DISCUSSION

In the present study, karyotypic analysis revealed normal karyotype in 30% of patients with AML. Normal karyotype was previously represented in 50% and 45% of patients with AML **[18,19]**.

In MDS, karyotypic analysis revealed normal karyotype in 56.3% of patients. Boultwood et al. reported normal karyotype in 50% of patients **[20]** while Chen et al. **[21]** and Pinto et al. **[22]** reported normal karyotype in 63% and 70% of patients with MDS, respectively.

In this study, conventional cytogenetics (CC) failed in 10% of patients. This figure is similar to previously published data **[23]**.

Cytogenetic findings in group 1 (AML patients):

1- Chromosome 11q23 abnormalities:

Structural abnormality of the 11q23 band (11q23 positive) bearing the MLL gene rearrangement (MLL+) is a recurrent chromosome change in leukemia described in AML and ALL with a peak incidence in infant leukemia. It occurs in up to 80% of children under one year of age who have leukemia **[5-24]**.

A proposal by the WHO specifies a separate category for AML with 11q23 positive/MLL+ **[25]**. This notion has been supported recently by biologic studies; microarray analyses have shown that MLL+ acute leukemias (ALs) have a peculiar gene profiling pattern that distinguishes them from all other ALs and that MLL+ leukemic blasts resemble very immature progenitor cells **[26]**.

Extensive cytogenetic and molecular studies have shown that 11q23/MLL is a highly promiscuous locus; more than 50 chromosomal loci have been described as 11q23 chromosome partners, whereas more than 30 MLL partner genes have been characterized **[27]**. To overcome this obstacle in studying MLL gene rearrangements, especially translocation, by FISH technique one can use break apart (split signal) FISH probe which is designed to hybridize upstream and downstream of the common breakpoint cluster region of the MLL gene. In that way MLL gene rearrangements are detected regardless of gene partners. FISH confirms the karyotypic results and helps to detect the precise nature of MLL and 11q23 abnormalities (translocation, deletion, or duplication). It may lead to reinterpretation of the karyotypic result **[28]**.

FISH collectively proved MLL gene rearrangements in 3 of 40 AML patients (7.5%) which is consistent with previously published data **[29,30]**. Another study detected MLL gene rearrangements at 4.7% frequency by FISH technique in adult de novo AML patients in which 11q23 band was cytogenetically normal **[31]**. Mrozek et al. **[32]** reported rearrangements of chromosomal band 11q23 involving the MLL gene at a 3-5% frequency in adult de novo AML.

Using southern blot technique, an Egyptian study published at the National Cancer Institute, revealed MLL gene rearrangements in 17.7% of pediatric AML patients **[33]**. The difference of the MLL gene rearrangement incidence in the present study may be due to different studied age groups as the incidence usually increases in pediatric patients while it is lower in the adults.

Conventional cytogenetics can discriminate between true 11q23 positive/MLL+ and rearrangements clustering within the 11q22-25 regions without MLL involvements **[34]**. Giugliano et al. **[35]** citated that 11q23 might involve genes other than MLL and Cox et al. **[36]** reported 45% 11q23 positive/MLL– in a series of AML patients.

Two studies reported many patients with MLL gene rearrangements in which the karyotype was normal **[37-38]**. Thus, FISH is more sensitive than CC in detecting MLL+ patients **[36-39]**.

In this work a strong association between AML subtypes M4 and M5 and MLL gene rearrangements was estimated. This is consistent with the study of Haferlach et al. **[40]**. Frenny et al. **[41]** reported high frequency of MLL gene rearrangements in M5a (50%) and M4 (20%) while Pan et al. **[39]** showed that MLL rearrangement is highly related to AML-M4/M5.

2- Other cytogenetic abnormalities:

The most frequently observed cytogenetic abnormality in this study was t (8;21) (q22;q22). It was detected in 8 patients (20%). One of these patients was firstly diagnosed as MDS because of the low number of blast cells in the BM. When cytogenetic studies were performed,

and t (8;21) detected, the diagnosis was changed to AML according to the WHO classification **[17]**. The rationale for the WHO classification that incorporated t (8;21) MDS into the category of acute myeloid leukemia with t (8;21) was supported by others **[43]**. The WHO system provides a reliable classification for the majority of AML patients. Further exploitation of cytogenetics and delineation of heterogeneous entities will probably extend its applicability and improve its clinical usefulness **[43]**.

Of the 8 patients with t (8;21) detected in this study, 5 were diagnosed as M2. This study included 12 AML-M2 cases, therefore, t (8;21) was detected in 5/12 (41.8%) of patients with AML-M2. Mitelman and Heim **[52]** showed t (8;21) in 15% of all AML subtypes and in 40% of M2. In this study, one of the patients with M2 having t (8;21) showed loss of Y chromosome; t (8;21) was recognized in one patient with M1, one with M4 and one with M5a. Published data reported t (8;21) predominantly in M2 and also in M4 and recognized that t (8;21) is frequently accompanied by the loss of sex chromosome; Y in males and X in females **[45]**. While others repored t (8;21) in 12.5% of patients with M2, 1.7% of patients with M1 and rarely in other FAB subtypes (M3-M7) **[46]**.

In this study t (8;21) was the sole abnormality in 4 patients (10%). Other studies reported this translocation as a sole abnormality in 20% of patients with AML and reported loss of Y chromosome to be a common additional abnormality **[47]**. In this work t (8;21) was detected as a part of complex karyotype in one patient which was diagnosed as M2. A previous study on pediatric AML patients reported favorable chromosomal abnormalities including t (8;21), t (15;17) and inv16 as a part of complex karyotype in 15/254 cases **[48]**.

In the present study, trisomy 8 was a recurrent chromosomal abnormality in AML. It was detected in 5 patients (12.5%); one patient with M1, one with M2, one with M4 and 2 with M5a. It was the sole abnormality in 3 patients (7.5%) and a part of complex karyotype in one patient. Heim and Mitelman **[45]**, reported +8 as the most frequent abnormality in AML that occurred as a sole abnormality in 5% of patients while, others recognized +8 in 12.7% of patients with AML that occurred as a sole abnormality in 5% **[49,50]**.

In this study, trisomy 21 was detected in 3 patients (7.5%); two patients were diagnosed as M2 and the third was diagnosed as M7 in an infant who had Down's syndrome. Wan et al. **[51]** detected +21 as a sole acquired karyotypic abnormality in AML-M2 and M4 and also reported that approximately 20% of leukemia in Down's syndrome was diagnosed as M7. The incidence of acute megakaryoblastic leukemia (M7) in Down's syndrome is estimated to be 400 times more than that in normal children **[52]**.

In this study the 3 patients with M3 showed a t (15;17) as a sole abnormality (7.5%). A previous study reported that all M3 patients have t (15;17) **[53]**. In agreement with the results of the current study, published data indicated that AML-M3 is associated with reciprocal chromosomal translocations always involving the RARa gene **[54]**, while some authors reported that 70% of patients with M3 had t (15;17) that represented 25% of all karyotypic abnormalities detected in AML **[23]**. The incidence of t (15;17) in different acute promyelocytic leukemia (APL) series varied so much. It may reflect differences in cytogenetic technique. In particular, the use of short term cultures instead of direct preparations seems to substantially increase the yield of chromosomally abnormal mitoses from patients with APL.

In this study two patients showed trisomy 19 as a part of complex karyotype, one patient was diagnosed as M1 and the other as M2. Similar findings have been previously reported **[55]**.

In this study, one patient diagnosed as M1 showed 7q- and double Philadelphia as part of complex karyotype. Complete or partial loss of long arm of chromosome 7 has been recognized in preleukemic MDS or unfavorable AML **[56]**. Monosomy 7 is associated with pediatric AML and MDS **[57]**.

Double Philadelphia was previously reported in a patient with AML belonging to myelomonocytic type of the FAB classification **[58]**. Some authors detected Ph chromosome in 1.2% of patients with AML and reported some patients with double Ph, but still one cannot exclude the possibility that the patient showed ++Ph was an acute blastic crisis (ABC) on top of chronic myeloid leukemia (CML) **[59]**.

In this study one patient diagnosed as M2 showed trisomy 10 as a part of complex karyotype. Others reported +10 as a rare recurring numerical abnormality detected in M0, M1 or M2 and sometimes M6 **[60]**.

In the current study, t (16;17) (q22;q11) was detected in one patient with biphenotypic leukemia M7/T-ALL. Others detected t (16;17) as a sole abnormality in AML-M6 **[61]**.

In this work, one patient diagnosed as biphenotypic leukemia showed complex karyotype in the form of 46, XY, add1q, t (8;15), add11p, -17, +mar. Marker chromosome was detected in another patient diagnosed as M2 as a part of complex karyotype. One patient with AML with variant translocation t (8;21;15) was previously reported **[62]**.

Published data detected monosomy 17 in patients with AML **[44]**. Monosomy 17 and del 17p in patients with AML and MDS as part of complex karyotype that included marker chromosomes was also reported **[63]**.

Some authors detected abnormalities of chromosome 1 in myeloproliferative disorders **[64]**. Others reported unbalanced translocation involving 1q in myeloid disorders **[65]**.

In this work, complex karyotypes were detected in 4 patients (10%), one diagnosed as M1, 2 as M2 and the fourth was diagnosed as biphenotypic leukemia. Others detected complex karyotype in 10% of AML with higher incidence in patients ≥60 years **[66]**. Other studies detected complex karyotype in AML including t (8;21) **[52-67]**. Published data described that 10-15% of patients with AML exhibit complex karyotypes and mentioned that many of these patients cannot be accurately described using conventional chromosome banding analyses due to the low resolution of the technique and the complexity of the rearrangements (e.g. additional material of unknown origin, marker chromosomes and ring chromosomes) that may need further investigations by FISH and CGH **[68]**.

In the current study, isochromosome 6p [i (6p)] and der 12 as parts of complex karyotype were detected in one patient diagnosed as M2. Isochromosome (6p) was previously reported in retinoblastoma tumor cells and in myeloid leukemia **[69-70]**.

Cytogenetic findings in group 2 (MDS):

In the present work 3/16 (18.8%) patients showed abnormalities of chromosome 7. Two patients diagnosed as RAEB and RA showed monosomy 7 as a sole abnormality. 7q- was detected in the third patient who was diagnosed as RAEB. In most of the previously reported series, patients with monosomy 7 were grouped with those with 7q- as both abnormalities lead to the loss of a putative tumor suppressor gene on 7q.

Monosomy 7 and 7q- have been previously reported as common cytogenetic abnormalities in MDS **[62-72]**, while a higher rate of -7 was reported in patients with RA and RAEB **[72]**.

In our series, one patient diagnosed as RA was pseudodiploid as it showed +19 and -22. Some authors detected +19 in patients with MDS, while others reported -22 in a patient with AML preceded by MDS at the time of progression **[73-74]**.

In the current study, trisomy 2 was reported in one patient which was diagnosed as RA in addition to 13q+. Published data reported +2 in MDS as a sole abnormality and suggested that +2 is an early chromosomal abnormality in leukemogenesis as it is found in combination with other chromosomal abnormalities in AML **[75]**. Others reported chromosome 13 abnormality in patients with MDS **[23]**.

In this work, del 9q was reported in one patient diagnosed as RA. Some authors reported del 9q as a sole abnormality to be rare in patients with MDS and AML **[60-76]**.

In our series, abnormalities in chromosome 5 (add 5q) and der 5 was detected in one 33 years old female patient diagnosed as MDS (RA). Some authors recognized 5q- as a common anomaly in patients with MDS with average age from 65 to 70 years **[73]**.

FISH results:

FISH studies revealed no MLL gene rearrangements in all patients with MDS which was consistent with published data. However MLL gene rearrangements, was reported in some patients with therapy related MDS **[77-78]**.

Conclusion:

FISH collectively proved MLL gene rearrangements in 3 of 40 AML patients (7.5%), all

of which were monocytic leukemias and in none of the MDS patients. FISH should be routinely used to screen for MLL gene rearrangements in AML patients because it is more sensitive and more specific than conventional cytogenetics.

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Thrombotic and Inflammatory Tendency to Particulate Matter of Woodsmoke

HASNAA A. ABO EL-WAFA, M.D.

The Department of Clinical Pathology, Faculty of Medicine, Sohag University.

ABSTRACT

Background: Wood is oldest of human fuels,it is literally true that exposure to woodsmoke is as old as humanity itself. Woodsmoke being a natural substance, it belived that it was considerd benign to human. The elevated level of particulate matter. Would be associated with increased blood levels of thrombotic and inflammatory markers especially in elderly individuals.

The aim of this study was to examine the effects of subchronic exposure to woodsmoke and biomass pollutants on blood coagulation factors, platelets activation and inflammatory markers in the farmers who live in rural areas and used to burn the wood, agricultural products and biomass as an alternative method for domestic heating and cooking.

Subjects and Methods: The study was carried out in the period between Jan. (2007) to Feb. (2008) on 51 exposed subjects to woodsmoke and 40 non exposed subjects as a control. The markers of thrombosis and inflammation were measured (Prothrombin time, activated partial thromboplastin time, thrombin time, fibrinogen levels, coagulation factor VII,VIII, IX D-dimer, von Willibrand factor, CRP, platelets aggregation by ADP and collagen were measured.

Exclusion criteria of the selected groups including: DM, liver and renal disease, Pregnancy, contraceptive bills and smoking, previous history of thrombosis and also family history.

Results: The exposure to particulate matter P.M. of woodsmoke affects blood picture components that leads to a significant increased in absolute neutrophilic count, increase in plateletes count and decrease in closure time of whole blood platelets activation by ADP and collagen ($p<0.05$). As regard to age and sex the results were a significantly increased in WBCs & neutrophiles count in exposed young <30 ys old groups of both sex in comparison to non exposed groups ($p<0.05$).

A significant decreased in closure time of whole blood platelet activation by collagen/ADP only when the exposed (young & old, <30 ys. & >30 ys. old) of both sex groups were compared to non exposed groups of the same age & sex ($p<0.05$). Coagulation profile: the following results were observed: only a.P.T.T clotting times showed a significant decreased when the exposed groups were compared to the non exposed groups ($p<0.05$). FVIII, FIX, & FVIII/vWF ratio showed a significant increase when the exposed groups were compared to non exposed groups ($p<0.05$). F VII & vWF showed a highly significant increase ($p<0.001$). As regard age & sex F VII: a significant increased when the exposed groups of both sex <30 ys & >30ys old) were compared to non exposed groups of the same age & sex ($p<0.05$). Inflammatory markers, Plasma levels of fibrinogen & D-dimer showed a highly significant increase when the exposed groups were compared to the non exposed groups ($p<0.001$), CRP represents a significant increased only ($p<0.05$). As regard age & sex: Plasma level of fibrinogen showed a significant increased when the exposed groups of both sex <30 ys old were compared to non exposed groups of the same age & sex ($p<0.05$). D-dimer level showed a highly significant increased when the exposed young male & female groups were compared to non exposed groups of the same age & sex ($p<0.001$). CRP like D-dimer.

Conclusion: Subchronic exposure to P.M. of woodsmoke and other biomass feul is considerd as one of the risk factors for thrombosis it leads to increased WBCs count, platelets count & activities, so formation what is called platelets-leukocytes aggregates & subsequent thrombosis also it increases the coagulation factors concentration F VIII, vWF, & FVII and plasma level of fibrinogen level and D-dimer. In males >30 ys old, leads to increase of factor VII concentration which is considerd as a risk factor for coronary artery disease.

Key Words: *Woodsmoke – Thrombosis – Inflammation.*

INTRODUCTION

Woodsmoke is a significant source of air pollution in many parts of the world specially in developing countries. Woodsmoke is generated using wood stoves, agricultural ash, in and outside the door **[1]**. Woodsmoke is a major ingredient tiny particles of soot and liquid pollution, it contains carbon monoxide and cancer –causing chemicals **[2]**.

The epidemiological data suggest a causal relationship between elevated wood smoke level and health effects **[3]**. The proposed mechanisms underlying this increase include effects on coagulation factors and inflammatory response **[4]**, increased blood levels of inflammatory cytokines and thrombotic markers specially in eldery. Also increase the oxidative stress **[5]**. P.M. exposure affect blood coagulation as they increase the plasma level of fibrinogen, homocysteine and high sensitive C-reactive protein **[6,7]**. Fine and ultrafine particles affect coagulation cascade, platelete function, leads to atherosclerosis and thrombosis **[8]**. On exposure to biomass smoke, platelet activation, aggregation and formation of platelet-leukocyte aggregates with thrombosis **[9]**.

The rising fossil energy costs has led to increase in the use of wood and other biomass fuels. While the commercial sources of wood combustion have been subjected to some regulation, there are still important unregulated sources woodsmoke including household heating stoves and fireplaces **[10]**.

Two of the principal gaseous pollutants in woodsmoke, CO and NOx, add to the atmospheric levels, haematological alteration occurred due to CO as formation of non functioning haemoglobin e.g. carboxyhaemoglobin **[11]**, why woodsmoke may be a special case requiring separate health evalution **[5]**. Wood consists primarily of two polymers: cellulose (50-70% Wt) and lignin(30% Wt), other biomass fuels also contain these polymersbut in different proportion **[10]**. Woodsmoke particles are generally smaller than 1μm, the range is (0.15-0.4 μm). Fresh woodsmoke contains a large number of ultrafine particles, less than 100μm which condense rapidly as they cool and age. These paticles evade the mucociliary system in the peripheral airway and exert pathogenic inflammatory response. About 5-20% of woodsmoke particulate mass consists of elemental carbon, the composition of organic carbon fraction differe according to fuel being burned and combustion conditions. Woodsmoke is also mutagenic and possibly carcinogenic, but less than coal smoke. A significant woodsmoke exposure, mostly in winter occur indoor and outdoor in all areas of developed world for residential heating **[12]**.

SUBJECTS AND METHODS

Population and study design: The study was carried out in the period between Jan. (2007) to Feb. (2008) on 51 farmers, whom were subjected to subchronic daily exposure to high concentration of woodsmoke (16 males and 35 females) their age ranged from 20 to 45 years old and 40 non exposed subjects as a control (14 males and 26 females), their age range from 20-49 years old. The exposed farmers were selected from the raural areas where burning agricultural biomass, ash and wood were used for indoor and outdoor ordinary human activities e.g. cooking, making bread, frying meat and heating in winter. The studied groups were subclassified according to the age and sex into young groups, whom age below 30 years and more old groups, whom age above 30 years, the exposure in the young groups were more than the old one, as the dueties and acivities in this age group were increase, also in raural areas most of the females above 30 are sitlle dawn, the exposure toparticulate matter P.M is less. The markers of thrombosis and inflammation were measured maximum after 12 hours of exposure to P.M. of woodsmoke. Evaluation the degree of thrombotic tendency and the rise in inflammatory markers by measuring (prothrombin time PT, activated partialthromboplastin aPTT, thrombin time TT, Fibrinogen Level, coagulation factors: FVII, FVIII FIX were done by clotting method on SYSEMEX DADE BEHRING system, vWF was done by latex agglutination method, the kit was supplied by DEAD BEHRING. Platelet activation by ADP/collagen & EPI/collagen was done by whole blood clotting method using PF-100 DADE BEHRING system. CRP was done by latex agglutination method, the kit was supplied by OMEGA. Complete blood count was done on H-MAX Coulter system. Liver and renal function were done on Beckman Synchron CX-9.

Exclusion criteria: Of the selected groups including: DM, liver and renal disease, family and previous history of thrombosis, pregnancy, contraceptive bills and smoking to exclude other risk factors for thrombosis tendency rather than exposure to P.M. of wood and biomass during normal human activities.

Subjects recruitment: Subjects were recruited from the near by villages through advertising them by the mean of the hospital lab. Technich-

iums whom living in nearby villages, also from medical outpatients clinic lab. Medical history and clinical examination were done to exclude cardiac, renal and hepatic disease.

Exposre measures: Atmosphere concentration of particulate matter (P.M.) due to wood burning was measured by the mean of (sequential air sampler, air trap for aerodynamic measurments) in the environmental sanitation center in sohag locality, the size was 2.5μm for fine P.M. and 2.5-10μm for coarse P.M, the conc. Was 265 mμg/m^3.

Blood measures:

About 10ml of venous blood was drawn from every subject within the frist 12 hour after P.M. exposure, 1.8ml was added to each separate citrated tubes (containing 0.2 sod. citrate, one for the coagulation profile and the other tube for the platelet activation). 3ml was drawn to K-EDTA tube for complete blood count performance. The remaining blood was deliverd to plain tube for chemical tests (the vacutainers were supplied by B.D.).

• For coagulation profile the citrated tubes samples were centrifuged in cooling centrifuge system at 4000 rpm for 10 min, at 20ºC to prepare P.P.P., then the poor plasma was deliverd to Sysmex Dead Behring fully automated autoanalyzer system for PT, aPTT, TT, Fibrinogen, coagulaion factors: VII, VIII, IX by clotting method and D-dimer by turbidimetric method. kits supplied by Dead Behring.

• Normal values for each: PT: 10.5-13 sec. & 130-70%, aPTT: 26-38 sec., TT: 14-17 sec, Fibrinogen 180-360 mg/L, coagulation factors: 70%-150%, D-dimer < 20ug/dl.

• For platelet activation the second citrated tube was deliverd to PFA-100 DEAD BEHRING as whole blood activation, using cartilage for ADP and collagen. The closure time was measured. The nomal closure time is for ADP/collagen & forEPI/collagen up to 175 sec.

• vWF was assyed by latex agglutination method supplied by Dead Behring.

• K-EDTA blood tube was subjected to H-MAX fully automated Coulter system for C.B.C.

• The remaining blood was left for clotting, centrifuged, the serum was delivered into Beckman Synchron CX-9 fully automated chemical analyzer for glucose, renal and liver functions. The remaining serum was subjected to CRP by latex agglutination method supplied by OMEGA.

Statistical analysis:

SPSS program was used for data analysis. *t*-test and two way ANOVA test were used (test of significant: *p*-value <0.0.

RESULTS

Environmental measures:

The mean concentration (±S.D) for coarse P.M in the burning field is 16.4±5μg/m^3, the mean concentration for fine P.M was 5±3μg/m^3 in the burning field. The data obtained from the Sohag Sanitation Center.

Time lag after exposure is 12 hours at least.

Subjects: 51 farmers, whom were subjected to subchronic daily exposure to high concentration of woodsmoke (16 males and 35 females) their age ranged from 20 to 45 years old with the mean age (31.6±7.4 SD) and 40 non exposed subjects as a control (14 males and 26 females), their age range from 20-49 years with the mean age (35.5±6.8 S.D). Age and sex of the studied groups were illustrated in Table (1).

Changes in blood picture: Neutrophilia was observed in 8 subjects (15.6%) of exposed group in comparison to 3 subjects (7.5%) of non exposed control group, the mean neutrophiles count was (4.8±2.4S.D) in exposed group versus (4.3±1.4 S.D) in non exposed control group (*p*-value <0.02). Absolute monocytes count was non significantly increased in exposed group versus control group (*p*-value >0.1). Absolute lymphocytes showed a non significant decrease in exposed group versus control group (*p*-value >0.05). Eosinophile and basophile counts were not affected in comparison of both groups (*p*-values >0.1 & >0.2 for both). When the blood picture variables were analysed as regard to age and sex, the young male and female groups <30 years old showed a significant increase in total WBCs and neutrophile counts in exposed groups versus non exposed groups (*p*-value <0.05, <0.005). Hb concentration and HT were significantly increased in exposed males (>30 ys & <30 ys) versus non exposed female groups of th same age (*p*-value 0.02). Reticulocytes count showed a significant increase in exposed

young female group versus control non exposed young one (p-value <0.05). Tables (2,3), Fig. (1) demonstrate the C.B.C changes.

