The Journal of the Egyptian Society of Haematology & Research

The Official Journal of the
Egyptian Society of Haematology & Research

Vol. 2, No. 2, September 2006

The Journal of the Egyptian Society of Haematology & Research

The Official Journal of the Egyptian Society of Haematology & Re search

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The Journal of the Egyptian Society of Haematology & Research

The Official Journal of the Egyptian Society of Haematology & Re search

Volume 2 * Number 2 * September 2006

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Journal of the Egyptian Society of Haematology & Research, Vol. 2, No. 2, September: 65-74, 2006

Role of High Dose Cytarabine in Remission Induction of Acute Non Lymphocytic Leukemia (Phase III Prospective Randomized Trial)

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ABSTRACT

Introduction: Acute myeloid leukemia (AML) is a heterogeneous group of diseases characterized by uncontrolled proliferation of clonal neoplastic hematopoietic precursor cells and impaired production of normal hematopoiesis. Series of studies had established the cancer and leukemia study group B regimen of continuous infusion cytarabine 100mg/m^{2}x7 days and an anthracycline x 3 days (3 and 7 regimen) as a standard induction therapy with an overall response rate of about 60%. However, the overall survival is still disappointing.

The Aim of this Study: Is to evaluate whether the use of higher doses of cytarabine in combination with mitoxantrone in induction therapy can improve the complete remission rates and overall survival compared with the standard dose cytarabine and doxorubicin (3 and 7 regimen).

Patients and Methods: The study includes 52 previously untreated adult AML patients. Their age ranged between 16-60 years. All cases were diagnosed by complete blood count, bone marrow aspirate, cytochemistry, immunophenotyping and cytogenetics and classified according to the FAB classification system. M3 cases were excluded. Patients were randomized to group A who received standard 3 and 7 induction regimen and group B who received Cytarabine 1gm/m^2 I.V infusion over 2 hours every 12 hours for the first 3 days, and Mitoxantrone 12mg/m^2 IV infusion over 2 hours on days 3,4 and 5 (HAM regimen). Patients who achieved complete remission in both groups received a similar consolidation therapy with two cycles of cytarabine 100mg/m^2 continuous infusion over 24 hours for 5 days and doxorubicin 25mg/m^2 IV for 2 days followed by two cycles of early intensification therapy with HAM regimen.

Results: 25 patients received 3 + 7 regimen and 27 patients received HAM. Complete remission rates were similar in both groups, 17/25 patients in arm A achieved CR (68%) and 18/27 (66.7%) for patients in arm B (p=0.986). After a median follow-up period of 96 weeks, the median duration of CR was 76 weeks (mean, 70 weeks; 95% confidence interval 43-98 weeks) in patients receiving arm A versus 52 weeks (mean, 60 weeks; 95% confidence interval 41-80) for arm B (p=0.44). The mean overall survival for patients who received the 3 and 7 regimen in this study was 12 months versus 13.5 months for those received HAM. This difference didn't reach statistical significance. As regard the toxicity, HAM regimen was associated with higher incidence of mucositis (p=0.005), diarrhea (p=0.005) and more prolonged thrombocytopenia (Days with platelet count <50,000/mm^3; p=0.001).

Conclusion: Standard doses cytarabine used in induction treatment is still the gold standard for AML patients with similar efficacy and less toxicity than higher doses. The use of HAM might be deferred till the consolidation/ intensification period when the patients' tolerance becomes better.

Key Words: *High dose cytarabine – Remission induction – AML – Phase III study.*

INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous group of diseases characterized by uncontrolled proliferation of clonal neoplastic hematopoietic precursor cells and impaired production of normal hematopoiesis leading to neutropenia, anemia, and thrombocytopenia. If untreated, patients usually die of infection or bleeding in a matter of weeks **[1]**. In the western population the overall incidence is 3.4 cases per 100 000 population; 1.2 cases per 100 000 population at age 30 and more than 20 cases per 100 000 population at age of 80 years. The median age is 20 years and has been increasing over the past decade **[2]**. Approximately 50 to 75% of adults with AML achieve complete remission (CR) with the deoxycytidine analog cytarabine and an anthracycline antibiotic, such as daunorubicin or idarubicin, or the anthracenedione mitoxantrone, which inhibit the enzyme

topoisomerase IIa. However, only 20% to 30% of patients enjoy long-term disease-free survival (DFS). The majority of patients die of their disease, primarily because of persistent or relapsed AML **[3]**. In an Eastern Cooperative Oncology Group (ECOG) analysis of the outcome of approximately 3000 patients with previously untreated AML entered on 5 successive clinical trials with cytarabine and daunorubicin for induction and with increasingly more intensive post remission therapy, 62% achieved CR, but 76% relapsed or died. The 5-year overall survival (OS) rate among 2000 patients younger than 55 years has improved from 11% in the 1970s to 37% in the 1990s **[4-6]**. The outcome for adults with AML depends on a variety of factors, including age of the patient, intensity of post-remission therapy, and biologic characteristics of the disease, the most important of which are the cytogenetics at presentation **[7-9]**. Other factors include the overexpression of transmembrane transporter proteins, which extrude certain chemotherapeutic agents from the cell and confer multidrug resistance, and mutations in or overexpression of specific genes such as CEBPA, BAX and the ratio of BCL2 to BAX, BAALC, EVI1, KIT, and FLT3 **[10-14]**.

During the past 35 years, a series of studies had established an induction regimen of the cell cycle-specific agent cytarabine 100mg/m^2 by continuous I.V infusion for 7 days and an anthracycline to become the standard of care for patients not participating in a clinical Trial **[15-16]**.

To improve the CR rates, studies have tested alternative and higher doses of anthracyclines or the anthracenediones **[17-20]**, higher doses of cytarabine **[21-23]**, new agents combined with cytarabine and/or daunorubicin such as etoposide, the purine analog fludarabine or the camptothecin topotecan **[24-26]** or sequential standard therapy followed by high doses of cytarabine **[27-28]**. Despite theoretic advantages, none of these approaches is definitively better than the standard regimen. Various strategies have been explored to eliminate minimal residual disease not apparent in the bone marrow of patients in CR which could contribute to relapse. Such strategies have included intensive consolidation therapy, high-dose chemotherapy, or chemo radiotherapy with either allogeneic or autologous hematopoietic stem-cell transplantation (HSCT), or low-dose maintenance therapy **[3]**. Although post remission therapy is a sine qua non for curing AML, fundamental issues remain unresolved. The optimal dose, schedule, and number of cycles of consolidation chemotherapy for most patients with AML who achieve CR have not been established. In younger patients, cycles of intensive consolidation chemotherapy, often with, but not limited to, high-doses of cytarabine, prolong DFS and OS **[20]**.

This study was planned to evaluate whether the use of higher doses of cytarabine in combination with mitoxantrone in induction therapy can improve the remission rate and overall survival compared with the standard dose cytarabine and doxorubicin (3 and 7 regimen). Correlation of clinical and biological factors which might affect response rate and survival were also studied.

PATIENTS AND METHODS

Fifty two patients, age 16-60Y, with previously untreated AML, presenting to the NCI in the period between October 2000 and December 2002 were included in this study. Patients with acute promyelocytic leukemia (M3), secondary leukemia, previous chemotherapy, CNS involvement, Prior history or clinical evidence of congestive heart failure, unstable angina, myocardial infarction or Performance status >3 were excluded.

Pretreatment evaluation included:

1- History and physical examination.

2- Complete blood count with differential and platelet counts.

3- Bone marrow smears stained with Romanovesky stain for bone marrow cellularity and percentage of blasts.

4- *Cytochemistry:* Myeloperoxidase (or Sudden Black) supplemented by Periodic acid shiff, acid phosphatase; acid esterase and non-specific esterase when necessary.

All cases were classified according to the French, American, British (FAB) classification.