Blood platelets changes: Blood platelet count was significantly increased in the exposed group versus non exposed control group (p<0.05), MPV and Pct showed a non significant variation (p>0.05). Platelet activation by ADP/collagen and EPI/collagen showed a significant decrease in closure time in exposed group in comparison to non exposed group (p-value <0.05). When analysed as regard to age and sex, platelet count showed a non significant variation (p>0.05), MPV represents a significant increase in exposed young male and female groups when compared to non exposed young male and female groups (p<0.05). Platelet crite and PDW showed no significant variation as regard to age and sex (p>0.05). Whole blood platelet activation closure time by ADP/collagen were significantly decreased in exposed young male and female <30 ys when compaired to the same age & sex groups (p<0.05), also a significant decreasewas found in closure time of platelet activation by ADP/collagen in exposedyoung female below 30 years old in comparison to exposed young male group (p<0.05). Platelet activation closure time by EPI/collagen when analysed as regard to age and sex only a significant decrease was found when the exposed old female group more than 30 years was compared to non exposed old female group (p<0.05). Blood platelet changes were listed in Tables (4,5), Figs. (2,3).

Table (1): Age & sex of the studied groups.

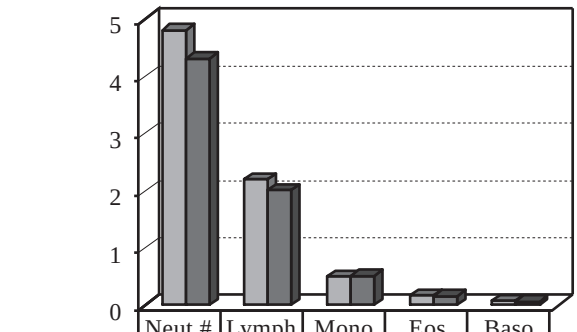
	Exposed group	Non exposed group
No. of subjects	51	40
Age: range	20-45	20-49
Mean±S.D	31.6±7.4	35.5±6.8
Sex	M:16 F:35	M:14 F:26
No. of subjects <30 Ys	M:6	M:6
age: (range)	(20-29 Ys)	(23-28Ys)
	24.2±5.3	25.7±2.6
mean±S.D	F:15	F:12
	(20-28 Ys)	(20-29Ys)
	23.5±4.4	26.7±2.8
No. of subjects >30 Ys	M:10	M:8
age: (range)	(30-45Ys)	(35-46Ys)
	38±5.5	41.2±5
Mean±S.D	F:20	F:14
	(32-45 Ys)	(31-49 Ys)
	37.7±6.8	38.5±5.7

M: Male. F: Female.

Table (2): The blood picture variables in studied groups.

Varibles	Exposed group range Mean±SD	Non exposed group range Mean±SD	p values
WBCs x 10^9/L	3.3-14 7.7±2.4	3.3-11 6.8±2.02	>0.07 NS
RBCs x 10^{12}/L	3.6-5.5 4.4±0.4	4.2-5.5 4.77±0.4	>0.06 NS
Hb g/dl	11-15.4 12.8±1	11.5-15.4 13.3±0.85	>0.06 NS
MCV fl	73-94 73.9±5.6	74.4-94 81.9±5.03	>0.07 NS
Ht%	31.4-47 38.4±3.2	34.9-45 39.2±2.65	>0.08 NS
MCH pg	25.8-34 28.7±2	26-34.4 28.4±1.9	>0.8 NS
MCHC g/dl	32-38.6 35±1.3	33.2-37.5 35.2±1.05	>0.9 NS
RDW%	11-17.4 13.±1.7	11-16 12.9±1.04	>0.06 NS
Neutrophiles# x10^9/l	1.3-10 4.8±2.1	1.7-7.9 4.3±1.43	<0. 02 S*
Lymphocytes # x10^9/l	0.66-4.5 2.18±0.8	0.8-3.5 2±0.71	>0.08 NS
Monocytes# x10^9/l	0.2-0.9 0.5±0.17	0.2-0.9 0.51±0.16	>0.1 NS
Eosinophiles# x10^6/l	0-0.7 0.16±0.14	0-0.7 0.15±0.12	>0.2 NS
Basophiles # x10^6/l	0-0.45 0.068±0.049	0-0.04 0.055±0.033	>0.1 NS

S: Significant p<0.05, NS: Non significant p>0.05.



	Neut #	Lymph #	Mono #	Eos #	Baso #
Exposed group	4.8	2.2	0.5	0.16	0.068
Non exposed	4.3	2	0.51	0.15	0.055

Fig. (1): The blood picture variables in studied groups.

Table (3): Blood picture variables in the studied groups as regard to age & sex.

	WBC x 10^9/L	RBC x 10^{12}/L	Hb g/dl	Ht %	Retic %	Differential count #: x10^9/L		
						Neut	Lymph	Mono
				Mean±SD				
Non exposed:								
I-M: <30Ys	6.2±2.1	5±1.2	14±1.7	44.3±4.4	1.2±0.2	3.5±1.3	2.1±0.1	0.5±0.2
II-F: <30Ys	6.9±3.4	4±0.7	11.2±0.8	34.2±3.1	1.0±0.7	3.7±2	1.7±0.3	0.49±0.1
III-M >30Ys	5.8±2.6	5.3±0.7	15±0.7	46.6±4.2	1.4±0.2	4.3±1.4	1.4±0.2	0.5±0.2
IV-F: >30Ys	6.4±2.7	4.3±0.8	12±1.2	38.3±2.6	0.7±0.3	4.4±2.4	1.8±0.6	0.4±0.1
Exposed:								
V-M: <30Ys	7.3±4.3	5.2±1.3	14±4 1.3	45.7±3.6	1.4±0.5	5.2±2.6	2.4±0.4	0.5±0.1
VI-F: <30Ys	8.4±3	3.8±1	11±0.7	35.7±3.5	1.5±0.4	7±2.8	2.2±0.4	0.5±0.2
VII-M >30Ys	7.4±1.8	5.3±0.9	15±1.2	48.6±4.8	1.4±0.3	6.4±1.8	2.5±0.5	0.4±0.2
VIII-F: >30Ys	7±2.3	4±0.8	12±1.7	34.4±3.6	1.5±0.4	6.4±3.5	2.4±0.3	0.5±-0.1
p-values:								
I versus V	NS	NS	NS	NS	NS	S*	NS	NS
II ver. VI	S*	NS	NS	NS	NS	HS*	NS	NS
III ver. VII	S*	NS	NS	NS	NS	NS	NS	NS
IV ver. VIII	NS	NS	NS	NS	S*	NS	NS	NS
V ver. VI	NS	NS	S*	S*	NS	NS	NS	NS
VII ver. VIII	NS	NS	S*	S*	NS	NS	NS	NS
V ver.VII	NS	NS	NS	NS	NS	NS	NS	NS
VI ver. VIII	NS	NS	NS	NS	NS	NS	NS	NS

NS: Non significant. S*: Significant. HS**: Highly significant. M: Male. F : Female.

Table (4): Blood platelets variables in the studied groups.

Platelet variables	Exposed group range Mean±SD	Non exposed group range Mean±SD	*p* value
Count x 10^9/L	177-447 305±72.4	212-397 276.03±48.4	<0.05 S*
MPV fl	7-15 9.5±1.8	7-12 8.36±1.02	>0.2 NS
Pct%	0.07-0.45 0.24±0.08	0.18-0.37 0.22±0.04	>0.3 NS
PDW (GSD)	14-23 16±1.5	14-19 16.4±1.11	>0.1 NS
Platelet activation by ADP/ collagen (closure time sec.)	124-135 132.3±2.6	143-148 145±4.5	<0.04 S*
Platelet activattion by EPI/collagen (closure time sec.)	145-152 146.4±3.3	148-159 154±3.7	<0.03 S*

*S: Significant. NS: Non significant.

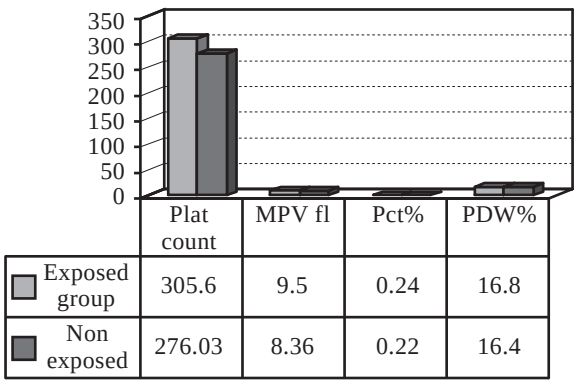


Fig. (2): Platelet variables in the studied groups.

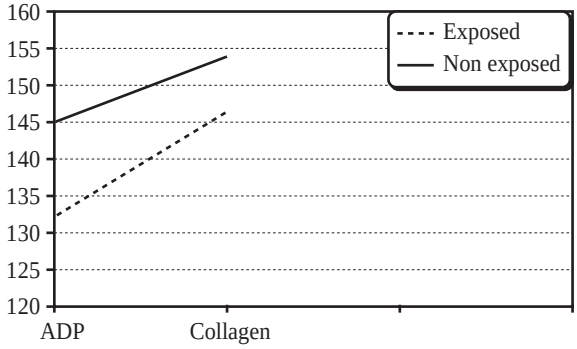


Fig. (3): Platelate activation in the studied groups.

Table (5): Platelet changes as regard to age & sex.

	Count x 10^9/L	MPV fl	PDW (GSD)	Plat aggr: ADP closure time (sec)	Plat aggr: collagen closure time (sec)
			Mean±SD		
Non exposed:					
I-M: <30Ys	260±54	8.2±1.1	16.6±2.3	145.4±4.6	152.2±4.2
II-F: <30Ys	340±66	10±1.6	17±1.5	145.3±5.2	148.3±5.7
III-M >30Ys	330±33	7.8±2.3	15±1.6	147.6±6.2	155.5±4.7
IV-F: >30Ys	350±65	9.9±3.2	16.4±2.4	144.3±4.3	157.5±3.6
Exposed:					
V-M: <30Ys	280±64	11.7±2.2	17.2±2.4	133.5±5.3	150.6±3.7
VI-F: <30Ys	390±43	13.4±1.1	18.6±2.1	126.2±2.5	146.2±5.2
VII-M >30Ys	340±36	10.2±2.5	16.7±1.7	134.4±3.7	151.4±6.2
VIII-F: >30Ys	378±65	12.3±1.9	17.2±2.8	129.2±2.5	148.3±6.3
p-values:					
I versus V	NS	S*	NS	S*	NS
II ver. VI	NS	S*	NS	S*	NS
III ver. VII	NS	NS	NS	S*	NS
IV ver. VIII	NS	NS	NS	S*	S*
V ver. VI	NS	NS	NS	S*	NS
VII ver. VIII	NS	NS	NS	NS	NS
V ver.VII	NS	NS	NS	NS	NS
VI ver. VIII	NS	NS	NS	NS	NS

NS: Non significant. S*: Significant. HS**: Highly significant. M: Male. F : Female.

Coagulation profile changes: The screening tests for haemostasis (PT, aPTT, TT) showed a significant decrease in clotting time of only a.P.T.T in exposed group versus non exposed control group (*p*-values <0.05). A significant increase was found in concentration of coagulation factors VIII and IX ($p<0.05$) and a highly significant increase in concentration of coagulation factors VII and vWF ($p<0.001$) when the exposed group was compared to non exposed group. FVIII/vWF ratio is significantly decreased in exposed group versus non exposed ($p<0.05$).

When the data were analysed as regard to age and sex, the results were: PT only showed a significant decrease in clotting times when the old exposed male group >30 ys was compared to non exposed old male group ($p<0.05$). a.P.T.T, T.T, FVIII represent a non significant variation ($p>0.05$). Coagulation factor VII represented a very characteristic finding that was significantly increased in concentration when both the exposed young male and female groups <30 ys old were compared to the non exposed same age and sex groups ($p<0.05$), also a significant increase was found when the exposed old male and female groups were compared to the same age and sex groups ($p<0.05$). Within the exposed subgroups a significant increase was found when the young male group was compared to young female group ($p<0.05$), also a significant increased was found when the old male group was compared to old female group ($p<0.05$). vWF was significantly increased when both the young exposed male and female groups were compared to the non exposed groups of the same age and sex, also when the old exposed male group was compared to non exposed old one ($p<0.05$).

A highly significant increase was found when the old exposed female group was compared to the old non exposed one ($p<0.001$). FIX was only significantly increased when the exposed young female group was compared to non exposed young one ($p<0.05$). Coagulation profiles are listed in Tables (6,7) & Figs. (4-8)

Inflammatory markers: Hyperfibrinogenaemia was observed in 8 cases (15.68%) of exposed group (fibrinogen level was >400 mg/L) and not observed in control group, there is a highly significant increased in fibrinogen level in exposed group versus control group (*p*-value <0.003). CRP level was increased in 21 cases (41.1%) of the exposed group, the level was more than 6 mg/L and in one case only (2.5%) of non exposed control group and showed a significant increase in exposed group versus non exposed control group (*p*-value <0.02). D-dimer level was more than 350 ug/dl in 16 cases of exposed group (31.3%), a highly significant increased in the level was observed in the exposed

group versus the control group (p-value <0.001), as regard to age and sex fibrinogen level was significantly increased when both exposed young male and female groups <30 ys old were compared to the non exposed groups of the same age and sex, also when the old exposed female group >30 ys old was compared to the non expose old one (p<0.05), a highly significant increase was found when the young exposed female group were compared to the young exposed males, also when the old exposed femalegroup was compared to the old exposed male group (p<0.005). CRP and D-dimer levels represent a highly significant increase when both exposed young male and female groups were compared to the same age and sex of non exposed groups, also when both the old exposed male and female groups were compared to the non exposed old groups of the same age and sex (p<0.005). A significant increase in the leveles of CRP and D-dimer was found when the young exposed female group was compared to the young exposed male group and when the young exposed female group was compared to the old exposed female group (p<0.05). No significant variation was found when the old exposed male and female groups were compared to each other and when the young and old male groups were compared to each other (p>0.05). Inflammatory markers were listed in Tables (8,9), Figs. (9-12).

Table (6): Coagulaton profile in the studied groups.

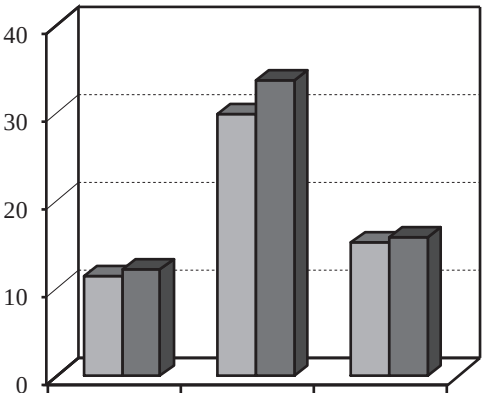
Varibles	Exposed group range Mean±SD	Non exposed group range Mean±SD	p values
PT sec.	9.9-13 11.3±0.72	11-13.9 12.17±0.8	>0.05 NS
aPTT sec.	22-37 29.8±4.3	23-42 33.7±3.7	<0.05 S*
TT sec	13.2-19 15.14±1.3	14-19 15.7±1.2	>0.05 NS
F VIII%	80-130 103±12.8	70-130 90.6±15.2	<0.05 S*
vWF%	110-156 133.2±10.3	70-120 89.05±16.98	<0.003 HS**
FVIII/vWF	0.72-0.83 0.77±0.08	1-1.08 1.01±0.89	<0.05 S*
F IX%	83-118 99.7±10.16	68-100 87.48±7.11	<0.05 S*
F VII%	120-210 144±11.9	65-100 82.87±8.96	<0.001 H*

NS : Non significant.
S* : Significant.
HS** : Highly significant.

Table (7): Coagulation profile as regard to age & sex.

	PT sec	aPTT sec	TT sec	F VII %	F VIII %	vWF %	FIX %
	Mean±SD						
Non exposed:							
I-M: <30Ys	11.8±0.5	31.2±3.2	14.4±1	85.6±8.7	80.5±10.6	75.5±7.5	105±10
II-F: <30Ys	12.2±1.3	34.4±2.7	16±1.3	70.4±.5.7	100±8.3	100.3±8.5	80.6±10.6
III-M >30Ys	12.4±0.4	30±4.2	15±0.9	95.6±7.4	120.4±11.6	90.5±11.3	85.7±12.6
IV-F: >30Ys	12.4±0.6	33.2±2.9	16.5±0.5	75.5±8.7	128.7±11.2	80.4±5.7	90±13.9
Exposed:							
V-M: <30Ys	10.4±0.8	28.5±3.2	14±1.3	200.5±11.8	95.6±10.2	120.4±10.6	110.6±8.6
VI-F: <30Ys	10.5±1.4	31.7±2.8	15±0.5	150.6±7.9	85.0±11.3	140.5±12.5	115.6±6.3
VII-M >30Ys	9.9±1.2	29.3±3.5	15±1.7	180.7±13.6	105.7±8.8	125.4±13.5	105.4±8.4
VIII-F: >30Ys	11±0.5	32.6±2.4	15±1.4	140.7±8.5	110.5±10.6	150.7±10.5	100.5±10.5
p-values:							
I versus V	NS	NS	NS	S*	NS	S*	NS
II ver. VI	NS	NS	NS	S*	NS	S*	S*
III ver. VII	S*	NS	NS	S*	NS	S*	NS
IV ver. VIII	NS	NS	NS	S*	NS	HS*	NS
V ver. VI	NS	NS	NS	S*	NS	NS	NS
VII ver. VIII	NS	NS	NS	S*	NS	NS	NS
V ver.VII	NS	NS	NS	NS	NS	NS	NS
VI ver. VIII	NS	NS	NS	NS	NS	NS	NS

NS: Non significant. S*: Significant. HS**: Highly significant. M: Male. F : Female.



	PT sec	aPTT sec	TT sec
Exposed group	11.3	29.8	15.14
Non exposed	12.17	33.7	15.7

Fig. (4): Coagulation profiles in the studied groups.

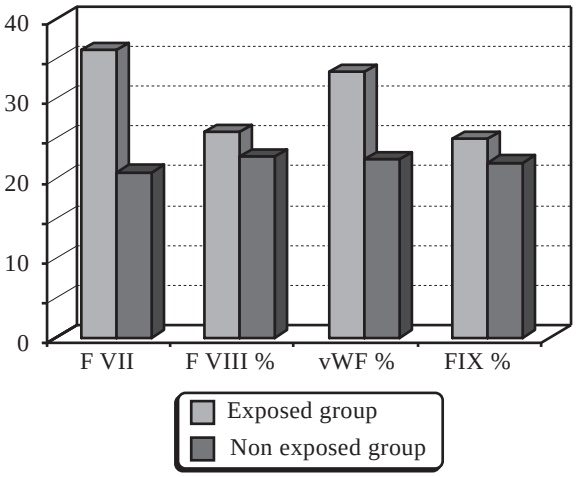
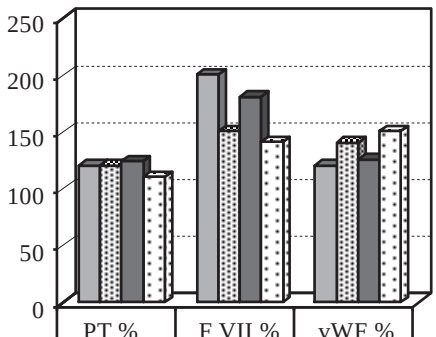


Fig. (5): Coagulation factors in the studied groups.



	PT %	F VII %	vWF %
Exp.M <30Ys	120	200.5	120.4
Exp.F <30Ys	120	150.6	140.5
Exp.M >30Ys	124	180.7	125.4
Exp.F >30Ys	110	140.7	150.7

Fig. (6): Coagulation variables in the exposed groups as regard to age & sex.

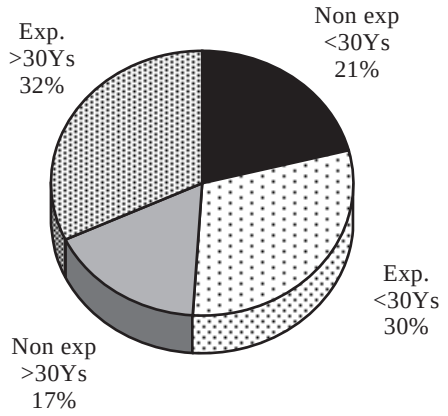


Fig. (7): vWF in the studied females groupes.

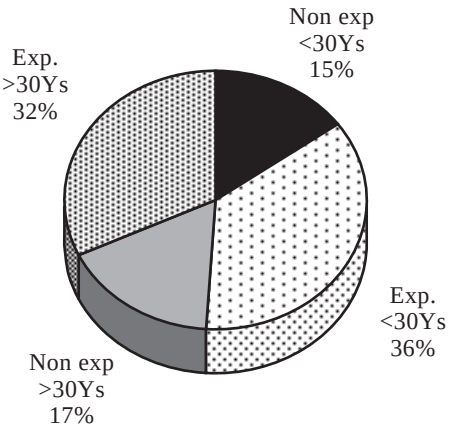


Fig. (8): Factor VII in the studied males groups.

Table (8): Inflammatory markers in the studied groups.

Varibles	Exposed group range Mean±SD	Non exposed group range Mean±SD	*p* values
Fibrinogrn mg/L	120-490 328.8±91	140-300 216.92±51.9	<0.003 HS**
CRP mg/L	0-36 12.11±6.52	0-12 3.7±2.21	<0.02 S*
D-dimer ug/dl	100-450 302.9±82.4	10-130 55.58±28.2	<0.001 HS**
vWF%	110-156 133.2±10.3	70-120 89.05±16.98	<0.003 HS*

S* : Significant.
HS** : Highly significant.

Table (9): Inflammatory markers as regard to age & sex.

	Fibrinogen mg/L	D-dimer ug/dl	CRP mg/L	vWF %
		Mean±SD		
Non exposed:	160.4±27.3	25.7±10.6	0	75.5±7.5
I-M: <30Ys	200.6±33.7	40.6±15.3	6.4±0.4	100.3±8.5
II-F: <30Ys	180.5±30.5	20±11.7	0	90.5±11.3
III-M >30Ys	260.4±35.4	65.7±14.8	0	80.4±5.7
IV-F: >30Ys		160±38.6		
Exposed:	195.5±34.5	210±37.8	4.2±2.6	110.6±8.6
V-M: <30Ys	320±40.4	180±28.9	16.3±4.4	115.6±6.3
VI-F: <30Ys	215±35.5	320±43.8	6.6±2.8	105.4±8.4
VII-M >30Ys	310±53.5		8.4±3.2	100.5±10.5
VIII-F: >30Ys		HS**		
p-values:	S*	HS**	HS*	S*
I versus V	S*	HS**	HS**	S*
II ver. VI	NS	HS**	HS**	S*
III ver. VII	S*	S*	HS**	HS*
IV ver. VIII	HS**	HS**	S*	NS
V ver. VI	HS**	NS	NS	NS
VII ver. VIII	NS	S*	NS	NS
V ver.VII	NS		S*	NS
VI ver. VIII				

NS: Non significant. S*: Significant. HS**: Highly significant.
M : Male. F : Female.

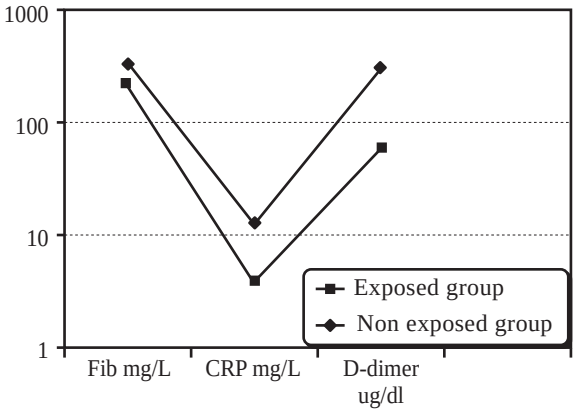


Fig. (9): Inflammatory markers in the studied groups.

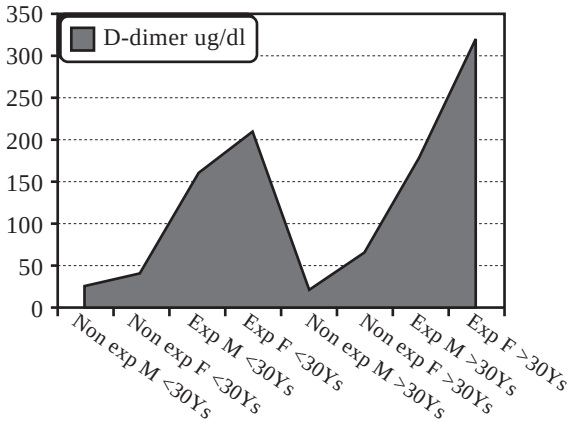
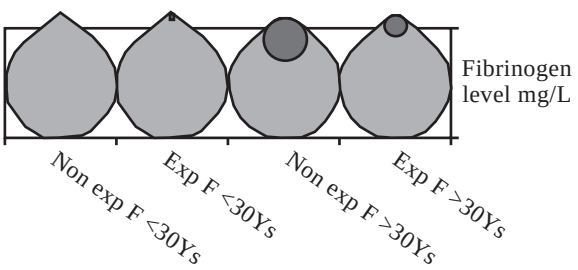


Fig. (10): D-dimer in the studied groups as regard to age & sex.



	Exp F <30Ys	Exp F >30Ys	Non exp F <30Ys	Non exp F >30Ys
Fibrinogen level mg/L	320	310	200.6	260.4

Fig. (11): Fibrinogen level in the studied females groups.

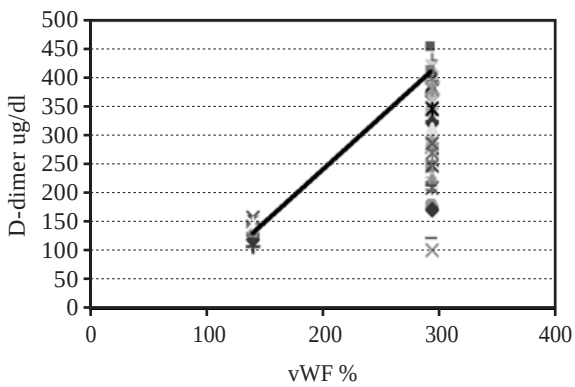


Fig. (12): Correlation between D-dimer & vWF in exposed group.

DISCUSSION

In the present study, exposure to P.M. of woodsmoke affects many of blood components that rinders most of them in the upper limit of normal or may exceed the upper normal range in certain condition, so these exposed groups are potentially at risk for thrombosis.

The total leukocytic count (WBCs) and specially, the absolute neutrophilic count is significantly increased, this is due to the release of inflammatory cytokines (IL-6) which affect the WBCs count and platelets count also was increased, this is in agreement with **[4]** who reported that bone marrow release of leukocytes and plateletes was an important component of the systemic inflammatory response.

MPV is significantly increased in the exposed groups, the newly released platelets are more large and giant than the old ones as reportedby.

The platelet activities are also significantly increased in the exposed group than the non exposed control group, this noticed by the decrease in the closure time of plateletes activation (by PF-100) by both ADP/collagen and EPI/ collagen, as the recently released platelets from the marrow megakaryocytes are more active as they contain most of its granule contents. Now there are two factors potentially increase the thrombotic tendency: the increase WBCs (neutrophiles) and increase both the platelet count and activities, these two components lead to the formation of platelet –leukocyte aggregates, which act as naidus for thrombus formation, this agrees with **[9]** who reported that a significant increase in leukocyte-platelet aggregates was found in women who used biomass as cooking fuel. In addition, they showed increased surface expression of CD11b/ CD18 in circulating neutrophiles and monocytes, also CD62P increased in expression.

The young exposed groups showed the more increase in WBCs and plateletes, as this young categories of people have more dueties and act more than the old one, so the exposure to pollutants of coocking fuel is more with more release of cytokines **[13]**.

Platelets activation by ADP/collagen in the PFA-100 showed more age and sex variation, than that by EPI/collagen, this is due to the fact that, ADP platelets aggregation occurs in two steps 1st receptors mediated process, 2nd the release of the endogenous ADP from the plateletes with subsequent aggregation, females below 30 years old whom are more exposed to pollutants and so the more cytokines released with more new platelets which contain all of its content granules so more activity, this is in accordance with **[14,15]**, who reported that amine polyester carbon particles affect platelets aggregation by ADP.

Coagulation profile response to P.M. of woodsmoke pollution affect aPTT that showed a significant shorting in clotting times this may be due to the increase concentration of the coagulation factors of the intrinsic pathway (F VIII, vWF, FIX) in the exposed groups, this not agree with who found that no association between pollution and a.P.T.T.

As regard to age and sex, P.T is significantly decreased in exposed males >30 years old group than the non exposed group, this may be due to the increase in concentration of factor VII **[16]**, who reported that factor VII was increased in response to P.M. and not agree with **[17]** who reported that no alteration in P.T and a.P.T.T with P.M. exposure.

vWF is highly significant increase in exposed females above 30 years old, as it is one of the acute phase reactant that is increases in response to the cytokines, this agree with **[5]**.

Inflammatory markers showed a highly significant incrase in plasma level of fibrinogen and D-dimer conentraion and significant increase in CRP level, these are acute phase reactant proteins that increase in inflammatory response as reported by **[12]**. Fibrinogen and D-dimer are rapid and early released inflammatory mediators than CRP (ultra sensitive CRP may be the more sensitive one as reported by **[18]**. Fibrinogen level is more affected by age and sex difference, it was increased more in exposed females groups below and above 30 years old than exposed males. This is similar to vWF response in female group, so the inflammatory response in females is exaggerated than male groups.

Conclusion:

* Exposure to P.M. of Woodsmoke is considered as a risk factor for thrombosis as it increase: WBCs (neutrophilic count), platelets

count and activities, coagulation factors (F VII, F VIII, vWF, F IX).

* Females below 30 years old are mor liable to thrombotic tendency as they are more exposed and have more activated platelets.

* Exposed males have the elevated level of factorVII (usually above 30 years old) are potentially at risk for thrombosis.

* D-dimer and CRP showed no sex variation, so can be used as as universal inflammatory marker with no sex affection D-dimer can be used as an indicator for thrombosis and inflammation irrespective to sex.

Recommendation:

Avoid the exposure to P.M of woodsmke as possible as we can by the use of protective mask and shorting the exposure times, increase the space between the exposure.

Alternate the traditional methods for cooking fuel to more automated machines.

Aknolowdgement:

To Dr. Heba, the resident doctor in our department for her hard work in this study. To Mr. Abd Elreheem, the technechium who recruited the farmers in the near villages and to all the haemostasis unit staff members in our department.

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Immunophenotyping and Immunoglobulin Heavy Chain Gene Rearrangement Analysis in Cerebrospinal Fluid of Pediatric Patients with Acute Lymphoblastic Leukemia

DOUAA SAYED, M.D.[1],*; HOSNY BADRAWY, M.D.[1],**; AMANY M. ALI, M.D.[2] and SANAA SHAKER, M.D.[1]

The Departments of Clinical Pathology[1], Flow Cytometry Lab, Molecular Biology Lab** and Pediatric Oncology[2], South Egypt Cancer Institute, Assiut University.*

ABSTRACT

Background: The diagnosis of CSF infiltration of hematologic malignancy has great prognostic and therapeutic implications both in symptomatic and asymptomatic patients. A diagnostic gold standard is not available and morphologic examination of CSF fails to demonstrate malignant cells in up to 45% of cases thought to be positive. Flow cytometric immunophenotyping and detection of clonal IgH genes rearrangement (IGHR) are considered to be more sensitive for detection of these malignant cells.

Aim of the Work: To assess the diagnostic accuracy of flow cytometric immunophenotyping and IGHR by real time PCR in comparison with classic cytology for diagnosing CNS infiltration in pediatric ALL.

Material and Methods: Forty five CSF specimens from pediatric patients with ALL were examined by flow cytometry (FCM) for immunophenotyping. Monoclonal antibodies were designed according to the BM or peripheral blood immunophenotyping at diagnosis. Twenty specimens were also examined for IGHR by light cycler. The results were compared with classic cytology.

Results: Twenty one samples were positive by FCM [21/45 (46.6%)]. Fifteen samples could not be analysed because of insufficient cell numbers. Twelve samples were positive by IGHR [12/20 (60%)] and only 10 samples were positive by cytology [10/45 (22.2%)].

Conclusions: The diagnostic value of FCM and IGHR are two to three times more sensitive than that of cytology. Therefore, immunophenotyping by FCM is recommended for routine diagnosis of CSF infiltration. Furthermore, IGHR analysis by real-time PCR appears to be a useful addition to morphological and FCM analysis of CSF in evaluation of CNS infiltration.

Key Words: *CSF – Flow cytometry – PCR – ALL.*

INTRODUCTION

Meningeal involvement is a frequent complication of hematological malignancies with an incidence of up to 25% in certain leukaemias and lymphomas **[1]**. The diagnosis of this involvement has great prognostic and therapeutic implications both in symptomatic and asymptomatic patients at high risk of such involvement **[2]**. A diagnostic gold standard is not available, and morphologic examination of cerebrospinal fluid (CSF) fails to demonstrate malignant cells in up to 45% of patients in whom meningeal involvement is thought to be present **[3]**. The major diagnostic problem in evaluating CSF involvement is distinguishing neoplastic infiltrates from inflammatory or infectious diseases **[4]**.

Flow cytometry (FCM) immunophenotyping is a valuable tool in the diagnosis and staging of lymphoproliferative disorders involving the lymph nodes, blood and bone marrow. While flow cytometric analysis is a standard procedure in the evaluation of blood and bone marrow cells, it is not generally applied to CSF samples in all clinical laboratories [5].

In addition, analysis of heavy chain gene (IgH) rearrangements by real-time polymerase chain reaction (PCR) is a powerful diagnostic tool for hematologists and oncologists. The detection of malignant cells by this technique has become the state of art for diagnosis, monitoring response to treatment and detection of minimal residual disease in leukemia and lymphoma **[6]**. The development of high technologies allows the application of real-time PCR assays in large prospective treatment studies for monitoring tumor cells in circulation as well as in bone marrow. Based on quantitative data

the kinetics of disappearance and reappearance of tumor cells can be followed up in "real-time". This allows developing new strategies to treat patients with an inadequate response to standard chemotherapy or at molecular relapse before symptoms or signs of clinical relapse occur **[7]**. However, this modality has not been widely applied to CSF specimens **[8]**.

In this study, we aimed to assess the diagnostic accuracy of flow cytometric immunophenotyping and IgH gene rearrangements (IGHR) analysis by real time PCR in comparison with classic cytology for diagnosing central nervous system (CNS) infiltration in acute lymphoblastic leukemia (ALL). In addition, we aimed at verifying the CNS status especially at first presentation, which is essential for risk stratification and proper treatment.

MATERIAL AND METHODS

Forty-five CSF specimens from pediatric patients with ALL were examined by FCM for immunophenotyping. In 12 patients, CSF analysis was performed because of neurological abnormalities (manifestation of increased intracranial tension, convulsion and cranial nerve palsy) and in 33 patients as part of their routine work up at first presentation; twenty-four patients at first presentation and nine patients at relapse.

Twenty large samples were divided into two tubes to send the second for the molecular biology laboratory for IGHR analysis by light cycler, 3 of them with neurological abnormalities. All specimens tested for IGHR were from B-precursor ALL patients.

The results were compared with classic cytology routinely done for all samples. Medical ethical committee of Assiut University approved this study.

Flow cytometry:

Cell counting and preparation:

CSF was centrifuged at 100 x g for 10 min. within 2 h of obtaining the sample. Cells were counted by hemocytometer.

For flow cytometry analysis, a minimum of 1 ml CSF containing at least one cell/10µl was needed. Flow cytometry could not be accomplished for samples of insufficient cell numbers (one cell/10µl of CSF).

The supernatant was discarded and the cell pellet washed with phosphate buffered saline (PBS) and then by hemolysite.

Antibody cocktails were added to each tube according to the manufacturer's recommendations. Immunophenotyping was performed by standard three-colour immunofluorescent staining using fluorescence-labeled monoclonal antibodies, directed against the following surface markers [CD45, CD34, Terminal Deoxynucleotidyl Transferase (TdT) and CD33 conjugated with FITC from Caltage laboratories (Austria), CD14, CD19 and CD10 conjugated with PE from BD Pharmingen (Becton Dickinson, Biosciences), CD3 and CD19 conjugated with CyQ from IQ Products (Groningen, Netherlands) CD4/CD8 and kappa/lambda dual colored monoclonal Abs from Becton Dickinson (Biosciences, San Jose, CA)].

The antibody cocktails were selected according to the patients' baseline bone marrow immunophenotyping results and in combinations show atypical pattern of antigen expression.

The following mixes were used:

1- Isotypic control.

2- CD45/CD14.

3- CD4/CD8/CD3.

4- Cyt TdT/CD10/CD19.

5- κ/λ/CD19.

6- CD34/CD19.

7- CD33/CD19.

Mix 6 was used if the BM lymphoblasts in the base line immunophenotyping showed expression of CD34. Mix 7 was used only if the lymphoblasts in the BM base line immunophenotyping showed aberrant myeloid expression.

Flow cytometric analysis:

The flow cytometer (FACSCaliber; Becton Dickinson) was calibrated using CALIBRITE-3 beads FACSComp software. Data acquisition and analysis was performed using CellQuest software (Becton Dickinson).

Polymerase chain reaction:

DNA extraction from CSF was done by High pure template kit (Roche diagnostic, Mannheim, Germany).

Principle:

The extracted DNA was analysed for IgH chain gene rearrangements as follow:

First round PCR using consensus primers to amplify variable segments (VH)-joining segments (JH) of IgH gene to obtain sufficient product for second round PCR.

Second round PCR of the first round product using allele specific primers to amplify complementary determining region (CDR) of JH of IgH gene. This was performed to further specify the target gene.

Equipment:

Light Cycler Instrument (Roche Diagnostics, Mannheim, Germany).

Reagents:

A- Primers **[9]** are represented in Table (1) (a and b).

B- Light Cycler^{R} FastStart DNA Master^{plus} SYBR Green I.

Master mix component for IgH first and second round PCR amplification were done as manufacturer instruction.

Amplification was done using thermal cycler. The thermal profile includes:

• Initial denaturation step at 94ºC for 5 minutes.

• 40 cycles of amplification:

Denaturation at 94ºC for 30 seconds.

Annealing at 56ºC for 30 seconds.

Extension at 72ºC for 45 seconds.

PCR protocol on LightCycler for SYBR Green detection is represented in Table (2).

Quantification program: Amplification curves were obtained and the fluorescence values versus cycle number were displayed.

Melting curve program: Assessment of the specificity of the amplified product was achieved by performing a melting curve analysis. The resulting melting curve allows discrimination between primer dimmer and specific product.

Monitoring PCR with the SYBR Green 1 dye:

SYBR Green 1 dye binds to the minor groove of dsDNA. Fluorescence is generally enhanced by binding. During the various stages of PCR, different intensities of fluorescence signals can be detected, depending on the amount of dsDNA that is present. All DNA become single stranded after denaturation. At this stage of reaction SYBR Green 1 dye will not bind and the intensity of flouresence signal is low.

During annealing the PCR primers hybridize to the target sequence, resulting in small parts of dsDNA to which SYBR green 1 dye can bind thereby increasing fluorescence intensity.

In the elongation phase, the PCR primers are extended and more SYBR Green 1 dye can bind. At the end of the elongation phase all of the DNA has become double stranded and a maximum amount of dye is bound. The fluorescence is recorded at the end of the elongation phase and increasing of amount of PCR product can be monitored from cycle to cycle.

Melting curve analysis of amplicons with SYBR Green 1 detection:

Each dsDNA product has its own specific melting temperature (Tm) which is defined as the temperature at which 50% of the DNA becomes single stranded and 50% remains double stranded, the most important factors that determine that Tm of dsDNA are the length and the GC content of that fragment.

In going from low to high GC content a difference of up to 40ºC can be measured in Tm. Checking the Tm of a PCR product can thus be compared with analyzing a PCR product by length in gel electrophoresis.

RESULTS

In this work, 45 CSF-samples were examined. Twenty-one samples were positive by FCM [21/45 (46.6%)]. Fifteen samples [15/45 (33.3%)] could not be analysed because of insufficient cell numbers. Twelve samples were positive by IGHR [12/20 (60%)] and only 10 samples were positive by cytology [10/45 (22.2%)]. A total of 26/45 positive samples were detected; 8 samples were positive for both FCM and cytology, five samples were positive for both IGHR and cytology (Table 3). Thirteen samples [13/45 (28.9%)] were positive by FCM and negative by cytology and three cases were

positive by IGHR and not by cytology or FCM (two could not be analysed due to insufficient cell numbers and one was negative) (Table 4). Two samples were positive for both cytology and FCM and not by IGHR and two cases were positive for cytology but not by FCM (Table 4). The first was negative for all markers by FCM and positive by PCR and the cells in the second were reactive T lymphocytes and negative for malignancy by FCM.

The gated cells in the FCM analysis varied greatly from 20 to 9808 cells; analysis of cases with large number of cells (Fig. 1) was not difficult as those cases with very low count number that needs aberrant phenotype to ensure the malignant involvement (Fig. 2).

The percentages of positive cases by the three methods in relation to the clinical manifestations are show in Table (5). All the 15 samples that had no sufficient cells to analyse were from cases at presentations. There were two patients with CNS manifestation; one showed negative results with cytology and FCM and the other was positive by cytology and negative for malignancy (all the cells were reactive T lymphocytes) by FCM. These two were cases diagnosed as toxic encephalitis by MRI.

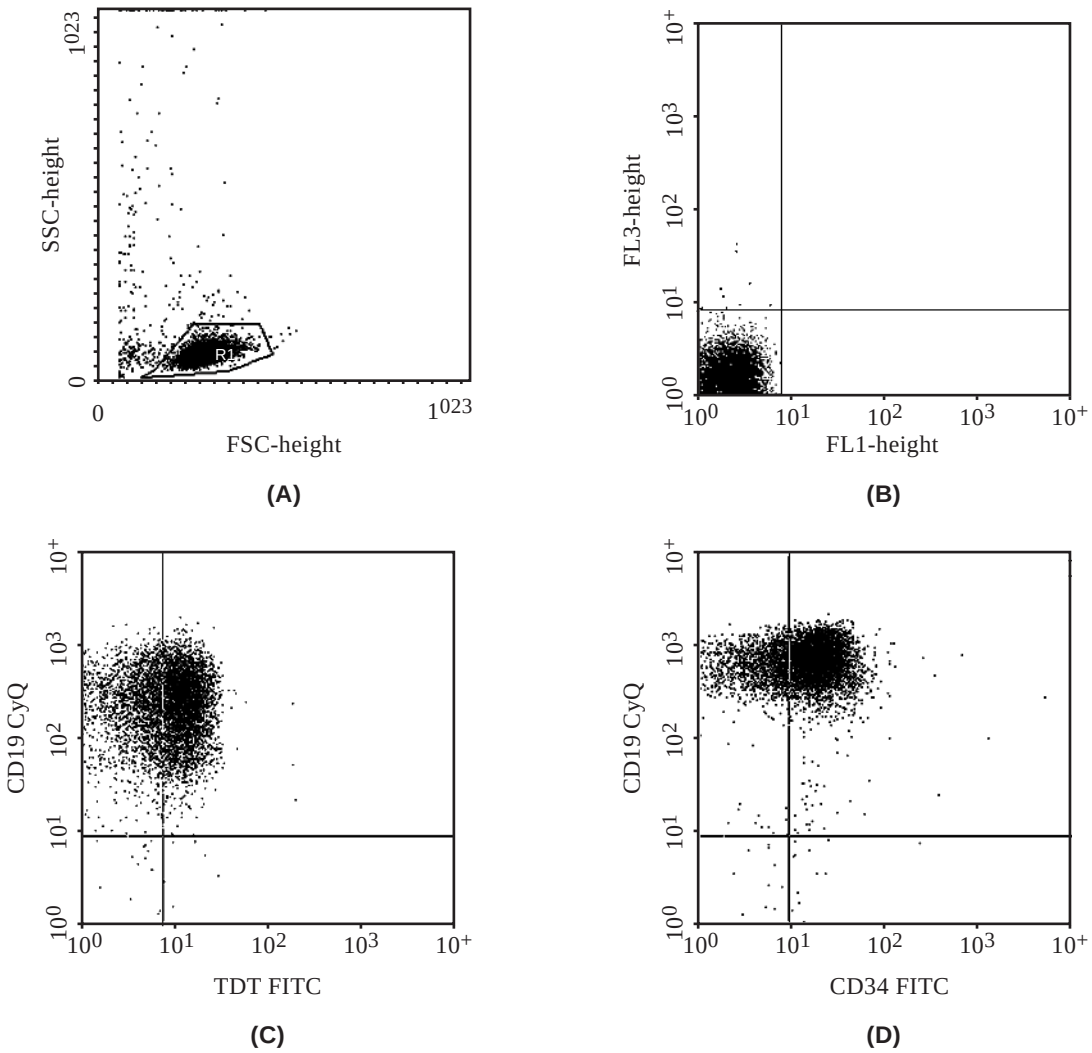

Fig. (1): (A) Dot plot of FSC versus SSC showing a distinct population (R1). (B) Dot plot of FL1 versus FL3 gated on R1 showing clear isotypic control (C) Dot plot of TdT-FITC versus CD19-CyQ gated on R1, showing that the gated cells co-express both markers. (D) Dot plot of CD34-FITC versus CD19-CyQ gated on R1 showing that the gated cells express CD34 also.