5- *Immunophenotyping:* Immunophenotyping on peripheral blood and bone marrow samples was done at diagnosis using flow cytometry to detect the phenotype.

6- *Cytogenetic analysis:* Done on bone marrow samples for chromosomal study.

7- Serum chemistry including hepatic and renal profiles, tumor lysis panel including serum sodium, potassium, magnesium, calcium phosphorus and uric acid during therapy.

8- *Multiple drug resistant gene detection: Indirect staining:* 100µl whole blood were lysed using lysis solution (Becton and Dickenson) for 10 minutes cells were washed once in phosphate buffered saline (PBS), resuspended in 100 (l. CD_4 E_3 moAb (DaKo) added and incubated for 10 minutes at 4ºC. 10µl of secondary antibody (antimouse IgGFITC) was added for 30 minutes. The cells were washed twice in PBS, suspended in 500µl and analyzed on the flow cytometry.

9- Base line chest X-ray, abdominal ultrasonography, echocardiography and ECG. Computer tomography (CT) was done if clinical situation arises.

Treatment plan:

Remission induction:

Patients were randomized into two arms *Arm A: Received the conventional 3 and 7 regimen consisting of Cytarabine 100mg/m^2 I.V continuous infusion over 24 hours for 7 days, and doxorubicin 25mg/m^2 intravenous (IV) shot for 3 days. *Arm B: Received Cytarabine 1gm/m^2 IV infusion over 2 hours every 12 hours for the first 3 days, and Mitoxantrone 12mg/m^2 IV infusion over 2 hours on days 3,4,5. Bone Marrow aspirate (BMA) was done on Day 14. Partial remission or stationary disease by BM aspirate on day 14 warranted a second induction with the same regimen.

Consolidation therapy:

Patients who achieved complete remission received consolidation therapy consisting of: 2 cycles of Cytarabine 100mg/m^2 continuous I.V infusion over 24 hours for 5 days and doxorubicin 25mg/m^2 IV shots for 2 days followed 3-4 weeks later by two cycles of Cytarabine 1gm/m^2 IV infusion over 2 hours every 12 hours for the first 3 days and Mitoxontrone 12mg/m^2 IV infusion over 2 hours on days 3, 4, 5. Patients who achieved complete remission and were eligible for bone marrow transplantation were referred for Allogenic BMT. Toxicity and adverse effects were reported according to WHO criteria. Response rate was reported according to CALGB response criteria **[16]**.

Statistical analysis:

Statistical analysis was done using IBM compatible computer and according to the following tests.

-Statistical tests:

Descriptive statistics was presented in frequency tables, means, and standard deviations whenever appropriate.

-Analytical tests used included:

- Chi-square test for comparing two quantitative variables. Comparison of means of two groups is done by student's *t*-test for unpaired series and by Paired *t*-test when a subject is taken as his own control.
- Survival analysis and analysis of duration of complete remission were done using Kaplan Meier analysis.
- Correlation between quantitative variables is done by the *r*-test diagrammatically represented by scatter dot diagram.

Significance level of 0.05 was used in all statistical tests.

RESULTS

I- *Patient Characteristics:*

A total of 52 patients were included in this study. Twenty five patients in arm A and 27 patients in arm B. The study included 22 females (42.3%) and 30 males (57.7%) with homogenous distribution of patient characteristics between both arms (Table 1). The mean age in arm A was 27.4±9.46 and in arm B was 31.12±10.45. At the time of diagnosis 40.4% of patients had a total leucocytic count below 25x10^9/L compared to 34.6% and 25% for those who had TLC between 25-100x10^9/L and 100x10^9/L respectively. Hemoglobin level more than 8mg/dL was encountered in 36.5% of patients, and 63.5% had a hemoglobin level below 8mg/dL. 61.5% of patients had a platelet count more than 25x10^9/L compared to 38.5% having a platelet count less than 25 x 10^9/L. The mean percentage of blasts in marrow was 65.5±19.9 and 59.9±25 in group A and B respectively. In group A: The most commonly encountered FAB subgroup was M1 (40%), followed by M2 (28%) while in group B: M1 and M2 were equally

encountered in (33.3%) of cases. Chromosomal analysis was obtained in 24 patients (Table 2). Fifteen cases (62.5%) showed karyotypic abnormalities. Numerical abnormalities were encountered in 6 cases: Hyperploidy in 3 cases and hypoploidy in 2 cases. Structural abnormalities were encountered in 10 cases. Only one case showed concomitant numerical abnormality. The expression of MDR-1 gene was tested in 37 patients, 23 found to be positive (62.2%) and 14 (37.8%) were negative.

II- *Response rate, duration of response and overall survival:*

Complete response (CR):

Complete remission was achieved in 35 out of 52 patients (67.3%). Among those who received arm A, 17/25 achieved CR (68%) compared to 18/27 (66.7%) among those who received arm B. The difference was not statistically significant (p=0.986) Table (3).

Early death:

Early death was encountered in 6 out of 25 patients (24%) of patients of group A, while in group B, 7 out of 27 patients died early (25.9%). The difference was not statistically significant (p=0.986). Septicemia (39%) was the leading cause of death in our patients followed by cerebral hemorrhage (21%).

Duration of complete remission:

After a follow-up period of 96 weeks the median duration of CR was 76 weeks with a mean duration of 70 weeks (17.6m) (95% confidence interval 43-98 weeks) in patients receiving arm A treatment. As for patients receiving arm B the median duration of CR was 52 weeks with a mean of 60 weeks (15m) (95% confidence interval 41-80 week). No statistically significant difference was noticed between the tow groups Fig. (1).

Overall survival:

The median overall survival for patients receiving SDAC in this study was 12 months versus 13.5 months for those receiving HDAC this difference does not reach statistical significance. The number of cases surviving at 48 weeks were 10 cases (40%) in arm A and 14 (56%) in arm B, while at 96 weeks it was 5 (20%) cases in arm A and 3 (12%) in arm B Fig. (2).

Analysis of important prognostic factors among all patients revealed that none of the factors listed in Table (4) showed statistically significant influence on complete remission except the pretreatment total leucocytic count. There was statistical significant difference in CR between patients with leucocytic counts <25,000/mm^3 compared to patients with higher counts. The only prognostic factor that impacted the DFS and OS was performance status (PS). Patients with PS I (ECOG) at presentation showed statistically significant longer duration of complete remission and significant improvement of survival as shown in Tables (4-6).

III- *Toxicity:*

Scoring of treatment toxicity was done according to WHO criteria. Alopecia was observed in 21 patients (40.4%) grade I in 11 (21.2%) patients and grade II in 10 (19.2%) patients. The difference between group A and B was not statistically significant (p=0.1). Mucositis was encountered in 69.2% of patients. Grade I in 11 patients (21.2%), grade II in 19 patients (36.5%) and grade III in 6 patients (11.5%). The incidence and severity of mucositis were significantly higher in group B compared with group A (p=0.005). Twenty nine (55.8%) patients developed nausea and vomiting, grade I in 38.5% and grade II in 17.3% of patients. Although the incidence and severity were higher in group B rather than group A, yet the difference did not reach statistical significance (p=0.2). Diarrhea was observed in 24.4% of all cases, grade I in 22.2%, and grade III in 2.2% of patients. According to the type of regimen, higher incidence of diarrhea was observed in those who received arm B (53.3%) compared with 10% among the group of patients who received arm A and the difference was highly significant (p=0.005). Cholestatic jaundice developed in 3 (12%) patients receiving arm A and also in 3 (11.1%) receiving arm B. Table (7).