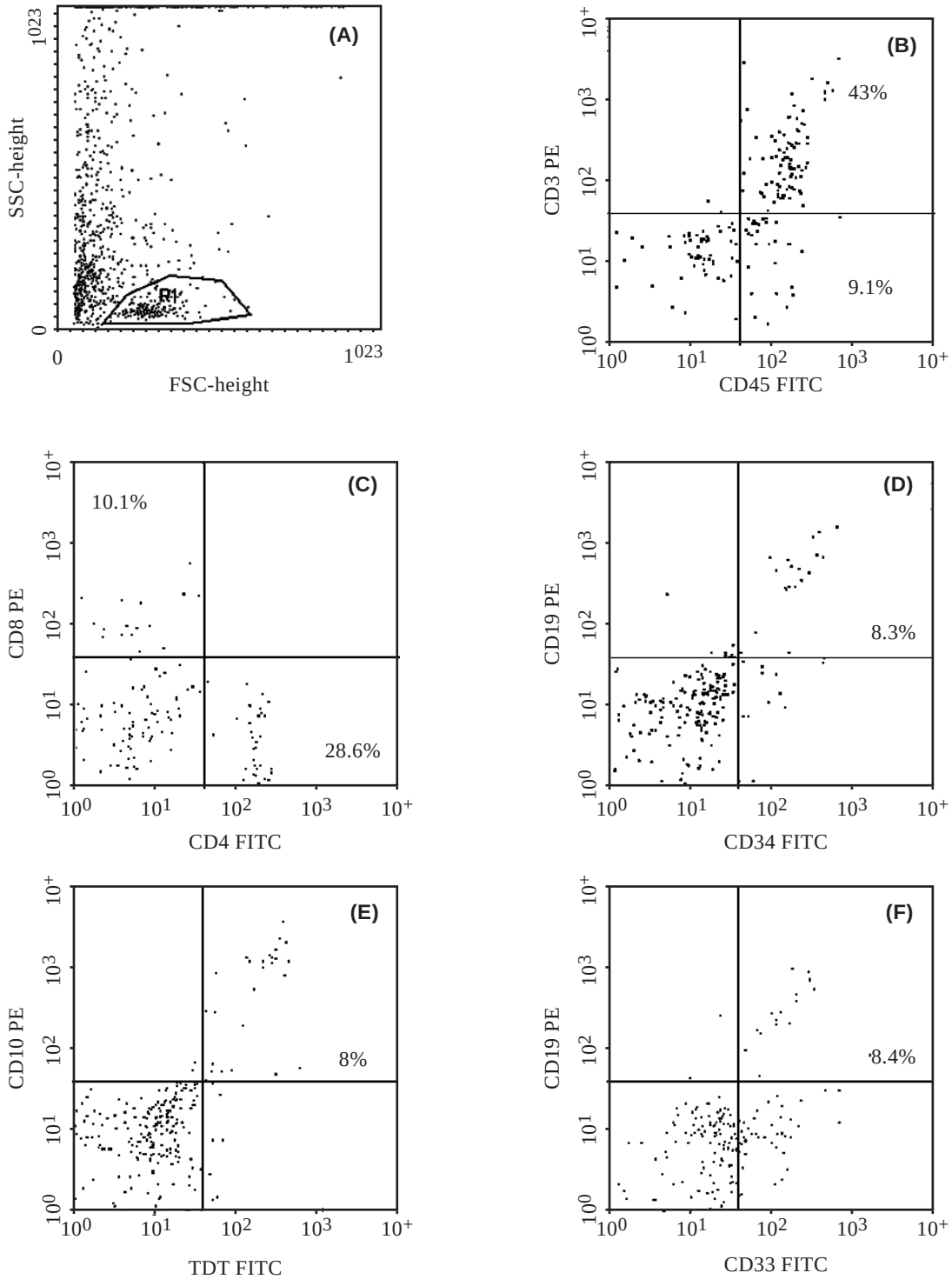

Fig. (2): (A) Dot plot of FSC versus SSC showing a distinct population (R1). (B) Dot plot of CD45-FITC versus CD3-PE gated on R1 showing showing 2 population groups the first is of T lymphocytes (43%) that coexpress CD45 and CD3 and the second is of malignant population that express CD45 and not CD3 (9.1%). (C) Dot plot of CD4-FITC versus CD8-PE gated on R1 showing that the T lymphocytes are reactive and distributed into CD4 cells (28.6%) and CD8 cells (10.1%). (D) Dot plot of CD34-FITC versus CD19-PE gated on R1 showing that 8.3% of gated cells co-express both markers (lymphoblast cells) which are the malignant population. (E) Dot plot of CDTdT-FITC versus CD10-PE gated on R1 showing that 8% of the gated cells co-express both markers (lymphoblast cells) which are the same malignant population. (F) Dot plot of CD33-FITC versus CD19-PE gated on R1 showing that the same population 8.4% of the gated cells express aberrant myeloid marker.

Table (1-A): Sequences of the consensus primers for IgH used in the first round PCR.

Sequences of the consensus primers for IgH	Size	Name	Length	GC %	Tm ºC
5GCC CAG GAC TGG TGA AGC3	376bp	VH4/6 outer	18	66.7	65
5ACC TGA GGA GAC GGT GAC3	376bp	JH	19	63.2	65.1

Table (1-B): Sequences of the allele specific primers for IgH used in the second round PCR.

Sequences of the allele specific primers for IgH	Size	Name	Length	GC %	Tm ºC
5ATC TAT TAT AGT GGG AGC ACC3	177bp	VH4/6 inner	21	42.9	57.9
5ACC CCG TAC CAG CTG CCT CC3	177bp	JH	20	65	68.6

IgH : Heavy chain gene.
PCR: Polymerase chain reaction.
Tm : Melting temperature.
bp : Base pair.
VH : Variable segments in heavy chain gene.
JH : Joining segments in heavy chain gene.

Table (2): PCR protocol on Light Cycler for SYBR Green detection.

Analyis mode acquisition mode	Cycles	Segment	Temperature	Hold time
Pre-incubation:				
None	1		95ºC	10 min.
Amplification:				
Quantification none	45	Denaturation	95ºC	1s
None		Annealing	60ºC	10s
Single		Extension	72ºC	20s
Melting curve:				
Melting curve none	1	Denaturation	95ºC	0s
None		Annealing	65ºC	15s
Continuous		Melting	95ºC	0s
Cooling:				
None	1		40ºC	30s

PCR: Polymerase chain reaction.

Table (3): Detection of CNS infiltration by IGHR and FCM in comparison to cytology in ALL patients.

	IGHR analysis		Immunophenotyping by FCM		
	Positive	Negative	Positive	No cells	Negative
Cytology:					
Positive	5/20	2/20	8/45	0/45	2/45
Negative	7/20	6/20	13/45	15/45	7/45

IGHR: Heavy chain gene rearrangements.
FCM: Flow cytometry.

Table (4): Positive CSF samples for malignant involvement by the three methods in 45 ALL patients.

No	Clinical manifestation	Cytology	FCM	IGHR
1	CNS (M)	-ve	+ve	ND
3	CNS (M)	+ve	+ve	+ve
5	RW up	-ve	+ve	ND
6	CNS (M)	-ve	+ve	ND
8	RW up	+ve	+ve	+ve
9	CNS (M)	-ve	-ve	+ve
10	CNS (M)	-ve	+ve	ND
11	Relp	-ve	+ve	ND
13	Relp	+ve	+ve	ND
14	Relp	+ve	+ve	+ve
16	CNS (M)	-ve	+ve	ND
22	CNS (M)	+ve	+ve	ND
23	RW up	-ve	No cell	+ve
26	Relp	+ve	+ve	+ve
27	CNS (M)	-ve	+ve	+ve
28	Relp	+ve	+ve	-ve
29	CNS (M)	+ve	-ve	ND
33	RW up	-ve	No cell	+ve
35	RW up	-ve	+ve	+ve
36	RW up	+ve	+ve	-ve
38	CNS (M)	-ve	+ve	ND
40	Relp	-ve	+ve	ND
41	Relp	-ve	+ve	ND
42	CNS (M)	+ve	-ve	+ve
44	Relp	-ve	+ve	+ve
45	Relp	-ve	+ve	+ve

CNS (M) : Central nervous system manifestation.
RW up : Routine work up of the ALL patient at first presentation.
Relp : Relapsed ALL.
FCM : Flow cytometry.
IGHR : Heavy chain gene rearrangements.
+ve : Positive.
-ve : Negative.
ND : Not done.
No cell : Insufficient cell number.

Table (5): Distribution of the CSF positive cases according to the clinical manifestation in ALL patients.

	Cases at presentation	Relapsed cases	CNS manifestation	Total
Cytology:	2/24 (8.33%)	4/9 (44.44%)	4/12 (33.33%)	10/45 (22.2%)
FCM	4/24 (16.66%)	9/9 (100%)	8/12 (66.66%)	21/45 (46.6%)
IGHR	4/11 (36.36%)	4/5 (80%)	4/4 (100%)	12/20 (60%)

CNS : Central nervous system.
IGHR : Heavy chain gene rearrangements.
FCM : Flow cytometry.

DISCUSSION

Hematological malignancies comprise many prognostically distinct subtypes, thus, a uniform approach to therapy would be inappropriate **[10]**. Instead, emphasis is placed on a strict assessment of risk at the time of diagnosis, so that only patients at high risk for relapse are treated with more intensive therapy, while at those lower risk may have less toxic treatment. CNS involvement is important for the prognosis and treatment; it requires CNS-directed therapy including irradiation and high dose chemotherapy **[11]**. Leukemic blasts in CSF can be found in one third of patients at diagnosis, the majority have no neurological symptoms **[12]**. Accurate CNS assessment at presentation or relapse is very essential for treatment stratification. Recent treatment protocols offer high dose chemotherapy and CNS radiotherapy only for patients with CNS infiltration and reduce treatment for patients without CNS infiltration to decrease the toxicity of treatment and late effect of systemic chemotherapy and CNS radiotherapy **[13]**.

The cohort of patients characterized by risk factors as high risk seems to be 4-5 folds larger than the subgroup, which will actually develop CNS disease. More sensitive and specific laboratory methods would be crucial to detect occult CNS infiltration and if validated in clinical trials, to ensure optimal treatment while reducing unnecessary therapies [5,14]. The early detection of CSF involvement allows targeted approaches and the use of intrathecal drugs represent a critical step in the treatment of the disease **[15]**.

Until now, no absolute standard has been established to diagnose the involvement of CNS and all methods conventionally applied are associated with problems. All methods used for detection of leptomeningeal seeding including cytology, flow cytometry and the DNA based examination have pitfalls that need to be considered. Conventional cytology is positive only when large numbers of neoplastic cells are present in the CSF. The interpretation of the results is based on morphology and therefore, poor fixation or cell debris may lead to negative findings at time when the malignant cells are actually present in the CSF. In addition, some examiners may consider atypical morphology as inconclusive evidence for malignancy while others may read it as positive cytology **[8]**. In our study only 10 cases were positive by cytology, one of them was false positive; the cells were reactive as proved by FCM. That indicates that cytology alone cannot asses the CSF infiltration.

Many studies were published about the superiority of FCM in detecting CSF residing abnormal cells as compared to conventional cytomorphology **[2,4,5,14-19]**. Our data are in accordance with them and show high sensitivity of FCM in detecting CSF infiltrating malignant cells even in the absence of positive cytomorphology, which were about 29% in our study and between 27 and 78% in the others. In spite of differences as regards conventional cytology performances, all studies clearly showed that FCM is able to increase dramatically the detection of occult CSF infiltration. Taking in consideration that the previous studies were based upon analysis of heterogeneous cohorts of cases or focused on lymphomas.

As regards the percentages of positive cases by FCM in relation to the clinical manifestations, we noticed that they were twice those detected by cytology in cases at first presentation and reached the level reported in the literature **[12]**.

Taken together, our and literature results indicate FCM as the first choice technique, probably due to its intrinsic capability of exploring a large series of cell specificities at a single cell level. It can offer a unique and objective method by the combination of different strategies as aberrant immunophenotype **[15]**. It is a simple, quick and reliable technique. Results were available within 2h, which can speed up the therapeutic management; this may be a cost-reducing factor **[18]**. The advantages of flow cytometry include the ability to enrich for possible malignant cells via gating techniques in samples with mixed cell populations and the rapidity of the analysis **[5]**. One of the strengths of FCM is its sensitivity. In our results, CSF involvement could be demonstrated by FCM and not by cytology in cases with very low cell counts that accounted for only 0.86% of the total number of cells. This is similar to the 1.4% reported by Frensh et al. **[17]** and 0.9% reported by Finn et al. **[16]**. This can be attributed to the simultaneous evaluation of multiple surface markers on each cell.

However, FCM could still be falsely negative in some cases that can be detected by other

methods. This may be explained by lack of CD34 or CD10 in some cases, loss of some antigens in cases of relapsed ALL or dilution of malignant B cells by large numbers of polyclonal normal B-lymphocytes **[14]**. It is also important to recognize, that while FCM could detect 13 cases of CSF infiltration with negative cytology, a total of 15 cases (33.3%) could not be analysed due to insufficient number of cells. This figure is slightly higher than that reported by French et al. **[17]** (29%). This may be due to a higher number of cases at presentation in relation to relapsed and symptomatic ones in our study.

The DNA-based molecular techniques do not require intact cells. DNA is stable and can be recovered from CSF even after tumor cell lysis, probably making it a more sensitive indicator of malignancy than FCM and cytology requiring presence of intact tumor cells **[20]**. The detection of clonal Ig gene rearrangements using PCR technique offers an alternative because of its high sensitivity **[21-22]**. The rearrangement of variable, diversity and joining segments (VDJ) of IgH genes generates unique DNA junctional sequences that are specific by its size and sequence to each B cell clone **[23]**.

Many attempts have been made to use PCR for identifying IGHR in cells from CSF **[8,20,21, 24,25,26]**. Several factors contribute to this diversity, including the presence of somatic hypermutations that prevent binding of the PCR primers and the fact that the consensus primers for any particular assay are not complementary to all V regions. Finally, as with all PCR protocols, false-positive results due to technical problems as contamination are possible **[20]**.

Some authors used one round of PCR amplification to determine the clonality of the B-cell population in the CSF **[21,22]**. However, the relatively low sensitivity of this method can produce false negative results because of the small number of cells usually present in most CSF samples. So others applied the highly sensitive semi-nested PCR **[27]** in the search for IgH gene rearrangements in CSF sample cells. This technique has proved to be sufficiently sensitive to produce a detectable band from a single B cell **[24]**. However to achieve a reliable detection of monoclonality, previous methods require the presence of approximately >1% of clonal B cells to show a clearly visible rearranged band **[28]**. The sensitivity of PCR methods for Ig gene rearrangement is limited by the separating power of the gel and the discriminating power of the eye to recognize a faint band of clonal B cell superimposed upon a diffuse smear, which is generated by the reactive polyclonal B cell population present in all tissues **[29]**.

PCR analysis of the IgH gene typically involves the use of a consensus primer pair, with the upstream primer being homologus to a V segment and the downstream primer annealing to one of the J segments **[30]**. Usually a single J region primer is sufficient to recognize all six possible J segments, but no single V region primer recognizes all V segments, since there are many more V segments that are more heterozygous as compared with J segments. This is the primary explanation for the lack of a 100% diagnostic sensitivity of a single primer pair IgH PCR assay **[31]**.

In this study, we used a real time PCR assay to evaluate infiltration of CSF by leukemic cells. This method was based on LightCycler technology and SYBR green dye for detection of the gene rearrangements. Real-time PCR permits accurate detection of PCR products during the exponential phase of the PCR amplification process, which is in full contrast to the classical PCR end point detection. Owing to the real time detection of fluorescent signals during or after each subsequent PCR cycle, detection of PCR data can be obtained in a short time and no post-PCR processing is needed, thereby drastically reducing the risk of PCR product contamination **[32]**.

Few studies have compared the sensitivity of IGHR with morphological analysis of CSF; all of them in lymphoma. Only one showed that IGHR analysis did not appear to be more sensitive than morphological evaluation, in which a low incidence of lymphomatous spread to spinal fluid was found (eight from 76 patients) **[25]**. Possibly, because of pretreatment of the majority of patients with steroids prior to lumbar puncture and the small CSF volume analyzed **[22]**.

Our results, which showed 60% infiltration of CSF by malignant cells, agree with the results

of Baehring et al. **[8]** who has indicated a sensitivity of IGHR analysis of 58%, however they worked on large B cell lymphoma not ALL. Our results also agree with the result of Ekstein et al. **[20]**, who has reported that in 60% of patients with active CNS lymphoma, the CSF was positive for the presence of IgH gene rearrangement, while in 95% of patients responding to treatment, the test was negative.

It is clear that the sensitivity of real-time PCR is superior to both cytology and FCM especially for those at first presentation and those with CNS manifestations. However, there were two cases (10%) positive for cytology and FCM and negative by real-time PCR. False negative results in PCR were reported in small samples, and in the presence of a low cell count. This may be due to difficulty to obtain DNA of sufficient quality. It was difficult to obtain enough DNA to run in 14% of CSF samples in Ekstein et al. **[20]** study. Others reported an even higher rate of inability to extract DNA from CSF (29%) **[25]**.

False positive results in real-time PCR could be eliminated by optimization conditions. The design of clone-specific primers and the annealing temperature are important steps for achieving accurate data. Melting curve analysis was found to be an essential tool for characterizing the PCR products. The sensitivity, specificity and predictive value of positive or negative results of IgH gene rearrangement by real-time PCR in the CSF require further evaluation.

In conclusions: The diagnostic value of FCM and IGHR are two to three times more than that of cytology. Malignant cells in CSF can be classified according to the immunological surface profile by FCM. Therefore, immunophenotyping by FCM is recommended for routine diagnosis of CSF infiltration combined with cytology to increase the diagnostic yield. Furthermore, IGHR analysis by real-time PCR appears to be a useful addition to morphological and FCM analysis of CSF in the evaluation of CNS infiltration in ALL. It is reliable; relatively sensitive and highly recommended if there are no sufficient cell numbers for FCM analysis in cases at presentation, in relapsed cases or in those with CNS manifestations with negative results for both cytology and FCM.

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Molecular Detection of Intron 22 Inversion of Factor VIII Gene in Egyptian Hemophilia A Patients

NAELA OMRAN, M.D.*; AZZA M. IBRAHIM, M.D.*; SAMIA H. RIZK, M.D.*; HODA ABD EL-GHANY, M.D.*; MONA AZIZ, M.D.*; HEBA HASSAN ABOU-ELEW, M.D.*; RANIA A. ZAYED, M.D.*; OLA M. IBRAHIM, Ph.D.** and NADIA ZAGHLOL, M.D.***

The Departments of Clinical Pathology, Faculty of Medicine, Cairo University, Child Health, National Research Centre** and Pediatric, Faculty of Medicine, Cairo University***.*

ABSTRACT

Hemophilia A (HA) is an X-linked bleeding disorder caused by a wide variety of mutations in the factor VIII (FVIII) gene, leading to absent or deficient factor VIII. We analyzed the FVIII gene of forty Egyptian patients with HA. The analysis included the investigation of intron 22 restriction fragment length polymorphism (RFLP) by PCR using Xbal restriction enzyme, screening of FVIII gene for other molecular abnormalities as deletions and point mutations by multiplex PCR and screening FVIII for a point mutation in exon 25 by using BsrDI restriction enzyme. Our research revealed the following molecular abnormalities in the studied HA patients: FVIII gene RFLP in intron 22 by using Xbal restriction enzyme: 30% Xbal (+), 67.5% Xbal (-ve), 2.5% intron 22 deletion, partial FVIII gene deletion in 10% of cases and exon 25 point mutation in 7.5% of cases. We concluded that the study of intron 22 polymorphism by Xbal restriction enzyme may be used to select the HA patients who have a severe disorder and are negative for the Xbal polymorphic marker, or who have intron 22 deletion to be investigated for FVIII gene intron 22 inversion.

Key Words: *Hemophilia A – Factor VIII gene – Intron 22 inversion.*

INTRODUCTION

Deleterious changes in the human FVIII gene reduce activity and/or circulating plasma levels of factor VIII (FVIII) protein causing (HA). Patients with HA are classified according to their plasma procoagulant levels of FVIII: severe (<0.01 IU/mL), moderate (0.01-0.05 IU/mL), or mild (0.05-0.4 IU/mL) **[1]**. The FVIII gene is located at Xq28 and is extremely large (~ 180 Kb) and structurally complex (26 exons); intron 22 of the gene contains a CpG island, which acts as a bidirectional promoter for two further genes, FVIIIA and FVIIIB. The CpG island and FVIIIA are contained within a stretch of DNA of approximately 9.5 Kb, which is repeated at least twice on the X chromosome, further towards the telomere and extragenic to the factor VIII gene, these homologues are known as int22h-1 (intragenic) and int22h-2 and int22h-3 (extragenic) **[2]**.

FVIII gene contains two types of polymorphism: single nucleotide polymorphisms (SNPs), and length polymorphisms, also known as variable number tandem repeat sequences (VNTRs), both types exist in the normal population **[3]**. One of the single nucleotide polymorphic sites is present within the int22h-1 sequence, it falls in the subcategory of restriction fragment length polymorphisms (RFLPs) and it resides within the recognition sequence for the Xba I restriction endonuclease, thus it is known as the Xba I RFLP **[4]**.

Abnormalities in FVIII gene include deletions, insertions, point mutations and inversion. FVIII gene inversions are rare defects in hemophilia, except for an inversion involving intron 22 of the FVIII gene, which causes severe HA, and which is found in 40-50% of patients with severe disease **[6]**. The intron 22 inversion arises through homologous recombination between int22h-1 and int22h-2 (proximal) or int22h-3 (distal) during meiosis. The distal inversion is more common than the proximal inversion, which may be explained by the greater genetic distance between the factor VIII gene and the distal int22h-3 homologue facilitating the formation of the loop required for alignment to take place **[4]**.

Here, we investigated intron 22 restriction fragment length polymorphism (RFLP) by polymerase chain reaction using XbaI restriction enzyme in hemophilic patients aiming to determine patients who are more likely to have intron 22 inversion, the most common genetic abnormality encountered in patients with severe HA. In addition, screening FVIII gene for other molecular abnormalities as deletions and point mutations by multiplex PCR technique followed by agarose gel electrophoresis was done and also, a point mutation in exon 25 was screened using BsrDI restriction enzyme.

MATERIALS AND METHODS

Patients:

The study included 40 patients attending the out patient clinic of the National Blood Transfusion Centre with clinical and laboratory confirmation of HA with age range from 4 to 19 years and 10 age and sex matched controls after obtaining their informed consent. 14 patients were classified as severe HA, 22 patients as moderate HA and the remaining 4 were mild cases. Moreover, the patients were defined as familial (positive) or isolated (sporadic) according to their available pedigree information.

DNA extraction and analysis:

DNA was extracted from peripheral blood samples using the salting out technique according to Miller et al. **[5]**.

I- Determination of the factor VIII gene restriction fragment length polymorphism in intron 22 using XbaI restriction enzyme:

PCR amplification was performed in a final volume of 50 ul (1 ul DNA, 25 ul Taq PCR Master Mix [Promega cat no.M7502], 1 ul primer; each sense and antisense [Qiagen Operon] and 22 ul distilled water).

The nucleotide sequence of the sense primer is 5`CACGAGCTCTCCATCTGAACATG 3`and the antisense primer is 5`GGGCTGCAGGGGGGGGGGACAACAG 3`.

The PCR reaction was carried out in the DNA thermal cycler (Perkin Elmer 9600) and the computerized thermocycler was programmed for the following conditions: 33 cycles of 1 minute at 94ºC, 1 minute at 58ºC and 1 minute at 72ºC.

17 ul of the amplified product were mixed with 1 ul XbaI restriction enzyme (Fermentas Life Sciences Cat no. number ER0683) with recognition sequence 5`T^CTAGA 3` 3` AGATC^T5` and the mixture was incubated at 37ºC for 16 hours.

The product was analyzed by gel electrophoresis using 4% agarose gel (Promega cat no. V 3121) and ultraviolet light transillumination.

II- Factor VIII gene mutations screening by multiplex PCR technique:

Multiplex PCR allowed simultaneous and rapid amplification of multiple exons of the FVIII gene, followed by detection of the amplified PCR product using agarose gel electrophoresis. The procedure was carried out as mentioned in the previous step.

The sequence of the oligonucleotide primers used to amplify 11 exons of the factor VIII gene is given in Table (1).

III- Assessment of a point mutation in exon 25 using the restriction enzyme BsrDI:

A mutant primer was used to detect nucleotide change from ATG to GTG.

PCR amplification was done using both the mutant primer and the 3`primer of exon 25 in a reaction mixture of 50 ul volume (25 u Taq PCR Master Mix, 1 ul from each of the 3 diluted sense and antisense primers [primer sequence is shown in Table (1)], 1 ul DNA, 21 ul DW).

The PCR cycles proceeded as follows: 33 cycles of 1 minute at 94ºC, 1 minute at 58ºC, and 1 minute at 72ºC and a final extension of 6 minutes at 72ºC.

PCR amplification was followed by digestion of the amplified exon 25 by the BsrDI restriction enzyme (Fermentas Life Science Cat no. ER 1261) which recognizes the sequence 5`GCAATGNN^ 3` 3` CGTTAC^NN 5`.

Then analysis of the product was done by agarose gel electrophoresis and ultraviolet light transillumination.

Table (1): Oligonucleotide sequence of the primers used to amplify factor VIII Gene.

Exon	Sequence	PCR size (bp)
2	5`TGAAGTGTCCACCAAAATGA 3` 5` TACCCAATTTCATAAATAGC 3`	207
3	5` GTACTATCCCCAAGTAACCT 3` 5` TATTCATAGAATGACAGGAC 3`	208
4	5`ACAGTGGATATAGAAAGGAC 3` 5` TGCTTATTTCATCTCAATCC 3`	295
5	5` CTCCTAGTGACAATTTCCTA 3` 5` AGCAGAGGATTTCTTTCAGG 3`	187
6	5` CATGAGACACCATGCTTAGC 3` 5` CTGGTGCTGAATTTGGAAGA 3`	220
7	5` TCAGATTCTCTACTTCATAG 3` 5` GAAACTGTGCAAGGTCCATC 3`	225
12	5` CTAGCTCCTACCTGACAACA 3` 5` GACATCACTTTGATTACATC 3`	283
22	5` TCAGGAGGTAGCACATACAT 3` 5` GTCCAATATCTGAAATCTGC 3`	288
23	5` CTCTGTATTCACTTTCCATG 3` 5` GATATTGGATGACTTGGCAC3`	214
24	5` GCTCAGTATAACTGAGGCTG 3` 5` CTCTGAGTCAGTTAAACAGT 3`	249
25	5` GAATTTCTGGGAGTAAATGG 3` 5` GCTTACCTTTACTTTGCCAT 3` 5`GGATTCCTGCAAGTGGACTTC CAGAAGGCA3`	322

RESULTS

Analysis of the clinical data showed that 77.5% (31) of the patients had one or more relatives affected on the maternal side of the family so their mothers could be obligate carriers; this is of importance for genetic counseling for the future risk of giving birth to a hemophiliac son as obligate carriers may transmit the hemophiliac gene to 50% of their children, males or females. On the other hand, 22.5% (9) of the patients had negative family history for the disease, these are considered as isolated cases which could be explained by the fact that isolated cases of hemophilia may result from transmission of the hemophilia gene through asymptomatic females in whom the gene has remained undetected; from a new mutation in the mother, resulting in her being a carrier; or a new mutation in the hemophiliac (true de novo mutation). The existence of somatic mosaicism has also to be taken in consideration.

Study of the intron 22 RFLP by the XbaI restriction enzyme showed that 27 out of 40 patients (67.5%) were negative for the XbaI polymorphic marker; (11 patients had marked severity, 14 cases had moderate severity and 2 cases had mild severity), while 12 patients (30%) were positive for this marker; (3 cases had marked severity, 7 cases had moderate severity and 2 cases had mild severity) and one patient (2.5%) had absent DNA polymorphic fragment indicating deleted intron 22. No significant relation was found between the XbaI polymorphic status and none of the clinical or laboratory data (p value >0.05) Fig. (1).

FVIII gene screening revealed that 10% (4/40) of hemophilia A cases had partial gene deletions. The deletion represented 28.5% (4/14) of severe hemophilia A patients and were found in exon 24, exons 23-24, exons 12-22 and a fourth deletion in one or more of the exons 2, 3, 6 or 7, however the identity of this deletion could not be determined precisely as the 4 amplified PCR fragments had the same electrophoretic migration site on the agarose gel (4%) used to analyze the PCR products. A significant relation was found between the presence of FVIII gene deletion and the severity of the disease where all patients had a severe disorder (p value ≤0.01) Figs. (2,3).

Assessment of the point mutation affecting exon 25, at codon 2238 where the amino acid methionine is replaced by valine using PCR technique where the BsrD I restriction enzyme and a mutated primer creating an artificial site for the enzyme were used, fragments of 140 bp and 29 bp were obtained for the normal gene, when A→G mutation was present, the sequence was not recognized by the enzyme and only a fragment of 169 bp was obtained (Fig. 4). Our research revealed that 3 patients (with a diagnosis of moderate hemophilia) out of 40 patients (7.5%) were found to have this point mutation, however, no significant association was found between this point mutation and the clinical or laboratory data of these patients (p value >0.05), nor with FVIII gene deletion (p value =0.86) or XbaI polymorphic status (p value =0.69).

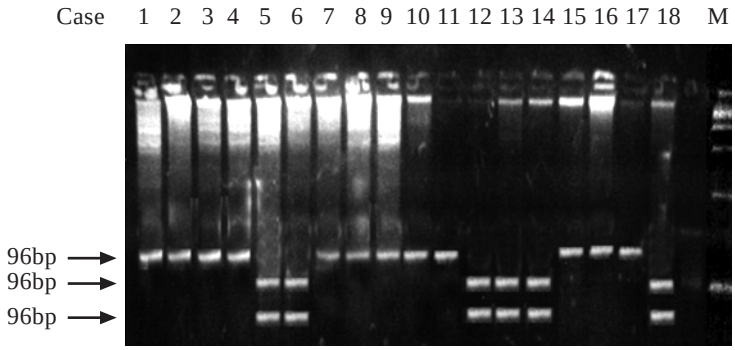

Fig. (1): Characterization of the polymorphism in intron 22 of the factor VIII gene using xbai restriction enzyme.

Cases 5, 6, 12, 13, 14 and 18 are XbaI (+) where 2 DNA fragments of 68 and 28bp were obtained, while the XbaI (-) ve cases showed single DNA fragment of 96bp.
M: PCR marker (50-100-150-200-250-300-350-400-450-500-550-600-650-700-750-800 bp).

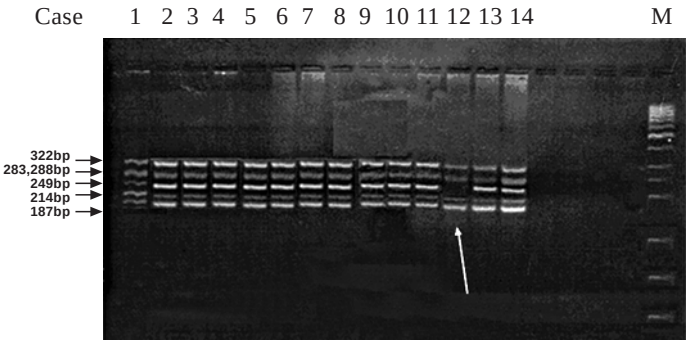

Fig. (2): Agarose gel electrophoresis 4% analysis of multiplex PCR products resulting from amplification of the part of factor VIII gene using primers for exons 5, 12, 22, 23, 24 and 25.

The fragments corresponding to exons 5, 12, 22, 23, 24 and 25 are identified on the left side.
Case 12 shows absence of DNA fragment corresponding to exon 24.
M: PCR marker (50-100-150-200-250-300-350-400-450-500-550-600-650-700-750-800 bp).

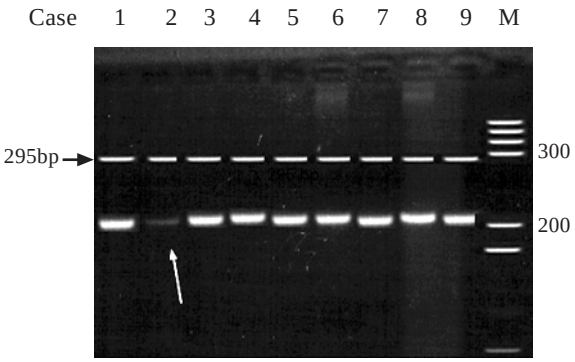

Fig. (3): Agarose Gel Electrophoresis 4% analysis of multiplex PCR products resulting from amplification of the part of factor VIII gene using primers for exons 2, 3, 4, 6 and 7.

The fragment corresponding to exon 4 is present in all cases while the other fragment represents exons 2, 3, 6 and 7 where the electrophoretic migration site was the same (207, 208, 220, 225 bp respectively).
Case 2 shows a faint DNA fragment, denoting the deletion of one or more of either exons 2, 3, 6, or 7.
M: PCR marker (50-100-150-200-250-300-350-400-450-500-550-600-650-700-750-800 bp).

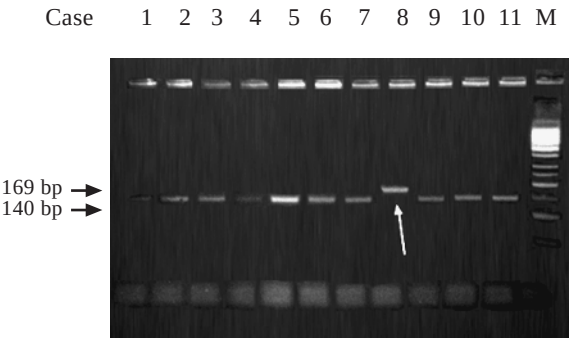

Fig. (4): Characterization of the mutation at exon 25, codon 2238.

After digestion with BsrDI, fragments of 140 and 29 bp were obtained for the normal nucleotide sequence. When the nucleotide substitution A_G is present, digestion with BsrDI results in a fragment of 169 bp as seen in case 8.

M: PCR marker (50-100-150-200-250-300-350-400-450-500-550-600-650-700-750-800bp).

DISCUSSION

Hemophilia A is an X-linked recessively inherited bleeding disorder characterized by deficiency of procoagulant factor VIII with an incidence of 1/5 000-10.000 male births. Except for the common inversion mutation in intron 22 of FVIII gene, most other changes are point mutations and deletions **[6]**.

The inversion of intron 22 was found in 40-50% of patients with severe HA **[7]** with a prevalence of about 47.5 to 53% in India population **[8]** and 40-50% in European **[9]**.

Direct mutation detection is essential for carrier detection and prenatal diagnosis in the family members of Egyptian hemophilic patients and this if achieved will decrease the incidence of the disease. In addition, it will avoid the hazards complicating therapy, including disease transmission and the development of inhibitors to FVIII substitutes.

In order to find mutations in a gene as large as FVIII gene, a simple method has to be applied. The use of PCR to search for RFLP of intron 22 is known to be useful to select cases likely to have intron 22 inversions. This was followed by screening for small mutations and deletions in FVIII gene using multiplex PCR followed by agarose gel electrophoresis and assessment of a point mutation in exon 25 using the restriction enzyme BsrDI.

The intron 22 inversion arises through homologous recombination between int22h-1 and int22h-2 (proximal) or int22h-3 (distal) during meiosis **[7,10]**. Thus, in haemophilia A families in which the intron 22 inversion is the causative gene defect, the defect can often be shown to have originated in an unaffected male relative, and in sporadic cases, this is often the patient's grandfather on his mother's side of the family **[11]**. This explains the presence of 22.5% of the cases included in our study with no family history for hemophilia A.

The work of several authors agreed with our findings regarding the study of the intron 22 RFLP by the XbaI restriction enzyme; El Maari et al. **[12]** studied intron 22 by RFLP, they showed that 52% of cases were negative for XbaI polymorphic marker FVIII gene haplotype study, they studied intron 22 inversion by Southern blotting technique and the study revealed that 29% of cases had intron 22 inversion. At the same time the haplotype of factor VIII gene revealed that the prevalence of one haplotype in which XbaI is negative was higher in the inversion patients. Also, when intron 22 polymorphism was studied **[13]**, int22h-1 gene deletion was reported and was associated with factor VIIII gene inversion. The author concluded that deletions involving intron 22 of the factor VIIII gene are associated with increased incidence of gene inversion and are associated with severe hemophilia A. These results had led us to speculate that the study of intron 22 polymorphism by XbaI restriction enzyme may be used not only to track the abnormal gene during family study, but also to select hemophilia A patients who have a severe disorder and negative for XbaI polymorphic marker, or who have intron 22 deletion to be investigated for intron 22 inversion.

On the other hand, a similar study was performed **[14]** and the results disagree with this hypothesis as they found no association between the presence of the intron 22 inversion and RFLP haplotype.

Multiplex PCR screening for FVIII gene revealed that 10% of the studied cases had partial gene deletion and this was associated with severe disease as FVIII gene deletions have a high probability of destroying the genetic function, removing part of FVIII protein, or introducing a frameshift, all of which are associated with a severe disease **[6]**. Exon 24 deletion was also reported **[15]** and was associated with a severe disease. The worldwide database for hemophilia A in 1995 also reported deletions affecting exon 24; 11 to 22, 23-24 all were associated with severe disease. This was also consistent with the results of a previous study where three partial deletions of factor VIII gene were characterized: a deletion of at least 7 Kb eliminates exons 24 and 25 and a deletion of 16 Kb deletes exons 23-25 and a 5.5 Kb deletion eliminates exon 22. In all these deletions non sense codons were generated resulting in a severe disease **[16]**.

The point mutation detected in exon 25, codon 2238 in three of our patients was also reported in a former study **[17]** that stated that this point mutation is responsible for the disease.

The results of FVIII gene mutation screening by multiplex PCR and the screening for exon 25 point mutation suggest that the spectrum of gene defects in Egyptian hemophiliacs is as heterogeneous as reported in other populations. In addition multiplex PCR followed by agarose gel electrophoresis offers a simple and sensitive way for mutation screening which is essential for carrier detection and prenatal diagnosis of this fatal disorder.

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Screening for Beta Thalassemia in Damietta

AMAL EL-BESHLAWY, M.D.*; MONA EL-GHAMRAWY, M.D.*; AZZA MOUSTAFA, M.D.**; NADIA MOSALEM, M.D.** and FATMA EL-RIES, M.Sc.***

The Departments of Pediatrics, New Children Hospital, Cairo University, Clinical Pathology, Cairo University** and Pediatrics, Damietta General Hospital.****

ABSTRACT

Introduction: Beta thalassemia is not only an important public health problem in Egypt, but also a socioeconomic one. A carrier rate of 9-10.2% was estimated in different geographical areas of Egypt.

Objective: To detect ß-thalassemia carriers among a random sample of population in Damietta Governorate, to help in the prevention of further births of ß-thalassemia patients. Increasing awareness about the disease was our second objective.

Patients and Methods: One thousand and four random subjects in Damietta were included in this study. Their mean age was 18.6±13.4 years. All studied population was subjected to a complete blood picture and high performance liquid chromatography (HPLC), using Variant 11 Rad, was done for those with evidence of microcytosis (MCV<80fl).

Results: In our study, 633 subjects (63.05%) had microcytosis, for whom HPLC was performed. Among our studied population (n=1004), 46 subjects (4.58%) had HbF>1%, 26 subjects (2.59%) had HbA2 >3.5% and 6 subjects (0.6%) had both increased HbF and HbA2.

Conclusion: The carrier rate of ß-thalassemia in Damietta was found to be 7.77%. This highlights the need for immediate implementation of a national preventive program all over Egypt. Increasing population awareness and premarital screening remain the best ways to reduce the disease incidence.

Key Words: *Beta-thalassemia – Carrier – HPLC.*

INTRODUCTION

Thalassemias are common autosomal recessive disorders especially in populations of Mediterranean, Middle Eastern and Far Eastern descent **[1]**. Beta thalassemia has an estimated frequency of 3-10% in certain regions **[2]**. In Egypt, it is the most common chronic hemolytic anemia (85.1%). The Hematology Clinic Study Group at New Children Hospital of Cairo University reported a carrier rate varying between 6 and 10%. Moreover, 1000 children affected with thalassemia are expected out of 1.5 million live births per year in Egypt **[3]**.

The determination of prevalence of beta thalassemia in endemic areas is important in order to develop programs for their control and management **[4]**.

Several tests have been proposed for detection of beta thalassemia variants including accurate measurement of mean corpuscular volume (MCV), mean cell hemoglobin (MCH), osmotic fragility, estimation of HbA2, HbF and identification of Hb variants **[5,6]**.

Therefore, the aim of this study was to detect ß-thalassemia carriers in a random sample of population in Damietta Governorate for prevention of further births of ß-thalassemia patients with its physical, social and financial burden. Increasing awareness about the disease was our second objective.

PATIENTS AND METHODS

Patients:

One thousand and four subjects from Damietta Governorate were randomly selected to be recruited in this study in a two months' interval. All subjects were above one year of age and were apparently healthy. Their age ranged between 1.1 and 76.0 years with a mean of 18.6± 13.4 years.

Laboratory investigations were done for all our subjects. These included a complete blood picture (by Advia 120 automated cell counter). Red cell indices included hemoglobin concen-

tration (gm/dl), mean corpuscular volume (MCV) (fl), mean cell hemoglobin (MCH) (pg), mean cell hemoglobin concentration (MCHC) (g/dl) and red cell distribution width (RDW) (%). High performance liquid chromatography (HPLC), using Variant 11 Rad, was done for those with evidence of microcytosis (MCV <80fl). Our cutoff points for diagnosis of ß-thalassemia carriers were the presence of microcytosis (MCV <80 fl) associated with elevated levels of HbA2 (>3.5%) and/or elevated levels of HbF (>1%).

Sample preparation:

Whole blood specimens were collected in a vacuum collection tube containing EDTA. The 16mm sample tubes were loaded into the Variant 11 sample racks in random order and placed on the VARIANT11 Sampling Station conveyer belt. Special rack inserts were used for 13mm tubes and special adapters for 10 mm pediatric tubes.

Procedure:

The VARIANT 11 ß-thalassemia Short Program is intended for separation and area percent determinations of HbA2 and HbF as an aid in the identification of abnormal hemoglobins in whole blood, using ion-exchange HPLC. The VARIANT 11 Clinical Data Management (CDM) software performed reduction of raw data collected from each analysis. One-level calibration was used for adjustment of the calculated HbA2/F values. To aid in the interpretation of results, windows (e.g. ranges) had been established for the most frequently occurring hemoglobins based on their characteristic retention times.

Statistical methods:

All numerical data were expressed in the form of mean and standard deviation.

RESULTS

Statistical analysis of data of all our studied subjects is shown in Table (1). Fig. (1) shows the scatter distribution of MCV and HbA2 among our subjects. Applying our cutoff value for microcytosis to our studied population, 633 subjects (63.05%) had microcytosis, for whom HPLC was performed with their data shown in Table (2).

Among our studied population, 78 subjects (7.77%) were defined as beta thalassemia carriers by fulfilling our diagnostic cutoff values of microcytosis associated with elevated HbA2 and/or elevated HbF levels. The statistical data of this carrier group is shown in Table (3), forty six subjects (4.58%) had HbF >1%, 26 subjects (2.59%) had HbA2 >3.5% and 6 subjects (0.6%) had both increased HbF and HbA2 (Fig. 2).

Among our studied population, 195 subjects (19.42%) had borderline HbA2 levels (≥3 and ≤3.5%).

Table (1): Descriptive statistical data of studied subjects (n=1004).

Variables	Min	Max	Mean	SD
Age (years)	1.1	76.0	18.6	13.4
Hb (g/dl)	7.26	17.80	12.74	1.49
MCV (fl)	50.60	93.70	76.81	6.90
MCH (pg)	14.60	37.90	26.44	2.68
MCHC (g/dl)	26.40	38.10	34.37	1.40
RDW (%)	12.00	29.60	15.05	1.57

Table (2): Descriptive statistical data of subjects with MCV <80 (n=633).

Variables	Min	Max	Mean	SD
Age (years)	1.1	65.0	15.1	12.3
Hb (g/dl)	7.26	16.40	12.29	1.33
MCV (fl)	50.60	79.90	72.92	5.56
MCH (pg)	14.60	29.70	25.10	2.38
MCHC (g/dl)	26.40	38.10	34.37	1.53
RDW (%)	12.90	29.60	15.46	1.71
HbF (%)	0.00	7.60	0.46	0.73
HbA2 (%)	0.40	5.50	2.80	0.55

Table (3): Descriptive statistical data of carriers according to cut off point MCV <80 and HbF>1 and/or HbA2 >3.5 (carrier rate) (n=78).

Variables	Min	Max	Mean	SD
Age (years)	1.1	45.0	9.4	10.8
Hb (g/dl)	9.30	15.90	12.06	1.09
MCV (fl)	54.70	79.90	70.85	6.45
MCH (pg)	18.40	28.50	24.43	2.61
MCHC (g/dl)	29.50	37.40	34.45	1.46
RDW (%)	13.30	21.10	15.89	1.74
HbF (%)	0.00	7.60	1.58	1.50
HbA2 (%)	0.60	5.50	3.28	0.92

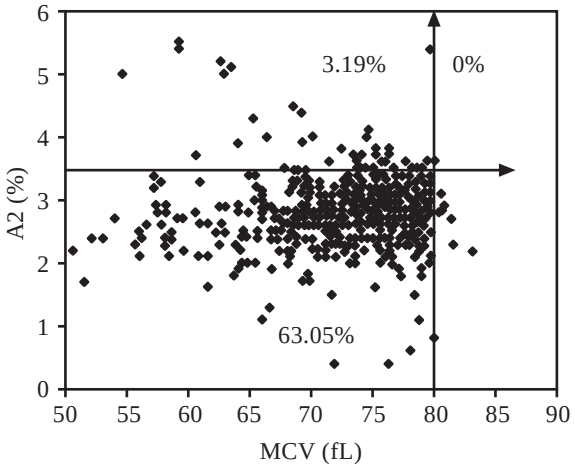

Fig. (1): Scatter distribution of MCV and A2 of 1004 subjects included in the study.

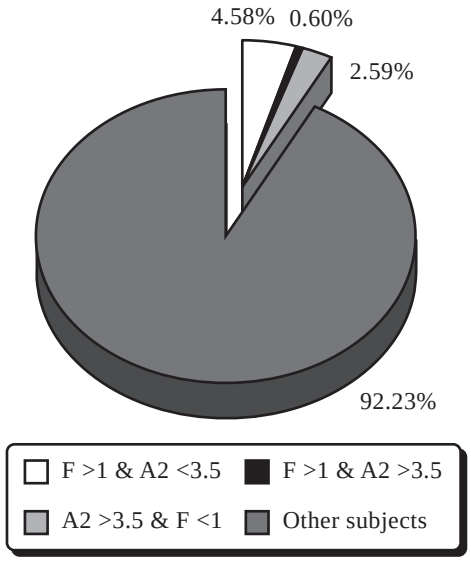

Fig. (2): Distribution of carriers among studied patients.

DISCUSSION

Beta thalassemia is not only an important public health problem but also a socioeconomic one. There are 270 million people who are carriers of globin gene mutations worldwide. Up to a half million infants are born annually with severe hemoglobinopathies **[7]**. The approach to deal with thalassemic problem is to prevent and control births of new cases, which requires an accurate identification of carriers **[8]**. Screening for beta thalassemia is extremely difficult, mainly because of the heterogeneity of beta thalassemias and the absence of a single pathognomonic finding to cover all beta thalassemia variants **[9]**.

In our study, the carrier rate found in Damietta (7.77%) was close to that reported by Tongsong et al. in 2000 (7.97%) **[10]**. On the other hand, a carrier rate of ≥9% was reported from different geographical areas of Egypt **[11]**, whereas a prevalence of beta thalassemia trait of 2.9% was reported in Bahrain **[12]** and of 1.84% in Gaziantep in Turkey **[13]**. This shows the regional difference in carrier rate of beta thalassemia as well as the need for standardized as well as accurate diagnostic tools.

Although, elevated HbA2 is a characteristic feature for beta thalassemia trait **[14,15]**, some individuals with beta thalassemia trait have normal indices **[16]** and HbA2 levels may be normal in the rare 'silent' beta thalassemia trait, so that an increased HbA2 can not be used as the sole discriminant for beta thalassemia trait **[17]**. In our study, 32 subjects (3.19%) showed both microcytosis and elevated HbA2 (HbA2 >3.5%) with a mean HbA2 value of 4.15% (ranging from 3.6 to 5.5%). This is slightly lower than that reported by other authors **[18]**. However, iron deficiency with its known reducing effect on HbA2 level may account for this difference. It was suggested that iron deficiency must be corrected before making any diagnostic or therapeutic decisions based on HbA2 **[19]**. Among our studied population, 195 subjects (19.42%) had HbA2 levels ≥3 and ≤3.5% which needs further investigations.