Hematological toxicity: The maximum nadir was reached after a mean of 15.48±2.46 days in arm A and 15.9±3 days in arm B. The difference was not statistically significant (p=0.5). The mean duration of neutropenia (neutrophils <500 mm^3) was (8.24±5.166) days in arm A, compared with (7.17±5.122) days in arm B. The difference was not significant (p=0.4). Also there was no statistical significant difference

in the mean duration of neutrophilic counts between 500-1000/mm^3 in both groups being 6.29±3.37 and 6.46±3.82 days for those who received arm A and arm B respectively (p=0.8). An attempt was done to correlate the duration of neutropenia less than 500x10^9/L and days of antibiotic and antifungal treatment, no significant impact was found. p value was (0.5) and (0.618) for days on antibiotic and antifungal therapy respectively. The mean duration of platelet count less than 50x10^9/L was 18±4.95 and 21±5.12 in arm A and B respectively. The difference reaches statistical significance (p=0.001). (Table 8; Figs. 3,4).

Table (1): Patient characteristics of both groups.

		Arm A (n=25)	Arm B (n=27)	p-value
Age (years)	<25	11 (44%)	9 (33.3%)	0.63
	25-45	11 (44%)	14 (51.8%)	
	>45	3 (12%)	4 (14.8%)	
Sex	Female	10 (40%)	12 (44.4%)	0.74
	Male	15 (60%)	15 (55.6%)	
PS (ECOG)*	I	13 (52%)	16 (59.25%)	0.36
	II	9 (36%)	8 (29.6%)	
	III	3 (12%)	3 (11.11%)	
TLC(x10^9/L)**	<25	9 (36%)	12 (44.4%)	0.8
	25-100	9 (36%)	9 (33.3%)	
	>100	7 (28%)	6 (22.2%)	
% of leukemic cells in BM	<50	5 (20%)	12 (44.4%)	0.06
	≥50	20 (80%)	15 (55.6%)	
CD34	+ve	10 (40%)	11 (40.7%)	0.87
	–ve	9 (36%)	11 (40.7%)	
	Not done	6 (24%)	5 (18.5%)	
MDR***	+ve	9 (36%)	14 (51.85%)	0.286
	–ve	8 (32%)	6 (22.22%)	
	Not done	8 (32%)	7 (25.92%)	
FAB****	M0	1 (4%)	0 (0%)	
	M1	10 (40%)	9 (33.3%)	0.5
	M2	7 (28%)	9 (33.3%)	
	M4	5 (20%)	4 (14.8%)	
	M5	2 (8%)	2 (7.4%)	
	M5b	0	3 (11.1%)	
Cytogenetics	Favorable	9 (36%)	5 (18.5%)	0.56
	Unfavorable	3 (12%)	7 (25.9%)	
	Not done	13 (52%)	15 (55.5)	

*PS: Performance Status (Eastern Cooperative Oncology Group).
**TLC: Total Leucocytic Count.
***MDR: Multiple Drug Resistance.
****FAB: French, American, British Classification System.

Table (2): Chromosomal analysis in all patients.

No. of studied cases	Chromosomal of analysis
5	46xy
4	46xx
4	t (8;21)
3	47xy, +8
1	45xx,-7
1	46xy,10q-
1	46xy,11q+
1	12q-,-17
1	Del 12q
1	Inv 16
1	45xy,-7
1	t (9;22), 7q-

Table (3): Overall response rate in both regimens.

	Arm A	Arm B	p-value
Complete response	17 (68%)	18 (66.7%)	0.986
No response	2 (8%)	2 (7.4%)	
Early death	6 (24%)	7 (25.9%)	
Total	25	27	

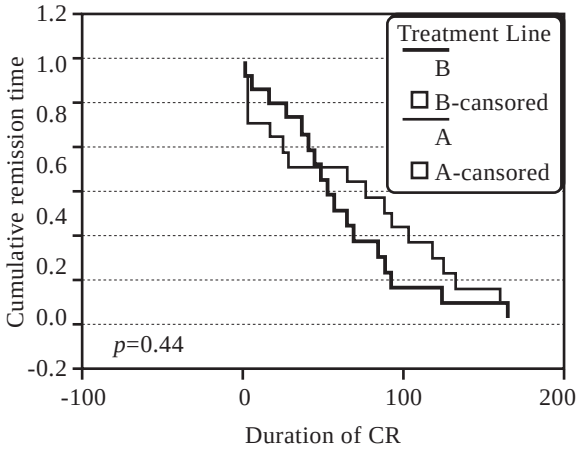

Fig. (1): Duration of remission according to type of regimen.

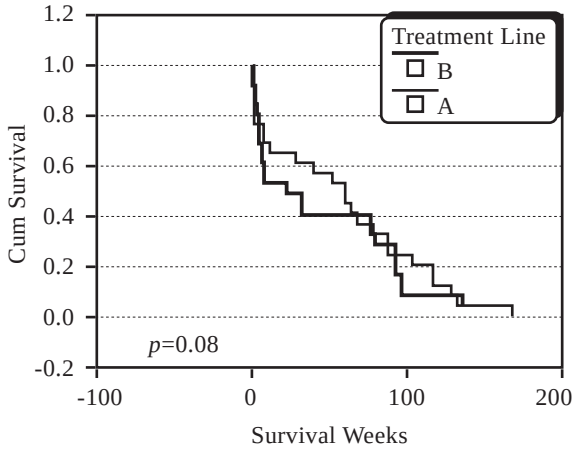

Fig. (2): Overall Survival according to type of regimen.

Table (4): Factors affecting complete remission rate.

Parameter	Complete remission	*p* value
Age (years):		
>25	15/19 (78.94%)	
25-45	15/27 (55.55%)	0.7
>45	2/6 (33.33%)	
PS (ECOG):		
I	20/30 (66.66%)	
II	12/19 (63.15%)	0.8
III	1/1 (100%)	
TLC (X10 ^{9}/L):		
<25	16/16 (100%)	
25-100	13/19 (68.42%)	0.03
>100	6/17 (35.29%)	
% of blasts in BM:		
<50	20/22 (90.9%)	
≥50	20/30 (66.66%)	0.7
FAB:		
Favorable (M1, M2)	25/31 (80.64%)	
Others	10/17 (58.82%)	0.2
MDR:		
+ve	13/23 (56.52%)	0.9
–ve	9/18 (50%)	
Cytogenetics:		
Favorable	10/14 (71.42%)	
Unfavorable	8/10 (80%)	0.5
CD34:		
+ve	15/21 (71.42%)	
–ve	10/21 (47.61%)	0.3

Table (5): Factors affecting duration of complete remission.

Parameter	Mean duration of CR (weeks)	95% confidence interval	Significance
PS (ECOG):			
I	79	(58,99)	Significant
II	31	(9,53)	(p<0.05)
III	68	(68,68)	
TLC (X10 ^{9}/L):			
<25	73	(50,95)	0.1
25-100	63	(32,95)	
>100	51	(11,92)	
FAB:			
Favorable (M1 & M2)	72	(51,93)	0.2
Others	50	(27,73)	
CD34:			
+ve	72	(45,98)	0.6
–ve	84	(52,116)	
MDR:			
+ve	77	(53,102)	0.7
–ve	73	(37,109)	
Cytogenetics:			
Favorable	83	(46-120)	0.4
Unfavorable	58	(32-84)	

Table (6): Factors affecting survival.

Parameter	Mean duration of CR (weeks)	95% confidence interval	Significance
PS (ECOG):			
I	65	(47,83)	
II	27	(9,45)	Significant
III	33	(0,94)	(p<0.05)
TLC (X10 ^{9}/L):			
<25	68	(45,90)	Not significant
25-100	51	(28,74)	
>100	29	(5,52)	
FAB:			
Favorable (M1, M2)	56	(38,74)	Not significant
Others	41	(21,61)	
CD34:			
+ve	57	(36,78)	Not significant
–ve	48	(22,74)	
MDR:			
+ve	49	(28,70)	Not significant
–ve	53	(22,84)	
Cytogenetics:			
Favorable	64	(36,93)	Not significant
Unfavorable	54	(26,82)	

Table (7): Non Hematological toxicity encountered in both groups of patients.

Toxicity	G0 No. (%)	G1 No. (%)	G2 No. (%)	G3 No. (%)	G4 No. (%)	p-value
Alopecia:						
Arm A	14 (56%)	8 (32%)	3 (12%)	0	0	0.129
Arm B	17 (63%)	3 (11.1%)	7 (25.9%)	0	0	
Mucositis:						
Arm A	13 (52%)	5 (20%)	4 (16%)	3 (12%)	0	0.005
Arm B	3 (11.1%)	6 (22.2%)	15 (55.6%)	3 (11.1%)	0	
Nausea/vomiting:						
Arm A	14 (56%)	8 (32%)	3 (12%)	0	0	0.245
Arm B	9 (33.3%)	12 (44.4%)	6 (22.2%)	0	0	
Diarrhea:						
Arm A	22 (90%)	3 (10%)	0	0	0	0.005
Arm B	13 (46.7%)	13 (46.7%)	0	1 (6.6%)	0	
Hepatobiliry:						
Arm A	22 (88%)	2 (8%)	0	1 (4%)	0	0.26
Arm B	24 (88.9%)	0	2 (7.4%)	1 (3.7%)	0	

Table (8): Hematological toxicity in both groups.

	Arm A	Arm B	p-value
Mean duration of days neutrophils <500 x 10^9/l	8.24±5.16	7.17±5.12	0.489
Mean duration of days neutrophils 500-1000 x 10^9/l	6.29±3.37	6.46±3.82	0.874
Mean duration of days platelet count ≤50 x 10^9/l	18±4.95	21±5.12	0.001

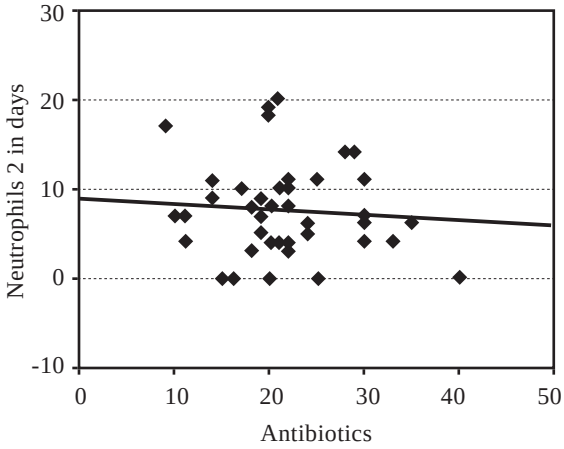

Fig. (3): Correlation between duration of neutropenia less than 500 in days and duration of antibiotic in days.

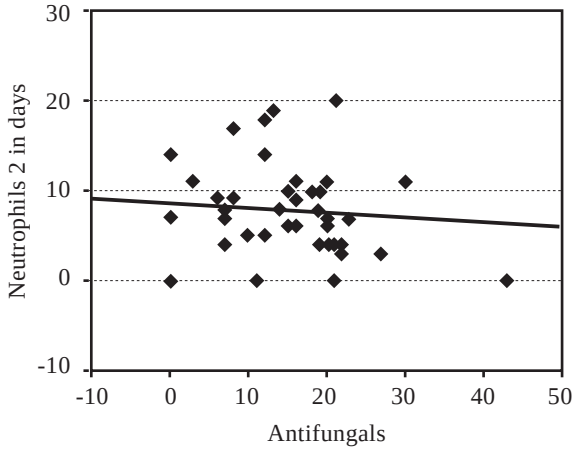

Fig. (4): Correlation between duration of neutropenia less than 500 in days and days of antifungal treatment.

DISCUSSION

The standard induction treatment of AML was established nearly 20 years ago with a combination of SDAC at 100mg/m^2/d plus 3 days of anthracyclines, either doxorubicin or DNR. Higher doses of cytarabine have been evaluated for induction therapy in AML since 1979 **[3]**.

The effectiveness of HDAC is presumed to be a result of higher intracellular concentration of cytarabine **[29]**. Uncontrolled studies have been reported using doses of 1.5 to 3g/m^2 every 12 hours for 4 to 6 days, with CR rates as high as 90% **[30-31]**. The small size and uncontrolled nature of these studies leaves some doubt about their significance.

This study included 52 patients diagnosed with AML. Patients were randomized into two treatment arms for induction. Arm A received the conventional 3 & 7 and arm B received HDAC and Mitoxontrone. Complete remission rates were similar between SDAC (68%) versus HDAC (66.7%). No statistically significant difference was noticed in duration of CR between the two treatment arms in this study (The median duration of CR was 17.6 and 15 months for SDAC arm and HDAC arm respectively). The median overall survival for patients receiving SDAC in this study was 12 months versus 13.5 months for those receiving HDAC this difference does not reach statistical significance.

Our results partially comply with previous studies. Bishop et al., 1996 **[21]**, randomized 300 patients with AML for induction with HDAC as 3gm/m^2 for 8 doses together with anthracycline and etoposide compared with SDAC with anthracycline and etoposide and reported similar response rates but longer duration of CR in the HDAC arm. However, there was no significant difference in overall survival. The only prognostic factors that were associated with improved incidence and duration of CR rates were performance status I (ECOG) and WBCs less than 25x10^9/L at presentation. The same findings were reported by Weick et al., 1996 **[22]**, who reported similar results. The HDAC arm was associated with higher toxicity and mortality. This is recently reconfirmed by Kern and Estey, 2006 **[32]** in their meta-analysis of randomized controlled trials which test induction with HDAC compared with SDAC and reported equal response rate and survival with longer DFS in the HDAC arm.

Furthermore in our study, patients who receive HDAC in the induction and consequently in the consolidation/early intensification therapy, did not show any change in the DFS or overall survival compared with the group who received HDAC in the consolidation/early intensification following SDAC in the induction therapy. This is in agreement with Bradstock et al., 2005 **[33]** who showed that Intensive induction chemotherapy incorporating high-dose cytarabine results in high complete remission rates, but further intensive consolidation treatment does not appear to confer additional benefit. However, it is different from the results of Weick et al., 1996 **[22]**, who reported improvement of survival with intensive post remission therapy.

Comparison of the of toxicity profile between the two treatment arms showed a statistically significant higher incidence of Mucositis and diarrhea in patients receiving HAM. Whereas the incidence of alopecia, nausea, vomiting and hepatobiliary toxicities were comparable in both groups. Similar results were reported by the previous studies **[22,32,33]**. The mean duration of neutropenia and requirement of systemic antibiotic and antifungal among patients who received SDAC and HDAC showed no statistically significant difference. Bishop et al., 1996 **[21]** reported no difference in the duration of severe neutropenia (neutrophils less than $0.5x10^9$/L). In their study there was statistically significant increase in the number of days of antibiotic use and in the organisms isolated by cultures. CNS toxicity was not encountered in any of our patients whether arm A or arm B. Mortality during induction was higher in the high dose Ara-c arm which is in agreement with the previous 2 studies **[22,32]**.

Although this study showed partial agreement with previous and current studies, yet we there was no DFS benefit in the HDAC arm. This could be multifactorial due to better disease biology in the SDAC arm compared with HDAC (more favorable Cytogenetics and less MDR-1 expression) or due to treatment effect. Also, the dose of Ara-c used in this study is lower than those used in previously mentioned studies both in induction and consolidation. Actually, Ara-c is a schedule dependent chemotherapeutic agent and the dose of $1gm/m^2$ might not be much different form $100mg/m^2$ given by continuous infusion. Etoposide was used as part of the induction treatment regimen in some studies which could have impacted their duration of DFS.

Also, the fact that the total group incidence of MDR expression in our patients was high (62%), despite of all being newly diagnosed patients compared to Del Poeta et al., 1994 **[34]** who reported a 20% positivity in de novo AML cases. Furthermore, the smaller number of our patients and the single institute nature of the study might have affected the results. In conclusion, HDAC failed to show improvement of response rates, DFS or OS and was associated with higher toxicity and mortality. Accordingly, SDAC induction treatment is still the gold standard for AML induction. The use of HDAC might be deferred till the consolidation period when the patient tolerance becomes better.