On the other hand, 52 subjects (5.18%) of our population were diagnosed as carriers by having microcytosis as well as elevated HbF (>1%), six of them had elevated HbA2 as well. This is in agreement with results of El-Beshlawy et al. (1992) **[18]** who reported elevated HbF in 17.5% of their carriers ranging from 2-7.4%. In another study, raised HbF was shown in 32 cases out of 69 cases of beta thalassemia heterozygote but no case showed raised HbF without the raise of HbA2 **[10]**.

The MCV is a key diagnostic indicator especially with all automated hematology analyzers now providing a measure of MCV that is both precise and accurate. Thalassemia carriers do not have significant anemia, but invariably have microcytosis (MCV < 80 fl) and hypochromia (MCH < 27 pg) **[20]**. It was reported that microcytosis was a consistent feature in all heterozygous beta thalassemia in several studies **[10,18]**. This was shown in our study, where the

mean MCV of beta thalassemia carriers was 70.85 fl±6.45 (ranging from 54.7 to 79.9 fl), in spite of their mean Hb level being 12.06g/dl± 1.09 (with a range of 9.3-15.9 g/dl). Among our subjects, 633 (63.05%) had microcytosis which highlights the importance of differentiating between different causes of microcytosis, especially iron deficiency being common in our community.

A reduced MCH <27pg was found among beta thalassemia carriers in previous studies [18,21,22]. This was also shown in our study where the mean MCH among carriers was 24.43 pg±2.61 (range 18.4 to 28.5 pg). However, the MCH can not be used as a discriminant factor due to the common association of iron deficiency which may significantly affect the MCH **[23]**.

Traditionally, electrophoresis has been the method of choice for identification and quantification of Hb variants. However, it is slow, labor-intensive and inaccurate in identification of low-concentration Hb variants (e.g. HbA2) or in the detection of fast Hb variants (HbH, Hb Barts) **[24]**. In our study, high performance liquid chromatography (HPLC) was used for quantification of HbA2 and HbF as it has relatively high sensitivity or specificity **[25]**. The simplicity of sample preparation, superior resolution of the method and accurate quantitation of Hb concentrations, combined with complete automation, make this an ideal methodology for routine diagnosis of Hb disorders in a clinical laboratory **[25]**.

The reliability of HbA2 measurement by HPLC without any false positive or false negative results is of great advantage **[26]**. However, HbA2 and HbF concentrations obtained should be interpreted together with other variables such as erythrocyte indices, iron studies or family studies in some individuals.

In conclusion, Beta thalassemia remains an important health problem in Egypt, including Damietta Governorate, with a high carrier rate which increases the possibility of a high birth rate of thalassemic patients. This necessitates the immediate implementation of a national preventive program all over Egypt. Increasing population awareness and premarital screening remain the best ways to reduce the disease incidence with potentially significant financial saving and social and health benefits.

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Molecular Cytogenetics of TEL/AML1 Fusion and other Abnormalities Involving TEL and AML1 Genes in Pediatric Acute Lymphoblastic Leukemia

SHERINE I. SALEM, M.D.* and HANY ABD EL-RAHMAN, M.D.**

The Department of Clinical Pathology and Pediatric Oncology**, National Cance Institute, Cairo University.*

ABSTRACT

Background: The TEL/AML1 fusion is the most common genetic abnormality found in childhood acute lymphoblastic leukemias (ALL). This cryptic translocation is not detected by conventional cytogenetic techniques but it can be readily detected using fluorescence in situ hybridization (FISH). We carried out cytogenetic and FISH studies on 50 children with ALL in order to measure the frequency of this translocation in the Egyptian population in comparison to other genetic subgroups, the incidence of TEL and/or AML1 gene alterations and their correlation with clinical evolution and prognosis.

Design and Methods: Bone marrow samples were obtained from 50 pediatric ALL patients who Karyotyping and FISH using probes for TEL/AML1, BCR/ABL and MLL were performed. The signal pattern of AML1 and TEL genes were analyzed using fluorescent in situ hybridization with a dual color DNA probe specific for the AML1 and TEL genes. Patients were treated according to the National Cancer Institute protocols and followed up for a period of 18 to 42 months (median 19 months).

Results: In the current study, successful karyotyping was obtained in 43/50 (86%); when adding FISH 46/50 (92%) cases showed informative results. Using FISH, TEL/AML1 fusion was detected in 7/50 cases (14%) while cytogenetics could not reveal the translocation (12;21). Normal karyotype was found in 13/50 (26%) when using conventional cytogenetics (CC) only, while when combining both FISH and CC two cases revealed TEL/AML1 gene fusion. Other chromosomal abnormalities that were frequently encountered in TEL/AML1 positive cases were either, deletion of chromosome 12p arm in 2 cases (4%), and polysomies of chromosome 21 in 2 cases (4%). These abnormalities correspond to lack of TEL signal and extra AML1 signals respectively as detected by FISH.

Also CC failed in 7 cases (14%), while when FISH was performed MLL gene translocation was detected in one case and TEL/AML1 gene fusion in 2 cases. Hyperdiploidy was found in 11 cases (22%), t(1;19) in 4 cases (8%), BCR/ABL, MLL gene translocation, t(8;14) in 3 cases (6%) each, hypodiploidy in 2 cases (4%) and AML1 gene amplification in one case (2%).

Best overall survival correlated with TEL/AML1 positive cases and high hyperdiploid cases with mean overall survival (OS) being 28.3 and 26.3 months respectively. Worst overall survival was associated with all other chromosomal abnormalities having mean OS of 14.6 months (p=0.0134). Cases with normal karyotype had a mean OS of 22.2 months, while cases with abnormalities of TEL or AML1 genes other than TEL/AML1 fusion had a mean OS of 21.8 months.

Conclusion: TEL/AML1 fusion is found in 14% in our series and had a favorable outcome. Other TEL and AML1 genes abnormalities are detected in 14/50 cases (28%) and associated with intermediate OS.

Key Words: *ALL – TEL/AML1 – AML1 amplification – TEL deletion – Fluorescence in situ hybridization.*

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common malignancy in childhood, accounting for approximately 20% of all childhood malignancies in the National Cancer Institute. It is well established that the identification of cytogenetic abnormalities is very useful for the risk stratification of childhood ALL. For instance, t(9;22), 11q23/MLL gene abnormalities, and hypodiploidy are known to confer a poor prognosis and t(12;21), hyperdiploidy are associated with a favorable outcome **[1]**.

The translocation (12;21) (p13;q22) which results in TEL/AML1 (ETV6/RUNX1) fusion, is a common genetic abnormality in acute lymphoblastic leukemia (ALL) **[2-5]**. It is detected in about 20 to 25% of cases **[6,7]**. Although it is difficult to identify by conventional cytogenetic techniques, TEL/AML1 fusion can be readily detected using fluorescence in situ hybridization (FISH) **[2,3]**. This translocation is

associated with an early onset of the disease, a Pre B-lineage immunophenotype and a favorable prognosis **[1,8]**. However, questions about the relationship between t(12;21) and favorable prognosis have arisen because several patients with TEL/AML1 fusion showed poorer clinical outcome if other gene rearrangements coexist **[9,10]**.

Additional cytogenetic abnormalities, associated with the t(12;21), are frequently found. The most common structural and numerical aberrations are 12p rearrangements and trisomy 21, respectively **[11-13]**. A double fusion signal, detected by FISH, is another common finding in patients with t(12;21) **[3,6]**. Deletions involving the untranslocated TEL allele, detected by cytogenetic and/or FISH techniques, are also often found in patients with TEL/AML1 fusion (3,15). Although deletions on the short arm of chromosome 12 are recurrent alterations found in a wide range of hematologic neoplasias, the leukemogenic role of the genes located there, TEL and p27, is unknown **[16]**.

Other studies reported intrachromosomal duplication of chromosome 21 and amplification of the RUNX1 (AML1) gene (iAMP21) as a recurrent chromosomal abnormality with an incidence of 1.5% in childhood B-lineage acute lymphoblastic leukemia (ALL) **[17]**. These patients have a median age of 9 years, a low presenting white blood cell count and a poor prognosis **[18]**. Thus, on the current U.K ALL treatment protocol, ALL 2003, these children are classified as high-risk and receive more intensive treatment **[19]**.

The RUNX1, formerly known as AML1 gene is also involved in many chromosomal aberrations associated with hematologic disorders. More than 40 different patterns of translocations or rearrangements involving the 21q22 region have been described **[20]** and amplification of the 21q22 region in pediatric patients with ALL has also been detected by comparative genomic hybridization (CGH) **[21]**. Thus, the AML1 gene could have an important role in the 21q22 amplification in childhood ALL **[21-22]**.

Twin studies on ALL patients **[23,24]** and retrospective analysis using neonatal blood spots (Guthrie cards) **[25-27]** have provided evidence that TEL/AML1 often arises prenatally, possibly as a first or initiating event. Consequently, "preleukemic" clone with TEL/AML1 can persist postnatally for extended periods, up to 14 years **[23]** and that at least one other postnatal genetic event is required for overt leukemia. Deletion of the nontranslocated TEL allele is the most common secondary abnormality found in newly diagnosed TEL/AML1-positive cases of ALL **[6,14,28]**. Studies on both singletons **[6,28]** and twins **[24]** with ALL indicate that such TEL deletions are subclonal or secondary to TEL/AML1 and almost certainly postnatal. Candidate preleukemic clones in normal cord blood with TEL/AML1 fusions retain the normal TEL allele **[29]**. Whether other genetic changes in addition to TEL deletion, e.g., kinase mutations **[30]**, are necessary for the clinical development of ALL remains to be established.

In the present study, we performed karyotyping and FISH with probes for BCR/ABL, MLL and TEL/AML1 rearrangements, for 50 childhood ALL cases. The aim of this study was to estimate the frequency of TEL/AML1 rearrangement among other genetic subgroups involving the above genes and to study the impact of any additional abnormalities and correlate with the clinical outcome.

MATERIAL AND METHODS

Patients:

Bone marrow samples were obtained from 50 (29 males and 21 females) pediatric newly diagnosed with B-lineage acute lymphoblastic leukemia between 2004 and 2006. The leukemia immunophenotype was determined by standard flowcytometric analysis using a panel of monoclonal antibodies. All patients were classified according to the WHO classification criteria.

Treatment protocol:

All Patients received the standard pediatric ALL chemotherapy protocol applied at the NCI, Cairo University. The protocol is composed of three phases, omitting the use of radiation therapy for CNS leukemia prophylaxis. The first induction phase is composed of the administration of the basic 4 drugs; Vincristine (VCR): IV, 1.5mg/m^2 and Daunorubicin: IV, 25mg/m^2 given on days 1,8,15. Prednisolone: PO, 40mg/m^2 started on day 1-28 then taper over 10 days. L-asparagenase: IM, 6000 u/m^2 alternating days, 3 times a week, for 9 doses, Triple intrathaecal: Methotrexate, Cytarabine and Hydrocortisone, given on days 1, 43. Etopside (VP16) and cyt-

arabin (Ara-C): 300mg/m^2 IV, each were given on days 22, 25, 29. Bone marrow examination for re-evaluation was done on day 43 to determine remission status. patients who achieved complete remission were promoted to the second phase of therapy (consolidation) and were offered high dose Methotrexate (HD-MTX) IV, 500mg/m^2 over 1 hour followed by 1500mg/m^2 over 23 hours given on days 44 and 51. The third continuation phase is based on using different drug combinations given on weekly bases for a total of 120 weeks. VP16 + Cytoxan each 300 mg/m^2 IV gave weekly starting from W1 till W61. Mercaptopurine (6MP) 75 mg/m^2 PO, for 7 days + Cytoxan 300 mg/m^2 IV 4 weekly from week 65 till week 117. 6MP IM 8 weekly starting from week 2 till week 118. MTX: 40 mg/m^2, IM + Ara-C: 300 mg/m^2 IV, 8 weekly start from week 3 till week 115. VCR IV, 1.5mg/m^2 + L-asparagenase:IM, 10000 u/m^2 once + Prednisolone: PO, 40mg/m^2 for 7 days given 4 weekly basis starting from week 4 till week 36, the coming weeks only VCR + Prednisolone were given on weeks 40, till week 120 on 4 weekly basis. HD-MTX: IV, 500mg/m^2 over 1 hour followed by 1500 mg/m^2 over 23 hours + 6MP: 75 mg/m^2 PO for 7 days on weeks 6, 14, 21, 22, 30, 38, 46, 54. VP16 + Ara-C each 300 mg/m^2 IV on weeks 7, 15, 23, 31, 39, 47, 55. 6MP: 75 mg/m^2 PO for 7 days + Ara-C: 300 mg/m^2 IV on weeks, 63, 71, 79, 87, 95, 103, 111, 119.

Follow-up:

By the end of the 120 weeks of continuation therapy, complete re-evaluation was performed by bone marrow analysis and CSF examination, then patients were put under follow-up once monthly by clinical examination + CBC. Complete remission is defined as the disappearance of organomegaly, normalization of hematological indices and bone marrow normocellularity with <5% lymhoblasts.

Patients were followed up for a period of 18 to 42 months (median 19 months).

Conventional cytogenetics:

Bone marrow or blood samples preserved on sodium heparin were cultured for each subject as previously described **[31]**. Chromosomes were incubated at 37ºC in culture media for 24 to 48 hours. Colcemid was added to arrest cells at mitosis, followed by addition of a hypotonic solution (KCl). Cells were then fixed with Carnoy Fixative, dropped onto clean slides, trypsin banded and karyotyped using an image analysis system (Vysis Quips XL Genetic work station) according to the international system of hemocytogenetic nomenclature **[32]**. Fifty patients with B-lineage ALL who were treated between 2004 and 2006 according to the NCI protocol were included in the study.

FISH:

FISH was performed using a selected panel of commercial probes for LSI TEL/AML1 ES Dual Color Translocation Probe (Vysis Inc., Downers Grove, IL, U.S.A), LSI BCR/ABL Dual Fusion Translocation Probe (Vysis) and MLL Dual Color Break Apart Rearrangement Probe (Vysis). FISH was performed according to the manufacturer's instructions. The image was analyzed using Vysis Quips XL Genetic work station. Metaphases and at least 200 interphase nuclei were analyzed for each case. Nuclei with ambiguous signals were excluded from analysis.

Statistical analysis:

Statistical Package for social sciences (SPSS) version 9 was used. Quantitative variables were summarized using mean, median, minimum and maximum values. Qualitative data were summarized using frequencies and percentage. Chi or Fisher's exact tests were used whenever appropriate to test the association between the different qualitative variables. Differences were considered significant at a p value of ≤0.05 and highly significant at a p value of ≤0.01.

RESULTS

The clinical and biological characteristics of the 50 patients are summarized in Table (1). The median age at diagnosis was 6 years (range 2 months -18 years) including 4 infants (<1 year). The immunophenotyping was precursor B ALL in all cases. Patients were subjected to conventional cytogenetics and FISH using probes to detect TEL/AML1, BCR/ABL and MLL rearrangements. Successful kayotyping was obtained in 43/50 (86%), while when using FISH 46/50 (92%) of cases showed informative results. Normal karyotyping was obtained in 13/50 (26%) of cases by conventional cytoge-

netics (CC), while in 11/50 (22%) when combining both CC and FISH. Chromosomal abnormalities were detected in 30/50 (60%) by CC while when combining both CC and FISH, 35/50 (70%) abnormalities were detected. High hyperdiploidy was detected in 11/50 (22%) of cases, TEL/AML1 in 7/50 (14%) and t(1;19) in 4/50 (8%) and t(8;14), BCR/ABL, MLL gene rearrangement in 3/50 (6%) each. BCR/ABL was detected as a part of complex karyotype (case 1), as a sole abnormality (case 4) and as double Philadelphia chromosome in a hyperdiploid karyotype (case 6) (Figs. 3,4). The MLL gene rearrangement was in the form of t(4;11) in one case (case 2) (Fig. 5), t(10;11) as a part of complex karyotype (case 28), while the third case had no mitotic figures and only FISH could detect the MLL translocation (case 29). Hypodiploidy (less than 45 chromosomes) was detected in 2 cases. One of the hyperdiploid cases had BCR/ABL gene fusion as well. Two cases having normal karyotype, showed TEL/AML1 when FISH was performed. FISH also revealed MLL gene translocation in one case and TEL/AML1 gene fusion in 2 cases with failed karyotype (Tables 2, 3).

In the TEL/AML1+ patients, 2 cases showed normal karyotype, in 2 cases karyotyping was not successful and 3 cases presented with abnormal karyotype (Fig. 1). Secondary chromosomal abnormalities were detected in 4/7 (57%). These abnormalities were +del 21 (extra fusion signal) in one cases (Fig. 2), +21 (extra AML1 signal) and del12p (deletion of untranslocated allele of TEL) in two case.

In TEL/AML1- patients, 14 cases showed either loss or gain of TEL and/or AML1 genes. Three cases showed TEL gene anomalies; case number 2 had TEL deletion together with MLL gene translocation, case number 38 showed monosomy 12 and hence one copy of TEL gene with a hypodiploid karyotype and case number 23 had trisomy 12 and thus 3 copies of TEL gene. Gain of the AML1 gene was detected in 10 cases; in cases 21 and 23 tetrasomy of chromosome 21 leading to 4 copies of AML1 gene. In case 32 there was amplification of AML1 gene and in cases (3, 5, 6, 11, 15, 17, 20, 25) trisomy of chromosome 21 and hence 3 copies of AML1 gene were detected as a part of a hyperdiploid clone with or without structural abnormalities. AML1 amplification was detected in a 6 year old boy with TLC $5.9x10^9$/L who did not achieve complete remission and died one month after start of therapy.

Ten cases showed numerical chromosomal abnormalities; one of them had TEL/AML1 gene fusion as revealed by FISH. Eleven cases showed structural chromosomal abnormalities, while 9 cases had both numerical and structural abnormalities. FISH revealed clonal chromosomal abnormalities in five more cases with either normal or failed karyotype.

The current study also revealed two cases with less common chromosomal abnormalities; del 6q, -20 (case 10) (Fig. 6) and del 9p (case 48).

Clinical correlation:

Age, sex and total leucocytic count (TLC) are summarized in Tables (4, 5 and 6). Response to induction chemotherapy and overall survival are presented in Table (7) and (Fig. 7). Complete remission was achieved in 41/50 (82%) cases with a mean OS of 20.6 months. The other 9 cases included 2 (4%) cases with normal karyotype, 2 (4%) cases with BCR/ABL, 2 (4%) cases with t(1;19), one (2%) case with t(8;14), one (2%) case with amplification of AML1 gene and one case with failed karyotype.

All 7 patients with TEL/AML1 fusion achieved complete clinical remission. They had a mean age of 4.3 years, range 0.2-18 years, TLC mean was $39.6X10^9$/L, range 2-$189X10^9$/L. Mean overall survival 28.3 months, (p= 0.0757), range 18-35 months and were all alive at the end of the study (Table 2).

The 10 cases with high hyperdiploid karyotype {not including t(9;22)} achieved CR with a mean OS of 26.3 months, range 17-42 months. They had a mean age of 10 years, range 2.5-18 years, TLC mean was $30.46X10^9$/L, range 1.4-$103X10^9$/L and were all alive in CR at the end of the study except two patients who died in CR.

Normal karyotype was encountered in 11/50 cases, 9 of them achieved complete remission, while one case entered into myelosuppression and died in CR (case 40) and another one (case 41) died shortly after diagnosis. Their mean OS was 22.2 months, range 1-30 months, they had

a mean age of 6.7 years, range 0.2-18 years, TLC mean was 64.0X10^9/L, range 3-289X 10^9/L.

Eighteen patients with t(9;22), MLL gene rearrangement, t(8;14), hypodiploidy, AML1 gene amplification were grouped together due to limited number of cases and their mean overall survival was 14.6 months (Fig. 8), significantly lower than the rest of the patients (p= 0.0134), range 1-44 months. They had a mean age of 9.7 years, range 0.3-18 years. TLC mean was 61.2X10^9/L, range 1.8-400X10^9/L. Cytogenetic results could not be obtained in 4 case (cases 14, 16, 19 and 47). Apart from TEL/AML1 positivity, 14 cases showed either AML1 or TEL aberrations as revealed by FISH. All cases achieved complete clinical remission, except for 2 cases (cases 6 and 32).

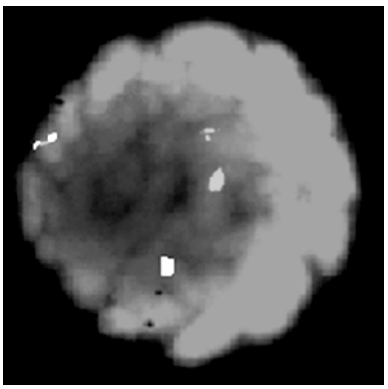

Fig. (1): FISH showing translocation TEL/AML1 using LSI TEL/AML1 extra signal dual color probe (vysis).

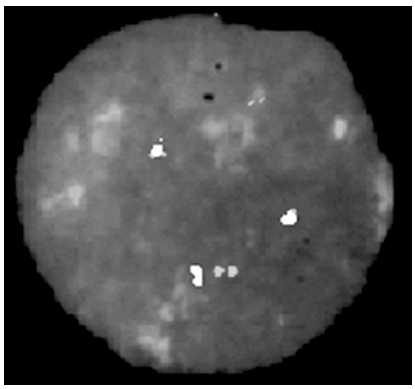

Fig. (2): FISH using LSI TEL/AML1 extra signal dual color probe (vysis) showing translocation TEL/ AML1 with an extra fusion signal denoting an extra derivative 21.

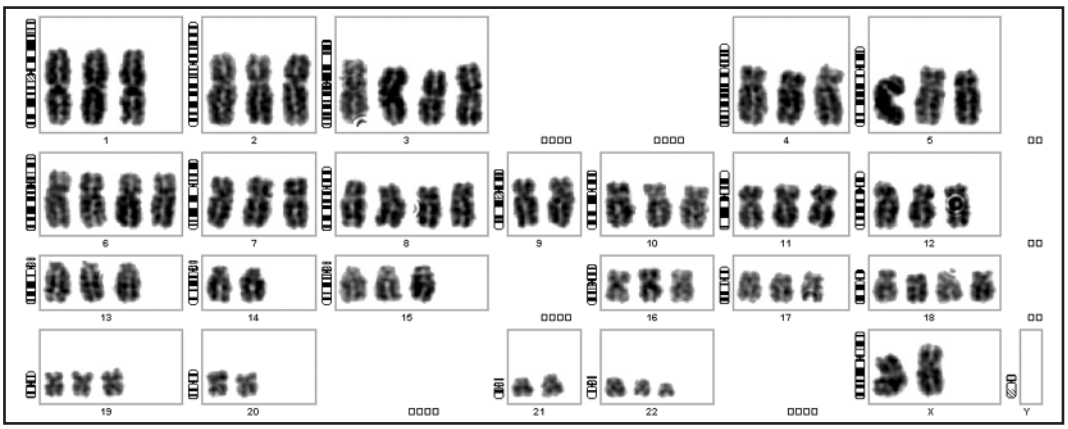

Fig. (3): Karyotype of case No. 6 showing High Hyperdiploidy and double Philadelphia chromosome.

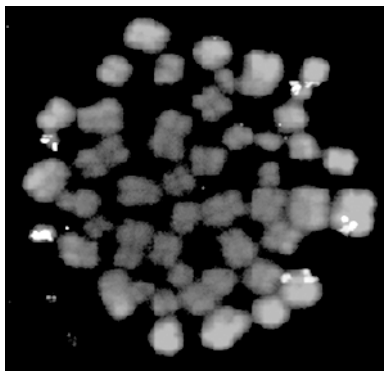

Fig. (4): FISH of the same case using BCR/ABL dual fusion probe showing high double Philadelphia chromosome.