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Journal of the Egyptian Society of Haematology & Research, Vol. 2, No. 2, September: 75-86, 2006

P53 Dysfunction in Chronic Lymphocytic Leukemia

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ABSTRACT

Introduction: Chronic Lymphocytic Leukemia (CLL) is characterized by a highly variable clinical course. Some of this variability can be attributed to the tumor suppressor protein p53 which regulates the transcription of a number of genes including the cycline-dependant kinase inhibitor of p21 and the antiapoptptic protein BCL-2. As p53 mutations occur in only 10% to 15% of patients with CLL, it is possible that p53 dysfunction occurs in the disease through alternative mechanisms. For example, 15 to 35% of patients have an extra copy of chromosome 12 which encodes the p53 inhibitory protein MDM2. Indeed over expression of MDM2 has been reported in CLL.

The Aim of this Study: is to determine p53 dysfunction in CLL patients as detected by impaired up regulation of p53 and p21 in response to chemotherapy, determine if p53 dysfunction is caused by MDM2 over expression, correlate CD38 and BCL-2 to the presence of p53 dysfunction and to correlate their percentage expression with other prognostic factors, treatment outcome and survival.

Results: This study included forty patients with CLL. In addition, ten subjects of matching age were used as a control group. Patients were grouped according to p53 response to chemotherapy into 3 groups: group "1", normal response (p53+, p21+). Group "2", Type A p53 dysfunction (p53+, p21–). Group "3", Type B dysfunction (p53–, p21–).

The highest p53 percentage expression was detected in group "2" (mean, 39.5±24.3SD) compared to group "1" (mean, 10.16±18.8SD) and group "3" (mean, 2.5±1.8SD) patients. The difference between groups was statistically highly significant.

P21 showed highest percentage expression in group "1" (mean, 16.1±6.6 SD) Compared to group "2" (mean, 2±1.3 SD) and group "3" (mean, 1.5±1.4 SD) patients (p<0.001).

Group 3 patients showed the highest MDM2 expression (mean, 2.36±2.21SD) Compared to group "1" (mean, 0.48±0.34 SD) and group "2" (mean, 0.23±0.36 SD) patients (p<0.001). On evaluating MDM2 by MFI, the highest value was detected in group "3" patients (a mean of 12.93±10.1 SD) Compared to groups "1" and "2" patients (mean, 6.95±6.03 SD and 1.25±0.22 SD respectively) (p=0.009).

There was no statistically significant correlation between CD34 expression and P53 function (p=0.2).

Among patients with advanced stages (III & IV) of the disease, a significantly higher percentage of patients was detected in group "2" and "3" compared to group "1" (p=0.008).

Patients with normal p53 response had a higher response rate and longer time to disease progression (mean 12 months ±5.1 SD) compared to patients with type A (mean 5.8 months ±2.3 SD; p=0.001) and type B (mean 9 months±4.4SD; p=0.08) p53 dysfunction.

The median overall survival for the whole group was 22.5 months (range 3-30 months). A higher percentage of dead patients was detected in group "2" (54.5%) and group "3" (45.5%) compared to normal response group (0%) patients (p=0.02). A negative correlation was found between percentage expression of p53 (r=–0.425, p=0.006), MDM2 (r=–0.61, p=0.07), BCL-2 (r=–0.09, p=0.57) and overall survival.

Conclusion: Causes of p53 dysfunction (other than p53 mutation) should be considered in CLL patients. High MDM2 expression level is associated with advanced stage of the disease, decreased response rate, TDP and overall survival. So MDM2 may be used as a prognostic and predictive marker for response and survival. Drugs that target MDM2-p53 interaction should be investigated for clinical applications in the treatment of CLL.

Key Words: *P53 dysfunction – CLL.*

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of predominantly non-dividing clonal mature B cells in the blood, bone marrow, lymph nodes, and

spleen and by a highly variable clinical course **[1]**. Some of this variability can be attributed to the tumor suppressor protein p53. Thus, p53 gene dysfunction in CLL is strongly associated with large cell transformation **[2]**, resistance to therapy with purine analogues **[3]**, and shortened patient survival **[4]**.

By triggering apoptosis or cell cycle arrest in response to DNA damage, p53 contributes to the cytotoxic action of many chemo therapeutic agents and protects the genome from mutagenic insult **[5]**. In quiescent cells, levels of p53 protein are low owing to its short half life. After DNA damage, the half life of p53 becomes prolonged **[6]**, and the protein accumulates in the nucleus **[7]**, where it regulates transcription of a number of genes, including the cyclin-dependant kinase inhibitor of $p21^{WAF1/CIP1}$, the proapoptotic protein BAX **[8]**, and the anti-apoptotic protein Bcl-2 **[9]**.

P53 mutations typically prolong the half life of the protein in the absence of DNA damage and are, therefore, associated with increased basal levels **[10]**. However, even when activated, mutant p53 protein cannot regulate gene expression because of its inability to bind to specific DNA sequence **[11]**. As P53 mutations occur in only 10% to 15% of patients with CLL **[12]**, it is possible that p53 dysfunction occurs in the disease through alternative mechanisms. For example, 15 % to 35% of patients have an extra copy of chromosome 12 **[4]**, which encode the p53 inhibitory protein MDM2. Indeed, MDM2 over expression has been reported in CLL and may be associated with triosomy 12 **[13]**.

The aim of this study is to determine p53 dysfunction in CLL patients as detected by impaired up regulation of p53 and p21 in response to chemotherapy, determine if p53 dysfunction is caused by MDM2 over expression, correlate CD38 and BCL-2 to the presence of p53 dysfunction and to correlate their percentage expression with other prognostic factors, treatment outcome and survival.

MATERIAL AND METHODS

The present study was carried out at the National Cancer Institute, Cairo University during the period between December 2003 and June 2006.

Forty patients with chronic lymphocytic leukemia (CLL) are included in the study. Their ages ranged between 35-79 years with a median of 66 years. In addition 10 normal subjects of matching age were used as a control group.

The studied groups were subjected to thorough history, physical examination, complete blood count, bone marrow examination and immunophentyping using flow cytometer partec III, to confirm the diagnosis of CLL using a wide panel of monoclonal antibodies purchached from DAKO (Denmark) and Santa Cruz Biotechnology (U.S.A), including FITC conjugated (CD45, CD5, CD3, CD4, CD20, FMC7, HLA-DR and Kappa light chains) and PRE conjugated (CD19, CD23, CD10, CD22, CD79b, CD8 and lambda light chains). Specific isotype control for FITC, PRE conjugated monoclonal antibodies were used. Results were expressed as a percentage of cells showing positive expression. The cut off values were calculated from the control group.

Other laboratory tests were also done including, liver and kidney function tests, Coomb's test, serum lactate dehydrogenase (LDH) level, B2 microglobulin and serum protein electrophoresis.

Radiological examination including chest X-ray, abdominal ultrasound and/or CT scan was done whenever needed for proper clinical staging of the disease.

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) **[14]**. All cases were staged according to Rai system **[15]**.

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

- *Chlorambucil (Clb) and prednisone:* Clb was given orally at a dose of 0.2mg/kg/day and prednisone 20mg/m^2/day for 14 days.
- *Cyclophosphamide, Vincristine, Prednisone (CVP):* Cyclophosphamide: 400mg/m^2 IV on days 1-3, Vincristine: 1.4mg/m^2 IV on day 1 and oral Prednisone 100mg/m^2 on days 1-5.
- *Fludarabine and cyclophosphamide (FC):* Fludarabine 25mg/m^2 IV on days 1-3 and

cyclophosphamide 250mg/m^2 IV on days 1-3 (for patients with refractory or resistant disease to CVP regimen).

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

- *Complete remission (CR):* Asymptomatic patients with no organomegaly or lymphadenopathy, lymphocyte count <$4x10^3$/µl, neutrophils >$1.5x10^3$/µl, hemoglobin (Hb) >11gm/dl, platelet count >$100x10^6$/µl and bone marrow lymphocytes <30%.
- *Partial remission (PR):* >50% decrease in organomegaly or lymphadenopathy plus one of the following: Neutrophils >$1.5x10^3$/µl hemoglobin (Hb) >11gm/dl, platelet count >$100x10^6$/µl.
- *Progressive disease (PD):*New lesion or >50% increase in organomegaly or lymphadenopathy, circulating lymphocytes revealing >50% increase.
- *Stable disease (SD):* Patients who do not fit the criteria for CR, PR or PD.

To screen for p53 dysfunction, CLL cells were examined (after 3 to 6 cycle of chemotherapy with a median of 4 cycles) for an impaired p53 response. To do this, we tested the effect of chemotherapy on the expression of p53 and other proteins reported to be transcriptionally activated (p21) or repressed (BCL-2) by p53. CD38 was also measured to be correlated with p53 dysfunction.

P53, MDM-2, BCL-2 and CD38 monoclonal antibodies were purchased from DAKO (Denmark). P53, MDM-2 and BCL-2 were measured intra-cytoplasmic using intrastain fixation and permebilization fit purchased from Dako. CD38 was measured as surface expression.

Results were expressed as a percentage of cells showing positive expression (Figs. 5-7). For MDM2 results were also expressed as mean fluorescent index, by dividing the mean fluorescent intensity of the monoclonal by that of the control. A cut off value of 10% and 30% were used for interpretation of BCL2 and CD38 positivity respectively **[16]**.

To establish the proper cut off value for either p53 or MDM2, a Roc curve was done (Figs. 2,3). Using this curve, a threshold of 4.95 and 0.09 percentage positivity was found to be appropriate for p53 and MDM2 respectively, above which the results were considered positive. A Roc curve was also done for MDM2 MFI and a cut off of 1.16 was established (Fig. 4).

P21 was measured by immunocytochemistry using mouse monoclonal antibody p21WAF1 Ab-5 (Clone HZ2) (Neo Markers) (REF: MS-387-Po, lot: 387 P405A). The detection kit used was Dako Envision system.

Quantification of positive cells was evaluated in 5 or more fields of each slide until a minimum of 1000 total cells had been examined. Percentage of cells showing p21 nuclear positivity was then calculated (Fig. 8).

A cut off values of 5% as previously reported in other studies **[17]** was used, above which the results were considered positive.

Statistical methods:

Data were analyzed statistically using SPSS (Statistical Package for Social Science) version 13. The following tests were done. Mean and standard deviation are descriptive values for quantitative data. Student t test for independent samples, and ANOVA (analysis of variance) for comparing means of more than two independents groups, post hoc test to detect the LSD (least significance difference). Chi-square compared impendent proportions. Pearson correlation coefficient (*r*) was used for correlation analysis. *p* value is Significant at 0.05. ROC curve was used for detection of the best cut off point.

RESULTS

This study included forty patients with chronic lymphocytic leukemia (CLL). Their ages ranged between (35-79) years with a median of 66 years. In addition 10 normal subjects of matching age were used as a control group.

The percentage expression of p53, p21, MDM2 (also by MFI), CD38 and BCL2 were significantly higher in the studied cases than that of the control group (Table 1).

A normal p53 response in which both p53 and p21 are positive in response to chemotherapy was observed in 6 out of the 40 studied

cases. Additional 6 cases showed p21 positivity but with negative p53 expression. P21 negativity was detected in 28 patients of them 13 patients had positive and 15 cases had negative p53 expression. So, patients were grouped according to p53 response to chemotherapy into 3 groups:

- *Group 1 (12 patients):* Normal p53 response (Positive or negative for p53 and positive for p21).
- *Group 2 (13 patients):* Type A p53 dysfunction (Positive p53, negative p21).
- *Group 3 (15 patients):* Type B p53 dysfunction (negative for both p53 and p21).

P53 percentage expression was highest in group 2 (mean, 39.5±24.3 SD) followed by group 1 (mean 10.16±10.8 SD). The lowest p53 percentage expression was detected in group 3 (mean 2.5±1.8 SD). The difference between groups was statistically significant (Table 2, Fig. 1).

P21 showed a mean percentage expression of 2±1.3 in group 2 and 1.5±1.4 SD in group 3. Those values were significantly lower than that of group 1 (mean, 16.1±6.6 SD) ($p<0.001$) (Table 2, Fig. 1).

To determine whether the type B p53 dysfunction was caused by MDM2 over expression, MDM2 expression was compared between groups.

The highest MDM2 percentage expression was detected in group 3 (mean, 2.36±2.21 SD) followed by group 1 (mean, 0.48±0.34 SD). Where as the lowest MDM2 expression was detected in group 2 (mean, 0.23±0.36 SD) ($p<0.001$) (Table 2).

Also on evaluating MDM2 expression by MFI, the highest MFI was detected in group 3 (mean, 12.93±10.1 SD) compared to a mean of 6.95±6.03SD and 1.25±0.22 SD for group 1 and 2 respectively. The difference between groups was highly statistically significant ($p=0.009$) (Table 2, Fig. 1).

The mean CD38 expression was 14.5±16.2 SD in group 1, 7.1±6.9 SD in group 2 and 8.6± 9.8 SD in group 3. The difference between the groups was statistically non-significant ($p=0.2$) (Table 2).

The mean BCL2 expression was 42.6±41.4 SD in group 1, 45.4±36.8SD in group 2 and 31.6±20.6 SD in group 3. The difference between the groups was statistically non – significant ($p=0.51$) (Table 2).

A negative significant correlation was found between the percentage expression of BCL-2 and CD38 ($r=–0.386$ and $p=0.014$).

Prognostic impact of p53 dysfunction:

A significantly higher percentage of patients with advanced stages (III & IV) of the disease was detected in group "2" (54.5%) and "3" (45.5%) compared to group "1" (9.5%) ($p=0.008$).

Pearson correlation coefficient showed positive correlation between p53 percentage expression and LDH level ($r=0.342$; $p=0.03$). No significant correlations were found with age ($r=0.221$; $p=0.1$), hemoglobin concentration ($r=–0.105$; $p=0.5$), total Leucocytic count ($r=–0.236$; $p=0.1$), platelet count ($r=–0.65$; $p=0.6$) or percentage of lymphocytes in peripheral blood ($r=0.019$; $p=0.6$).

As regard treatment outcome:

Patients with normal p53 response tend to have a higher complete response rate and less treatment failure with statistically significant longer time to disease progression (mean 12 months ±5.1 SD) than either those with type A (mean 5.8 months ±2.3 SD; $p=0.001$) or type B (mean 9 months ±4.4 SD; $p=0.08$) p53 dysfunction (Fig. 9).

As regard overall survival:

The median overall survival for the whole group was 22.5 months (range 3-30 months). A higher percentage of dead patients was detected in group "2" (54.5%) and group "3" (45.5%) compared to normal response group (0%) ($p=0.02$) (Table 3).

A negative correlation was found between percentage expression of p53 ($r=–0.425$, $p=0.006$), MDM2 ($r=–0.61$, $p=0.07$), BCL-2 ($r=–0.09$, $p=0.57$) and overall survival (Table 4).

A positive significant correlation was found between p21% expression and overall survival ($r=0.325$ and $p=0.041$).

Table (1): Comparison of profile of p53 and its related markers among control group and studied group.

	Percentage expression (Mean ± SD*)		*p*-value
	Cases (N=40)	Control (N=10)	
p53 %	16.8±21.9	0.9±0.2	0.02
p21 %	6.1±7.6	0±0	0.01
MDM2 %	1.5±1.6	0.038±0.04	0.05
MDM2 (MFI)**	7.34±8.44	1.18±0.16	0.05
Bcl2	37.9±32.2	0.8±0.2	<0.001
CD38	9.9±11.5	1.9±0.7	0.036

*SD: Standard Deviation. **MFI: Mean fluorescent intensity.