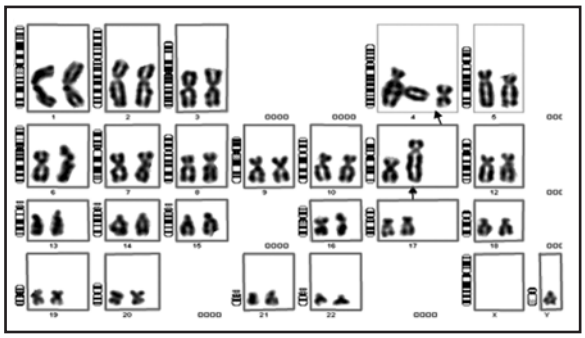

Fig. (5): Karyotype of case No. 28 showing t(4;11) (q21;q23).

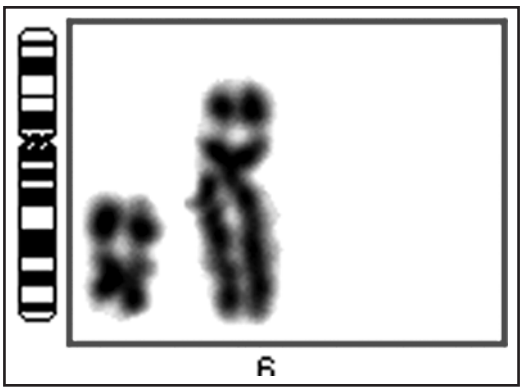

Fig. (6): Partial karyotype of case No. 10 showing deletion of the long arm of chromosome 6.

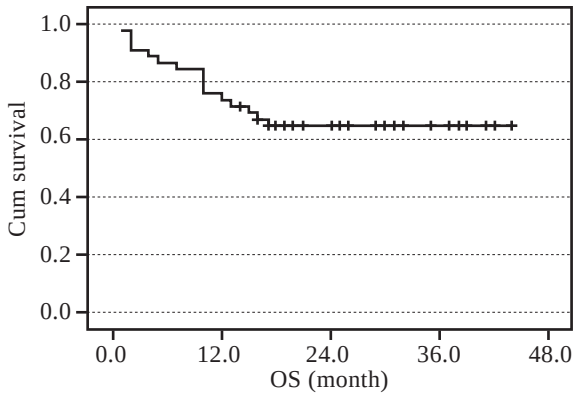

Fig. (7): Overall survival of all patients.

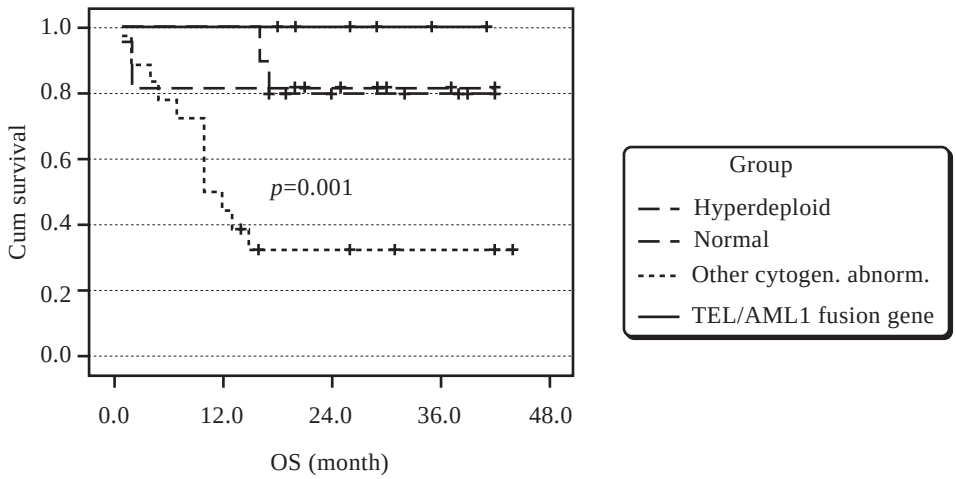

Fig. (8): Overall survival of different cytogenetics group.

Table (1): Clinical and biological characteristics of 50 B lineage ALL patients with different cytogenetic findings.

	Total	Normal karyotype	High hyperdiploidy	TEL/AML+	Others*
No. cases	50	11/50 (22%)	10/50 (20%)	7/50 (14%)	18/50 (36%)
Gender:					
Male	29 (58%)	7 (63.5%)	4 (40%)	5 (71.5%)	12 (66.6%)
Female	21 (42%)	4 (36.5%)	6 (60%)	2 (28.5%)	6 (33.3%)
Median	25.5	4	9	3.8	11
Age	0.2-18	0.2-18	2.5-18	2-10	0.3-18
Range		p=0.4719	p=0.4561	p=0.1117	p=0.4682
Age:					
<1	4 (8%)	1 (9%)			2 (11.1)
1-9.9 yrs	25 (50%)	7 (63.6%)	5 (50%)	6 (85.7%)	6 (33.3%)
10-18 yrs	21 (42%)	3 (27.3%)	5 (50%)	1 (1.3%)	10 (55.6%)
TLC ($x10^9$/L):	35 (70%)	6 (54.5%)	8 (80%)	6 (85.7%)	12 (66.6%)
<50	15 (30%)	5 (45.5%)	2 (20%)	1 (1.3%)	6 (33.3%)
≥50		0.6976	0.3745	0.6680	0.7012

*Cases with BCR/ABL, MLL gene rearrangement, hypodiploidy, t(1;19), t(8;14) and other less common cytogenetic abnormalities.

Table (2): Secondary chromosomal abnormalities in 7 TEL/AML1 positive precursor B-ALL patients.

No. cases	Age/ sex	TLC	Karyotype	TEL deletion	Trisomy 21	Induction response	Overall survival (month)	Status
9	3/M	189	46, XY			CR	26	Alive
13	2/M	6.2	49, XY, +8, +21, +mar		+	CR	18	Alive
31	4/F	11	46, XX			CR	29	Alive
37	3.8/M	20	47, XY, +21		+	CR	20	Alive
43	5/M	14	46, XY, del12p	+		CR	35	Alive
45	2/F	17	Not done			CR	41	Alive
46	10/M	20	Not done	+		CR	29	Alive

Table (3): Chromosomal abnormalities in 43 TEL/AML1 negative B-ALL patients.

No.	Age	TLC	Karyotype	BCR/ABL	MLL*	RR	OS	Status
1	18/M	1.8	46, XY, (1;10) (q12;q21), t(9;22) (q34;q11), -14, del17 (p11), +mar	+		CR	10	D
2	0.8/M	7.1	46, XY, t(10;11) (q22;q23), del (12) (p11), -17, -19, -20, +22, +mar, +mar		+	CR	12	D
3	2.5/F	19	55~56, XX, +X, +4, +6, +7, +8, +10, +11, +13, +21, +mar			CR	42	A
4	18/M	12.8	46, XY, t(9;22) (q34;q11)	+		R	4	D
5	7/F	24	62, XX, +X, +4, +6, +8, +10, +10, +11, +13, +14, +16, +17, +18, +20, +21, +22, +mar			CR	16	D
6	16/F	14.4	69, XXX, +3, +5, +8, t(9;22) (q34;q11)x2, +10, +18	+		R	7	D
7	0.3/F	17.8	46, XX, t(1;19) (q23;p13)			R	10	D
8	3/M		46, XY, der(19) t(1;19) (q23;p13)			CR	14	A
10	12/F	400	46, XX del6q, -20, +mar			CR	26	A
11	11/F	103	67, XXX,…			CR	24	A
12	1/F	21	46, XX, t(1;19) (q21;p13)			R	5	D
14	5.2/M	19.3	Failed			CR	14	D
15	16/F	96	55, XX, +3, +6, +8, +9, +11, +15, +20, +21, +22			CR	39	A
16	2.5/M	179	Failed			R	4	D
17	17/F	10.1	52, XX, +8, +11, +17 +18, +19, +21			CR	17	A
18	0.2/M	13	46, XY			CR	25	A
19	16/F	14	Not done			CR	16	A
20	3.5/M	6.2	56, XY, +6, +8, +10, +13, 14, +17, +18, +20, +21, +22			CR	19	A
21	4/M	7.1	65, XXY, +2, +3, +4, +6, +8, +9, +10, +11, +12, +13, +15, +16, +18, +19, +20, +21, +21, +22			CR	38	A
22	3/M	71	46, XY			CR	20	A
23	17/M	24.2	89, XXYY……			CR	19	A
24	4/M	14	46, XY			CR	17	A
25	18/M	1.4	52, XY, +4, +6, +10, +14, +18, +21			CR	17	D
26	4/F	13.6	57, XXX, +4, +8, +12, +14, +15, +16,+17, +20, +21, +mar			CR	32	A
27	15/M	102	45, XY, add1q, t(8;14), -11, -17, +mar			R	13	D
28	10/M	104	46, XY, t(4;11) (q21;q23)		+	CR	42	A
29	0.3/M	2.3	Not done		+	CR	10	D
30	3.5/M	44	46, XY, t(8;14) (q24;q32)			CR	44	A
32	6/M	5.9	47, XY, +mar			R	1	D
33	16/M	168	46, XY, t(8;14) (q24;q32)			CR	15	D
34	2/M	289	46, XY			CR	42	A
35	7/M	19	46, XY			CR	21	A
36	11/M	30	46, XY			CR	29	A
38	18/F	2.9	42, XX, -2, -12, -14, -19			CR	16	A
39	18/F	76	46, XX			R	2	D
40	18/M	32.9	44, XY, -8, -13			CR	2	D
41	18/M	66	46, XY			R	1	D
42	2/F	12	46, XX, t(1;19) (q23;p13)			CR	10	D
44	6/F	24	46, XX			CR	37	A
47	14/F	35	Not done			CR	15	D
48	16/M	97	46, XY, del9p			CR	31	A
49	3/F	41	46, XX			CR	20	A
50	2/F	61	46, XX			CR	30	A

*All three cases showed MLL gene translocation.

Table (4): Age distribution of patients with positive karyotype at presentation.

Age	Case summaries		
	N	Mean	Std.deviation
Group:			
Normal	11	6.7	6.3
Hyperdeploid	10	10.0	6.5
TEL/AML1 fusion gene	7	4.3	2.8
Other cytogen. abnorm.	18	9.7	7.3
Total[a]	46	8.2	6.6

[a]: p=0.195 (NS).

Table (5): Tolal Leucocytic Count (TLC) of patients with positive karyotype at presentation.

TLC	Case summaries		
	N	Mean	Std.deviation
Group:			
Normal	11	64.000	78.2573
Hyperdeploid	10	30.460	37.1892
TEL/AML1 fusion gene	7	39.600	66.0660
Other cytogen. abnorm.	18	61.272	96.6858
Total[a]	46	51.928	77.1209

[a]: p=0.699.

Table (6): Sex distribution of patients with positive karyotype at presentation.

	Sex			
	F		M	
	No	%	No	%
Group[a]:				
Normal	4	36.4	7	63.6
Hyperdeploid	6	60.0	4	40.0
TEL/AML1 fusion gene	3	42.9	4	57.1
Other cytogen. abnorm.	6	33.3	12	66.7

[a]: p=0.57 (NS).

Table (7): Post induction Status of 50 B lineage ALL patients with different cytogenetic findings.

Status	Total	Normal karyotype	High hyperdiploidy	TEL/ AML +	Others *
Complete remission	41/50 (82%)	9/11 (81.8%)	10/10 (100%)	7/7 (100%)	12/18 (66.6%)
No response	*9/50 (18%)	2/11 (18.2%)	0/10	0/7	6 (33.3%)
Mean overall survival (months)	21.1	22.8 p= 0.6337	26.9 p= 0.1030	28.3 p= 0.0757	15.0 p= 0.0134

*The 9th patient had failed karyotype.

DISCUSSION

Since the t(12;21) is virtually undetectable with conventional cytogenetic procedures, the two preferred screening methods are those with reverse transcriptase polymerase chain reaction (RT-PCR) and fluorescence in situ hybridization (FISH). The latter technology has the advantage that it enables the identification and quantification of the most common and thus, most relevant secondary changes on a single cell level **[2,3,6,8, 33-35].**

The purpose of the present study was to identify the frequency of TEL/AML1 translocation among in comparison to other recurrent genetic abnormalities and to detect other abnormalities involving the TEL and AML1 genes either associated with or without the TEL/AML1 translocation in Egyptian children with B lineage ALL. We also aimed to demonstrate the usefulness of FISH in detecting such abnormalities. Finally we aimed to identify the correlation of such abnormalities with the overall survival.

A substantial increase in detection rate from 60% to 70% was observed using the combination of conventional G-banding and FISH analysis. Especially of note, FISH was useful to identify the cryptic gene rearrangements in cases with normal banded karyotype or in case of failure to obtain mitotic figures. A previously published Korean study reported successful CC in detecting chromosomal abnormalities in 49.2% of cases and when combining both CC and FISH the detection rate rose up to 73.8% **[42].**

The Children's Cancer Group (CCG) and the Pediatric Oncology Group (POG) agreed on that both Ph-positive ALL and extreme hypodiploidy were very poor risk factors and that TEL/AML1 fusion and triple trisomies of 4, 10 and 17 conveyed a very good prognosis **[34].**

In the current study, the frequency of TEL/AML1 translocation was found to be 14%. They all attained complete remission and have a mean overall survival of 28.3 months. Previously published studies reported the frequency of TEL/AML1 translocation to be around 25% in American, German, Italian and French populations **[34].** Two previous Egyptian studies reported the frequency to be 9.7% and 12.35% respectively **[35,36]** and others reported as 8.6% in

Indians **[37]** and in Japan 10% **[38]**. In Saudia Arabia the TEL/AML1 positivity was reported as 20% **[39]**. This difference may attribute to variability in inclusion criteria in the studies **[7, 8,33,34,40-41]**.

While in the present study, deletion of the non-translocated allele of TEL was detected in 2/7 cases of the TEL/AML1 gene fusion and a TEL deletion was found more often in the TEL/AML1+ cases than in the TEL/AML1- cases (28% vs 4.5%).

Similarly, another study detected 2/9 cases with TEL/AML1 fusion and simultaneous rearrangements of non-translocated TEL gene **[42]**. While other studies reported higher frequency of rearrangements of non-translocated TEL gene in more than 50% of the patients with TEL/AML1 fusion **[7,28]**. This supports the theory that the TEL/AML1 fusion gene acts in a recessive manner with regard to TEL gene, or that the secondary genetic changes including rearrangements of non-translocated TEL gene are needed in leukemogenesis by TEL/AML1 fusion **[11,15]**.

In the current study, the 2 cases with TEL/AML+ and TEL deletion achieved CR and are still in remission till the end of the study (35 and 29 months+) while others reported that one patient with non-translocated TEL deletion was classified into the high-risk group, whereas other patients with TEL/AML1 fusion alone were classified into low- or intermediate- risk group **[42]**.

Kempski et al. **[15]** did not find any difference in EFS between TEL-deleted (n=17) and non-TEL-deleted patients (n=5). On the contrary Attarbaschi et al. **[40]** studied 327 patients and found TEL/AML1 fusion in 94/327 (25%) and that cases with a TEL deletion had worse outcome than those without it.

In the current study, trisomy 21 was detected in TEL/AML1+ cases in 2/7 (28%) while in TEL/AML1- cases in 8/43 (18.5%). Others reported that the presence of trisomy 21 was not significantly different between the TEL/AML1+ and TEL/AML1- patients 13/94 (14%) vs 32/278 (12%) (p=0.785). The same study also detected no significant differences between TEL/AML1 patients with and without a trisomy 21 with respect to the presenting features and treatment outcome **[42]**.

In this work, AML1 gene amplification was detected as a sole chromosomal abnormality in one case which had a poor outcome. A previously published study identified 14 pediatric ALL cases with amplification or over-representation of 21q22 **[43]**. Another study reported that the amplification of AML1 is associated with a poor outcome **[18]**. A recently published study reviewed this novel cytogenetic finding (the intrachromosomal amplification of chromosome 21) which is typical for childhood B lineage ALL and requires high-risk therapy irrespective of other risk factors **[44]**.

In the present study, MLL gene rearrangement was detected in 3/50 (6%) A Korean study reported MLL gene rearrangement in 11.3% of cases for MLL **[42]**. Similar to our results, a Chinese study reported MLL gene rearrangement in 3/51 cases; one case showed t(4;11), another case showed MLL gene deletion as well as AML1 gene amplification as a large ring chromosome 21 and another case of MLL gene deletion together with t(12;21).

In the current study, BCR/ABL was detected in 3/50 cases (6%), one of them had double Philadelphia and hyperdiploid karyotype. Other studies reported 1.8% and 1.9% for BCR/ABL translocations **[42,45]**. Similar to one of our cases, a previously published study, reported a case of double Philadelphia chromosome, a hyperdiploid karyotype and duplication of chromosome 1 long arm **[45]**.

Among the TEL/AML1– patients included in this study, 14 cases showed either loss or gain of TEL and/or AML1 genes. Three cases showed TEL gene anomalies either deletion or trisomy and 10 cases with gain of AML1 gene either trisomy, tetrasomy or amplification. Our results agreed with what was published previously by Zang et al. **[45]**.

Finally, the current study confirms the well known important role of cytogenetics in risk stratification and outcome of pediatric ALL. Favorable outcome in childhood ALL includes hyperdiploidy, TEL/AML1 fusion versus unfavorable cytogentic abnormalities including the mixed lineage leukemia (MLL) and the translocation t(9;22) **[46-49]**.

In conclusion, TEL/AML1 gene fusion is a associated with a higher overall survival, similar

to high hyperdiploidy, than to normal karyotype and other chromosomal abnormalities such as BCR/ABL, MLL gene rearrangement, t(1;19), t(8;14) and hypodiploidy. Secondary chromosomal abnormalities are present in 4/7 of TEL/AML1+ cases. AML1 gene amplification is a less common chromosomal abnormality which is accompanied with poor outcome. Therefore, a larger scale study is recommended.

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Tissue Factor Pathway Inhibitor and P-selectin as Markers of Sepsis Induced Non Overt Disseminated Intravascular Coagulopathy

EMAN MOSAD, M.D.*; KHALID I. EL-SAYH, M.D.** and AZZA A. EL-TAYEB, M.D.**

The Department of Clinical Pathology, South Egypt Cancer Institute, Assiut University, Assiut, Egypt and the Department of Pediatric, Pediatric University Hospital, Assiut University, Assiut, Egypt**.*

ABSTRACT

Background: Inflammation and coagulation occur concomitantly in sepsis and are intimately linked. Proinflammatory mediators can stimulate tissue factor (TF) expression on endothelial cells and circulating monocytes. Expression of TF is a primary mechanism of inflammation induced coagulation activation. It is the most important initiator of thrombin formation. Thrombin activates platelet which leads to P-selectin translocation and glycoprotein (GP) IIb-IIIa activation. P-selectin was shown to up regulate TF generation in monocytes and also to initiate signaling pathways in leucocytes and activate the elaboration of cytokines. Tissue factor pathway inhibitor (TFPI) is an endogenous anticoagulant that modulates initiation of coagulation induced by TF. Disseminated intravascular coagulopathy (DIC) is a major factor influencing mortality in sepsis. The term non overt DIC refers to a state of affairs prevalent before occurrence of overt DIC. In these cases the DIC score is less than 5. It was suggested that initiation of treatment in pre DIC state (non overt DIC) has better outcome than overt DIC.

Objective: This study investigated the role of TFPI level, P-selectin and thrombin activation markers in non overt and overt DIC induced by sepsis and its relationship to outcome and organ dysfunction as measured by the Sequential Organ Failure Assessment (SOFA) score.

Study design: The study included 176 patients with mean age 3±2.5 years. They admitted to the intensive care unit (ICU) of the Assiut University Pediatric Hospital. They included 144 cases of non overt DIC [group I; GIT infection with dehydration (n =66) and group II; respiratory tract infection (n=78)] and 32 children with overt DIC [group I; GIT infection with dehydration (n=15) and group II; respiratory tract infection (n=17)]. Twenty three healthy children of matchable age were included as control.

Results: There was a significant difference in haemostatic markers; platelet count, partial thromboplastin time (PTT), fibrinogen, D dimer levels, platelet activation marker (P-selectin), thrombin activation markers (thrombin antithrombin complex and prothrombin fragment 1+2), TFPI and DIC score between overt and non overt DIC in both groups. Statistically significant differences in plasma levels of haemostatic parameters between the non overt DIC and the control in both groups were found. The highest significant level of TFPI was present in GIT infection. However, in respiratory infection the level of TFPI did not differ significantly from the control. It was noticed that P-selectin was positively correlated with DIC score, fibrinogen consumption, fibrinolysis (D. dimer), thrombin activation markers and TFPI. TFPI was significantly correlated with fibrinolysis, DIC score and prothrombin fragment 1+2. There was a significant difference between overt and non overt DIC in SOFA score in both types of infection. SOFA score was positively correlated with DIC score, thrombin activation markers, TFPI and P-selectin and negatively correlated with platelet count and fibrinogen in overt DIC Patients. In contrast SOFA score was correlated only with prothrombin fragment 1+2 and partial thromboplastin time in non overt DIC.

In conclusion, the plasma TFPI concentration and P-selectin increases in the majority of the patients with non overt DIC with GIT and respiratory tract infection and they are useful for predicting outcome in DIC patients in the ICU. To improve the outcome of DIC patients, there is a need to establish more diagnostic criteria for non-overt-DIC. Plasma levels of TFPI and P-selectin may be helpful in this respect.

Key Words: *TEPI – P-selectin – DIC – Overt.*

INTRODUCTION

Pediatric sepsis remains a leading cause of death in children. Eighty percent of deaths in children can be classified as sepsis deaths **[1]**. Sepsis is defined as systemic inflammatory response syndrome (SIRS) in the presence of documented or suspected infection. When sepsis is associated with acute organ dysfunction, the sepsis is considered severe **[2,3]**. It has been reported that inflammation and coagulation occur concomitantly in sepsis and are intimately linked. Acute inflammation as seen in association with sepsis lead to systemic activation of

coagulation system [4]. Pro inflammatory mediators such as endotoxin, tumor necrosis factor α (TNFα), lipoproteins and growth factors can all stimulate tissue factor (TF) expression on endothelial cells and circulating monocytes [5]. Intravascular expression of TF is a primary mechanism of inflammation induced coagulation activation. It is the most important initiator of thrombin formation [6]. Thrombin activates platelet [7]. This in turn leads to P-selectin translocation and to glycoprotein (GP) IIb-IIIa activation, rendering them able to accomplish their functions in inflammation and hemostasis [8] P-selectin was not only shown to up regulate TF generation in monocytes [9] but also to initiate signaling pathways in leucocytes and activate the elaboration of cytokines in a mechanism that involved P-selectin interaction with its receptors. This process contributes to microthrombi formation. If this process goes unchecked by natural anticoagulants such as tissue factor pathway inhibitor, thrombin will propagate uncontrolled coagulation leading to organ dysfunction as seen in severe sepsis [5].

Tissue factor pathway inhibitor (TFPI) is an endogenous anticoagulant that modulates initiation of coagulation induced by TF [10,11]. It inactivates both factors Xa and TF-VIIa complex [12]. High dose of exogenous recombinant TFPI may increase this threshold and protect against disseminated intravascular coagulation (DIC) and venous thrombosis [13]. DIC is a major factor influencing mortality in sepsis. Previous studies suggest that initiation of treatment in pre DIC state (non overt DIC) gives better outcome than overt DIC [14]. An evolving score based on prothrombin time (PT), platelet count, fibrinogen, partial thromboplastin time (PTT) and D-dimer in the first 48 hours of intensive care reflected clinical severity [15]. A score of 5 or greater could diagnostically define patients with a poor prognosis from haemostatic dysfunction. If the score ≥5 it is compatible with overt DIC. If the score <5 it is suggestive (not affirmative) for non-overt DIC [16]. The non-overt DIC has a prognostic relevance [17,18,19].