Table (2): Comparison of profile of p53 and its related markers among the three subgroups of the study group.

	Percentage expression (Mean ± SD*)			*p*-value
	Group 1 (N=12)	Group 2 (N=13)	Group 3 (N=15)	
p53 %	10.16±10.8	39.5±24.3	2.5±1.8	0.008* <0.001** <0.001***
p21 %	16.1±6.6	2±1.3	1.5±1.4	<0.001
MDM2 %	0.48±0.34	0.23±0.36	2.36±2.21	<0.001
MDM2 (MFI)	6.95±6.03	1.25±0.22	12.93±10.1	0.009
Bcl2	42.6±41.4	45.4±36.8	31.6±20.6	0.51
CD38	14.5±16.2	7.1±6.9	8.6±9.8	0.2

Comparison between groups: 1 & 3*, 2 & 3** and 1 & 2***.

Table (3): Comparison between percentage expression of P53 and different prognostic factors, treatment outcome and survival in the studied 40 cases.

	Group 1 (No. %)	Group 2 (No. %)	Group 3 (No. %)	*p*-value
Age (years):				
<60	4 (28.6)	4 (28.6)	6 (42.9)	0.8
≥60	8 (30.8)	9 (34.6)	9 (34.6)	
Stage (Rai):				
II	10 (52.6)	3 (15.8)	6 (31.6)	0.008
III & IV	2 (9.5)	10 (47.6)	9 (42.9)	
LDH:				
Low	10 (43.5)	5 (21.7)	8 (34.8)	0.07
High	2 (11.8)	8 (47.1)	7 (41.2)	
Response rate:				
Complete Remission	3 (60)	1 (20)	1 (20)	
Partial Remission	9 (36)	7 (28)	9 (36)	0.2
Stable Disease	0 (0)	2 (66.7)	1 (33)	
Progressive Disease	0 (0)	3 (42.9)	4 (57.1)	
TDP (months):				0.001*
(Mean ± SD)	12±5.1	5.8±2.3	9±4.4	0.08** 0.07***
Survival Status:•				
Dead	0 (0)	6 (54.5)	5 (45.5)	0.02
Alive	12 (41.4)	7 (24.1)	10 (34.5)	
OS (months):				0.001*
(Mean ± SD)	24.3±4.6	16.8±9.03	21.1±9.5	0.3** 0.17***

• At 30 months. TDP: Time to Disease Progression. OS: Overall Survival.
Comparison between groups: 1& 2*, 1 & 3** and 2 & 3***.

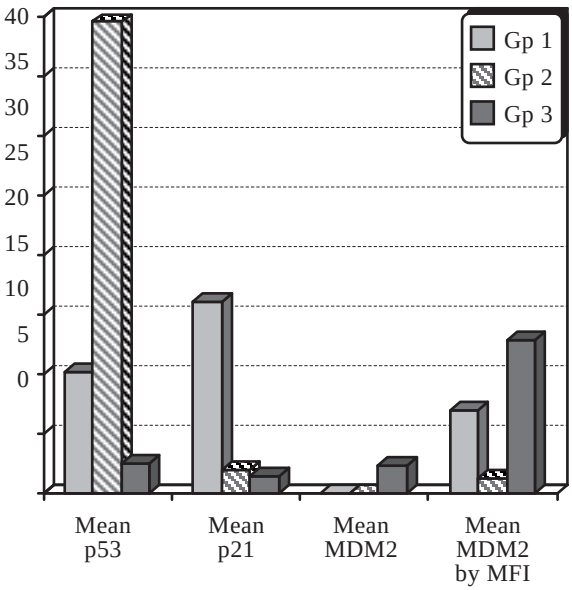


Fig. (1): Means of p53, p21 and MDM2 % expression in groups 1, 2 and 3 within the studied group.

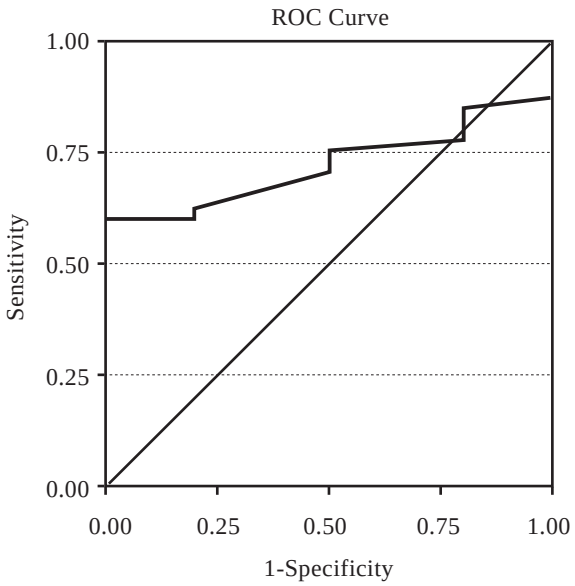


Fig. (2): ROC curve for p53 expression.

Diagonal segments are produced by ties.
1- Area under curve (AUC) = 0.72
2- Significance: <0.05 is significant.
3- The best cut off was 4.95% at which: Sensitivity = 60% and specificity = 100%.

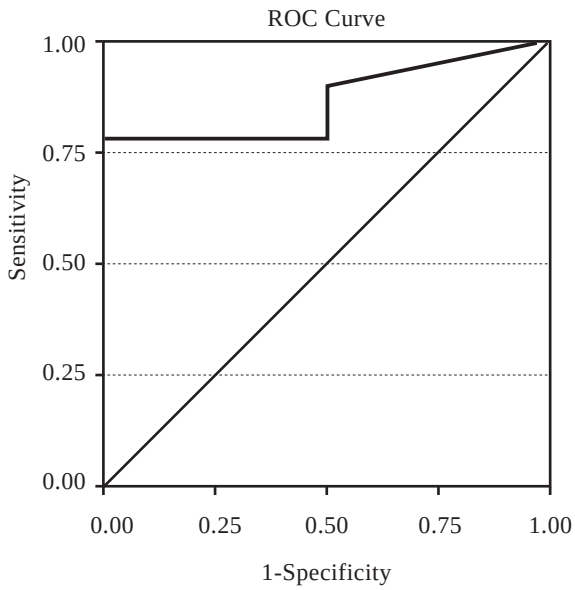


Fig. (3): ROC curve for MDM2 % expression.

Diagonal segments are produced by ties.
1- Area under curve (AUC) = 0.862
2- Significance: <0.05 is significant.
3- The best cut off was 0.09% at which: Sensitivity = 77% and specificity = 100%.