Our study aims to investigate the role of TFPI, P-selectin and thrombin activation markers in overt and non overt DIC in gastrointestinal and respiratory infections with severe sepsis in relation to organ dysfunction measured by SOFA score. We also aim to detect their usefulness as markers for diagnosis of non overt DIC.

PATIENT AND METHOD

The study protocol was approved by the Human Ethics Review Committee of Assiut University, and signed consent from patient's parents was obtained. The study included 176 patients with mean age 3±2.5 years. They admitted to the ICU of the Assiut University Pediatric Hospital. They included 144 cases of non overt DIC [group I; GIT infection with dehydration (n=66) and group II; respiratory tract infection (n=78)] and 32 cases with overt DIC [group I; GIT infection with dehydration (n=15) and group II; respiratory tract infection (n=17)]. Twenty three healthy children of matchable age were included as control.

The groups of patients fulfilled the diagnostic criteria of non overt and overt DIC of International Society on Thrombosis and Hemostasis (ISTH) [20-22]. The patients also had manifestation of systemic inflammatory response syndrome (SIRS), which manifests itself as age dependant changes in temperature, abnormal heart rate, elevated respiratory rate and changes in white blood cell count in peripheral blood. However, at least one of them must be either temperature or white blood count [1]. The control group is healthy volunteers of the same age from the healthy pediatric and circumcision office; they were not receiving any medication at the time of blood sampling.

DIC score is a score equal the sum of 5 variables. A score 5 or more means at least 2 elements are affected. The scoring system for DIC proposed by ISTH, suggested that if the score ≥5, compatible with overt DIC. If <5, suggestive of non-overt DIC [16].

DIC score:

Score	P.T sec	PTT sec	Platelet $x10^9$/L	Fibrinogen g/L	D.dimer n/ml
0	≤13.5	28-41	>150	>1.8	≤1000
1	>13.5	<28->41	≤150	≤1.8	≤2000
2	≥15	<24->46	≤100	≤1.5	≤4000
3	≥18	≥61	≤80	≤1	>4000

The degree of organ dysfunction in patients with DIC was assessed by the sequential Organ Failure Assessment (SOFA) score system [18].

Sequential Organ Failure Assessment (SOFA) score system:

	SOFA score				
	0	1	2	3	4
Respiratory PAO2/FO2 mm/Hg	>400	≤400	≤300	≤200	≤100
Coagulation Platelet Count x 10^9/L	>150	≤150	≤100	≤50	≤20
Liver Billirubin mg/dl	<1.2	1.2-1.9	2-5.9	6-11.9	>12
Cardiovascular hypotension	No hypotension	Mean bl.p <70	Dop ≤5	Dop >5, epi ≤0.1	Dop >5, epi >0.1
Central nervous system Glascow coma scale	15	13-14	10-12	6-9	<6
Creatinine mg/dl Or urine output ml/dl	<1.2	1.2-1.9	2-3.4	3.5-4.9 or <500	>5 or <200

Dop, dopamineEpi, epinephrine, and FO2, fraction of inspirited oxygen.
Adenergic agents administrated for at least 1 hour.

Method:

Prothrombin time (PT) was determined by the one-stage method of Quick **[19]** using thromborel S (Behringwerke, Marburg, Germany). Plasma fibrinogen was measured by clotting methods using (fibri-prest Diagnostica Stago). Plasma levels of TAT by Enzygnst TAT micro enzyme immunoassay (Dade Behring) Marburg/Germany, Prothrombin fragment 1+2 by Enzygnst enzyme immunoassay (Dade Behring) Marburg/Germany. D-dimer was the fibrin-related marker utilized and D-dimer levels. By an ELISA kit from Biopool International (Umeå, Sweden; Tintelize. Human soluble P-selectin was detected by ELISA kit from R&D system NE, USA. Total TFPI enzyme immunoassay (Asserachrom Total TFPI, Diagnostica Stago, Asnieres, France).

The non-overt DIC and overt scoring template was applied and the score derived for each patient assessed against the outcome parameters of SOfA score.

Statistical methods:

All data were analyzed using SPSS (Statistical Program for Social Sciences version 11 for windows, 2001, SPSS Inc., Chicago, IL, USA). Comparisons between means for continuous variables were done using independent sample *t*-test and simple ANOVA. (Between groups). Values are represented as mean ± SD. Relationships in variables were assessed by correlation test A p value <0.05 is considered to be significant. All p values were two-tailed.

RESULTS

Our results are shown in Tables (1-4) and Figs. (1-8).

This study comprised 176 (101 males and 75 females with a mean age (3±2.5) patients admitted to the ICU of the Assuit University Pediatric Hospital Underlying conditions included non overt DIC groups [GIT infection (n=66) and respiratory tract infection (n=78)], DIC groups [GIT infection (n=15), respiratory tract infection (n=17)] fulfilled the DIC diagnostic criteria of overt DIC criteria and non overt DIC of ISTH **[13-15]**. Hemostatic data for GIT infection patients with overt and non overt DIC are described in Table (1). There was a significant difference in GIT infection group Between overt and non overt DIC in platelet count, PTT, fibrinogen, D dimer levels, platelet activation marker (P-selectin), thrombin activation markers (Thrombin antithrombin complex and Prothrombin fragment 1+2), Tissue factor pathway inhibitor and DIC score (Table 1) (Figs. 1,2).

Similar differences in haemostatic markers Between overt and non overt DIC in respiratory infection group Table (2) (Figs. 3,4). We demonstrated statistically significant differences of plasma levels of haemostatic parameters between the non overt DIC groups compared to the control in prothrombin time, fibrinogen level, thrombin activation markers and DIC score in the two groups compared to the control (Table 3).

Fibrinolysis diagnosed by qauantitative D-dimer was manifested in GIT infection compared to the other two groups. Platelet activation marker (P-selectin) was significantly increased in GIT infection compared to respiratory tract infection and the control (Fig. 5). The highest significant level of TFPI was achieved GIT infection, however respiratory infection was not differ significantly from the control (Table 3) (Fig. 6).

DIC score is positively correlated with its variables (D. dimer, and PT) also, it was correlated with thrombin activation markers (Thrombin antithrombin complex and Prothrombin fragment 1+2) *p* values were (0,001) for both. It was noticed that P-selectin was positively correlated with DIC score, fibrinogen consumption, fibrinolysis (D. dimer), thrombin activation markers (Thrombin antithrombin complex and Prothrombin fragment 1+2) and TFPI. *p* values were (0.003, 0.03, 0.004, 0.02, 0.001, 0.04) respectively. TFPI was significantly correlated with fibrinolysis, DIC score and prothrombin fragment 1+2 *p* values were (0.002, 0.01, 0.01) respectively. (data not shown in the tables).

SOFA score:

There was a significant difference between overt and non overt DIC in SOFA score *p*= (0.001) Tables (1,2) in both types of infection. The correlation coefficients (r) of SOFA was positively correlated with DIC score, thrombin activation markers, TFPI and P-selectin and negatively correlated with platelet count and fibrinogen in overt DIC Patients. In contrast SOFA score was correlated only with prothrombin fragment 1+2 and partial thromboplastin time in non overt DIC. (Table 4) (Figs. 7,8) (50%) died in overt DIC group versus 9% in non overt DIC *p*=0.01.

Figures: Box plots indicate 25th percentile, median value, and 75th percentile (horizontal lines of rectangle from the bottom, respectively) and 10th and 90th percentiles (horizontal lines outside rectangle).

Table (1): The studied parameters in overt and non overt DIC in patients with GIT infection.

Haemostatic parameters	Overt DIC Mean±SD N=15	Non overt DIC Mean±SD N=66	*p* value
Platelet count x 10^9/L	96±28	320±165	0.001
Prothrombin time (seconds)	17±2.7	17±3	NS
Partial thromboplastin time (seconds)	47±4	39±14	0.001
Fibrinogen (g/L)	0.7±0.4	1.2±0.5	0.01
Ddimer (ng/ml)	2300±1240	691±311	0.005
DIC score	5.8±0.8	2.9±1	0.001
Thrombin antithrombin complex (ug/L)	35±9	21.9±18	0.001
Prothrombin fragment 1+2 (pmol/L)	563±179	284±185	0.001
P-selectin (ng/ml)	63±9.0	51±17	0.004
Tissue factor pathway inhibitor (ng/ml)	159±42	92±39	0.01
Organ dysfunction (SOFA score)	6.6±1.2	2±1.8	0.001

Table (2): The studied parameters in overt and non overt DIC in patients with respiratory tract infection.

Haemostatic parameters	Overt DIC Mean±SD N=17	Non overt DIC Mean±SD N=78	*p* value
Platelet count x 10^9/L	91±28	316±19	0.001
Prothrombin time (seconds)	16.7±1.9	17±2	NS
Partial thromboplastin time (seconds)	46±2	39±12	0.001
Fibrinogen (g/L)	0.7±0.4	1.4±0.8	0.01
Ddimer (ng/ml)	1908±1000	476±297	0.005
DIC score	6±0.9	2.2±1	0.001
Thrombin antithrombin complex (ug/L)	35±9	13±10	0.001
Prothrombin fragment 1+2 (pmol/L)	524±190	215±144	0.001
P-selectin (ng/ml)	64±5.0	36±19	0.001
Tissue factor pathway inhibitor (ng/ml)	151±41	85±24	0.01
Organ dysfunction (SOFA score)	4.8±1.2	1.8±1.8	0.001

Table (3): The studied parameters in non overt DIC in patients compared to the control.

Haemostatic parameters	Group I GIT infection Mean±SD N=66	GroupII Respiratory infection Mean±SD N=78	Control Mean±SD N=23
Platelet count x 10^9/L	320±165	316±19	269±91
Prothrombin time (seconds)	17±3*†	17±2*	13.5±0.6
Partial thromboplastin time (seconds)	39±14	39±12	41±1
Fibrinogen (g/L)	1.2±0.5*	1.4±0.8*†	3.0±0.5
Ddimer (ng/ml)	691±311*	476±297*†	100±70
DIC score	2.9±1*	2.2±1*†	0
Thrombin antithrombin complex (ug/L)	21.9±18*	13±10*†	2.6±0.9
Prothrombin fragment 1+2 (pmol/L)	284±185*	215±144*†	105±41
P-selectin (ng/ml)	51±17*	36±19*†	28±6
Tissue factor pathway inhibitor (ng/ml)	92±39*	85±24	72±22
Organ dysfunction (SOFA score)	2.3±1.7*	1.8±1.8*†	0

*: Significant compared to the control.
†: Significant difference between group 1 and 2.

Table (4): Correlation between haemostatic parameter and organ dysfunction (SOFA score).

Haemostatic parameters	SOFA score in overt DIC		SOFA score in non overt DIC	
	r	*p* value	*r*	*p* value
Platelet count x 10^9/L	-0.3	0.004	NS	NS
Prothrombin time (seconds)	NS	NS	NS	NS
Partial thromboplastin time (seconds)	NS	NS	-0.2	.01
Fibrinogen (g/L)	-0.4	0.005	NS	NS
Ddimer (ng/ml)	0.3	0.001	NS	NS
DIC score	0.6	0.001	NS	NS
Thrombin antithrombin complex (ug/L)	0.5	0.006	NS	NS
Prothrombin fragment 1+2 (pmol/L)	0.5	0.001	0.5	0.005
P-selectin (n/ml)	0.4	0.03	NS	NS
Tissue factor pathway inhibitor (ng/ml)	0.5	0.001	NS	NS

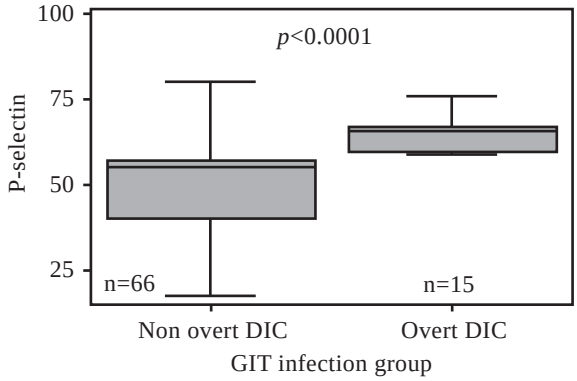

Fig. (1): P-selectin levels in GIT groups.

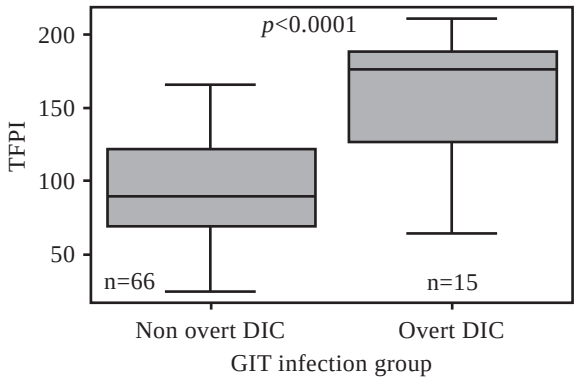

Fig. (2): TFPI levels in GIT groups.

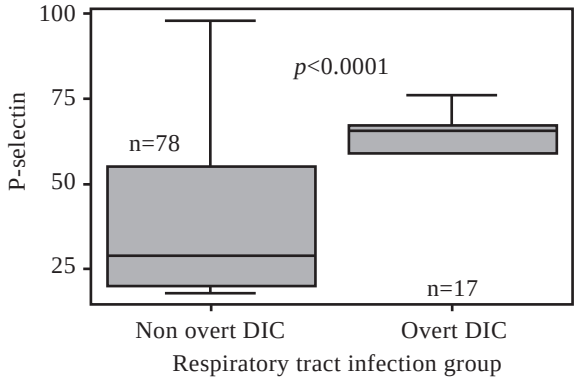

Fig. (3): P-selectin levels in respiratory groups.

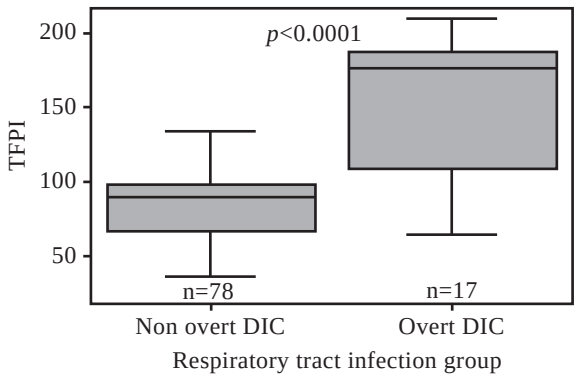

Fig. (4): TFPI levels in respiratory groups.

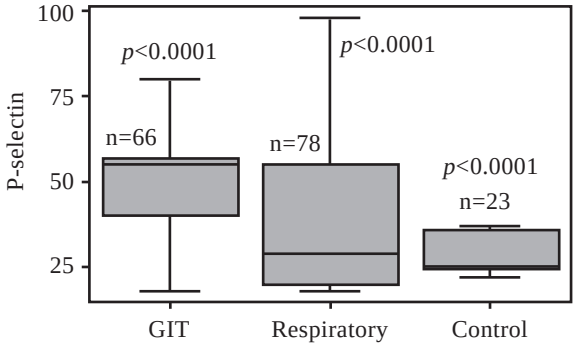

Fig. (5): P-selectin in non overt DIC groups compared to the control.

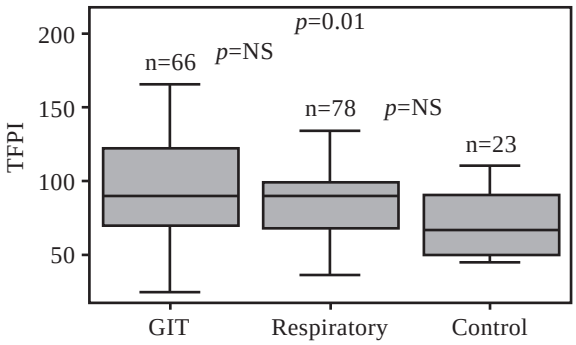

Fig. (6): TFPI in non overt DIC groups compared to the control.

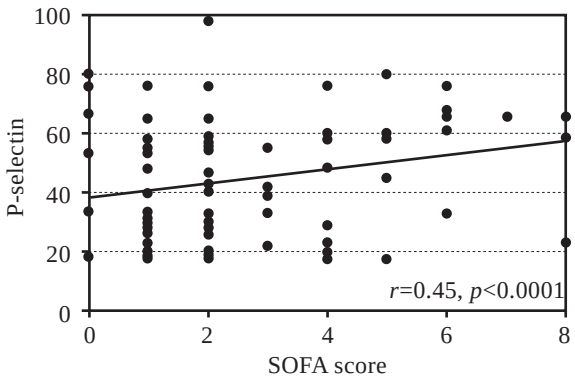

Fig. (7): Correlation between P-selectin and SOFA score in overt DIC.

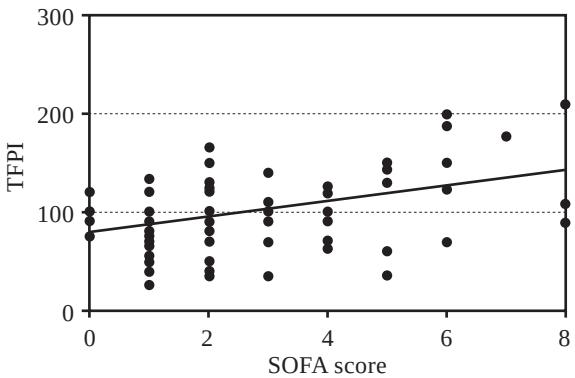

Fig. (8): Correlation between TFPI and SOFA score in overt DIC.

DISCUSSION

Sepsis with acute organ dysfunction is common, frequently fatal, and associated with a significant national health economic burden **[23]**. Abnormalities of coagulation and fibrinolysis are frequently observed in patients with sepsis. Endotoxins released from Gram-positive or negative bacteria can initiate the inflammatory cascade that characterizes sepsis which could change the properties of the vascular endothelium from anticoagulant to procoagulant. During infection and after stimulation with endotoxin or tumor necrosis factor, TF can be induced rapidly on blood mononuclear cells **[24]** and on vascular endothelium **[25]**. Fibrin deposition and complement activation can cause extensive vessel wall damage and may be associated with multiple organ failure **[26]**.

Generalized activation of coagulation depletes the body natural inhibitors including TFPI **[27,28]**. The previous studies have shown controversial results on the plasma TFPI concentrations in patients with DIC (overt and non overt) **[29,30]**. Our results showed that, plasma TFPI was elevated in patients with overt and non overt DIC. Simlar to our finding high concentration of TFPI has been reported in patients with septicemia **[31,32]**. Studies in primates showing that the coagulant response during bacteremia or endotoxemia could be completely blocked by monoclonal antibodies to TF or by infusion of the tissue factor pathway inhibitor (TFPI) which is capable of blocking the coagulant response completely and reducing the cytokine response **[33,34,35]**.

In our study TFPI remained within physiologic levels in non overt DIC in respiratory tract infection. Similar result was reported by Sabharwal et al. and Bajaj and Tricomi **[36,37]** who have studied the plasma levels of TFPI in patients with and at risk for acute respiratory distress syndrome (ARDS). They found that, the mean plasma TFPI levels in the patients at risk for ARDS group did not differ from the normal despite markedly increased TF levels in the patients with ARDS **[38,39]**. To explain the occurrence of non overt DIC in the presence of normal TFPI (as in respiratory group) or elevated TFPI levels (as in GIT infection), we have to take into account the following possibilities. The first, being that TFPI at physiologic concentration inhibits TF/VIIa effectively only after Xa, has been generated. Thus, TFPI does

not sufficiently prevent the coagulation process when continuing generation of TF occurs **[40]**. The second neutrophil elastase, a serine proteinase, cleaves TFPI. This impairs the ability of TFPI to neutralize both factors Xa and TF/VIIa. The third is that more recently Belaaouaj et al. **[41]** proved that matrix metalloproteinase derived from activated leukocytes cleave TFPI but not TF, factor VIIa, and factor Xa. Function analysis further demonstrated that matrix metalloproteinase-mediated cleavage of TFPI was accompanied by considerable loss of anticoagulant and anti-Xa activities **[41,42]**.

P-selectin had been established as a vascular adhesion molecule critical in the inflammatory response, in 1992. Palabrica et al. demonstrated that P-selectin also played a significant role in blood coagulation and thrombosis **[43]**. In our study P-selectin was elevated in patients with non overt DIC in both GIT and respiratory tract infection as well as in overt DIC. In accordance with our result Furie et al. **[44]** reported that P-selectin expression on platelets significantly increased in patients with severe SIRS in comparison with values in normal volunteers. During inflammatory states, intact endothelial cells release VWF and P-selectin from their Weibel-Palade bodies. Both molecules are ligands for GP Ib-IX-V. The newly released VWF binds platelets spontaneously **[45]**. P-selectin up regulate tissue factor generation in monocytes, and activates the elaboration of cytokines in a mechanism that involved P-selectin interaction with its receptor, PSGL **[46]**. A number of reports have secured that over expression of P-selectin can induce a pro coagulant state, that circulating micro particles bearing PSGL-1, the counter receptor for P-selectin, deliver tissue factor to the growing platelet thrombus **[44,45]**.

Our results showed that platelet counts and fibrinogen levels were significantly lower in overt than non overt group. DIC score, plasma levels of D-dimer and thrombin activation markers were significantly higher in patients with overt DIC than those with non overt –DIC and the later is higher compared to the control. In accordance with our results the plasma levels of, prothrombin fragment F1+2, TAT, and D-dimer were reported to be significantly higher in patients with systemic inflammatory response syndrome **[47,48]**. Prothrombin fragment F1+2, TAT reflect intravascular thrombin generation, but do not directly reflect microthrombi formation. D-dimer is considered to be the most useful marker for diagnosis of DIC and pre DIC **[49,50]**. These parameters are useful for the diagnosis not only overt DIC but also of non overt DIC. In our study prothrombin time did not differ significantly in overt and non overt DIC. In accordance with our results Hiedeo et al. **[51]** studied changes in prothrombin time in pre DIC and they concluded that prothrombin time was not a useful marker for diagnosis of pre DIC.

Fourrier et al. **[52]** identified consumptive coagulopathy as a strong predictor of death and multi organ failure in patients with sepsis in our study a significant difference was found in DIC score between overt and non overt DIC also a significant correlation was found between DIC score and SOFA score in overt DIC suggesting that the severity of DIC may be an important determinant of outcome **[47]**. In accordance with our results Toh and Downey demonstrated that, a score of 5 or greater could diagnostically define patients with a poor prognosis from haemostatic dysfunction, independent of developing overt DIC **[20]**. Different study designs by Toh and Downey et al. in different patient populations, found that a worsening coagulopathy augers a worse outcome in patients with severe sepsis and increased development of new organ failure **[22]**.

We use SOFA score to demonstrate the degree of organ dysfunction or failure as a determinant of outcome and we found that it was correlated significantly with TFPI and P-selectin only in overt DIC. To our knowledge there were no similar studies in the literature. Mean SOFA scores in overt DIC was 6.6±1.2 in GIT infection versus 4.8±1.2 in respiratory group. The Initial and highest scores of more than 11 or mean scores of more than 5 corresponded to mortality of more than 80% **[53]**. Other investigators performed serial SOFA scores to allow a more effective representation of the dynamics of illness including the effects of therapy compared with traditional outcome **[53]**. In accordance with our study Moreno et al. demonstrated that the initial SOFA score can be used to quantify the degree of organ dysfunction or failure present on admission **[54]**.

In conclusion, the plasma TFPI concentration and P selectin increases in the majority of the patients with non overt DIC with GIT and respiratory tract infection they are useful for predicting outcome in DIC patients in the ICU. To improve the outcome of DIC patients, there is

a need to establish more diagnostic criteria for non-overt-DIC. Plasma levels of TFPI and P-selectin may be helpful in this respect.

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