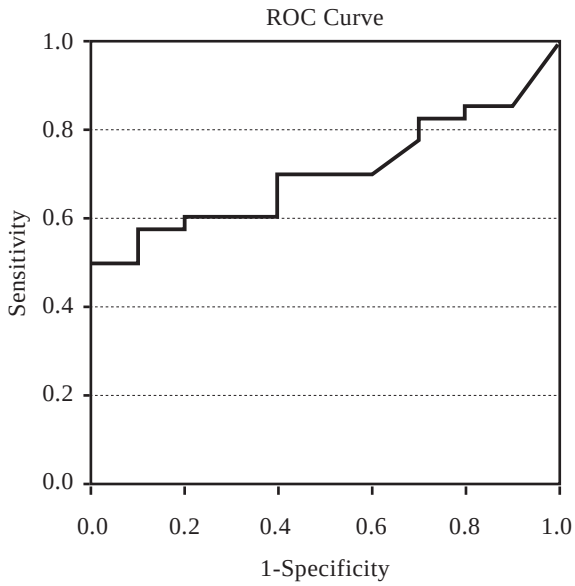
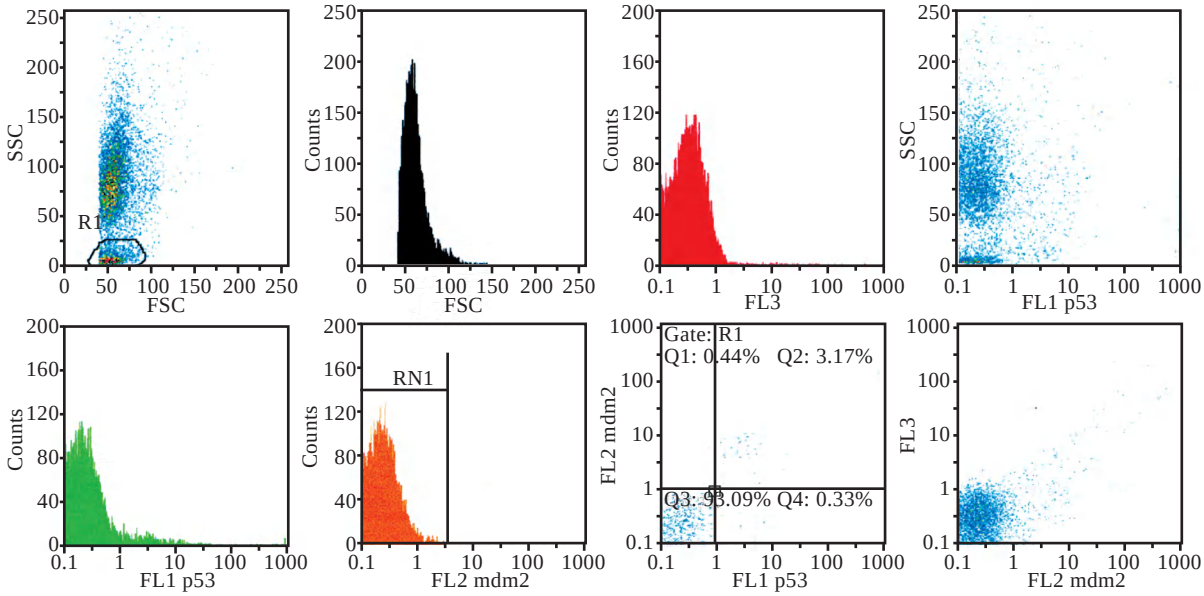


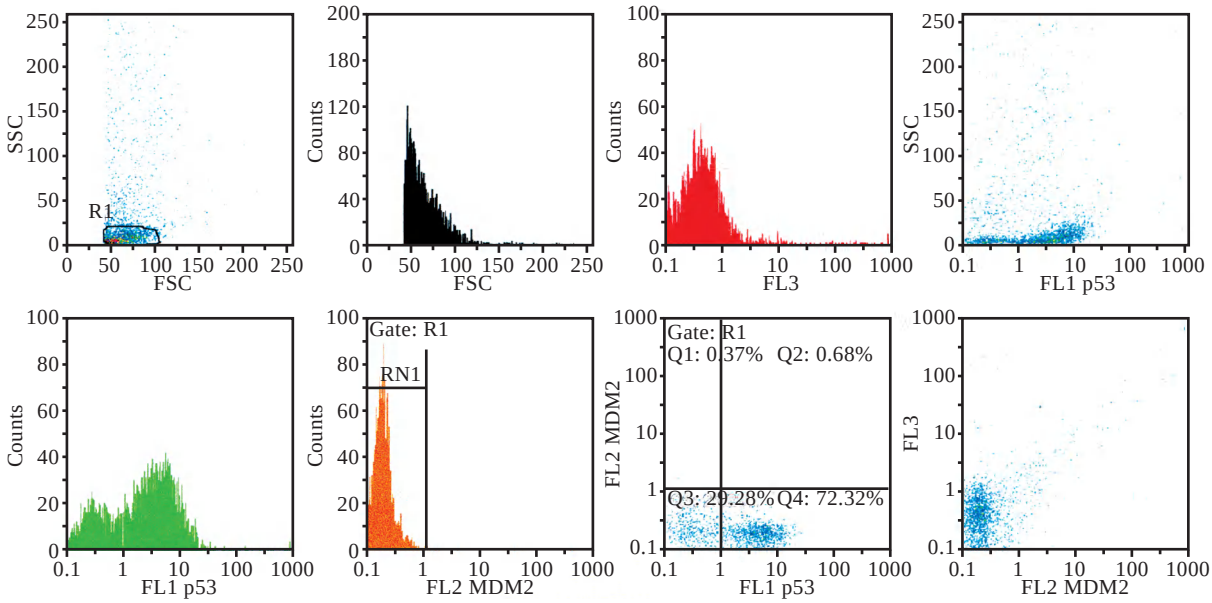
Fig. (4): ROC curve for MDM2 expression by MFI.

1- Area under curve (AUC) = 0.701
2- Significance: <0.05 is significant.
3- The best cut off was 1.16 at which: Sensitivity = 70% and specificity = 60%.



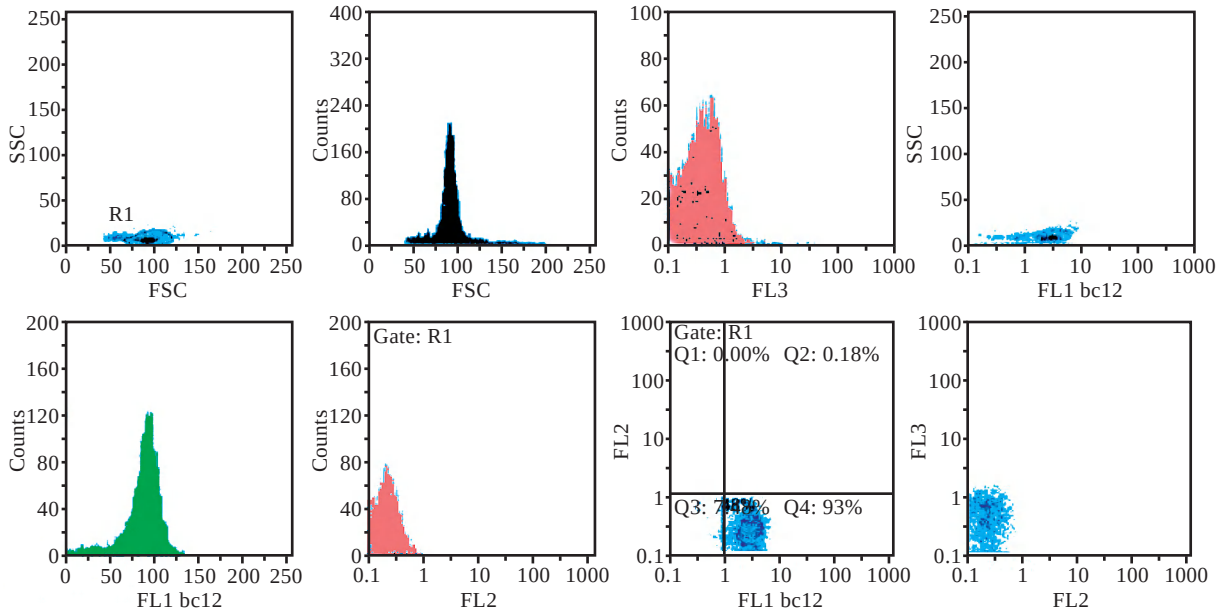
Region	Gate	Ungated	Count	Count/ml	% Gated	GMn-x	Mean-x	CV-x%	GMn-y	Mean-y	CV-y%
R1	<None>	160.7	1607	–	19.24	56.70	57.54	17.66	0.00	7.60	73.88
Q1	R1	61	7	–	0.44	0.13	0.20	131.31	1.33	1.76	109.79
Q2	R1	284	51	–	3.17	4.47	40.95	447.00	6.23	15.53	325.31
Q3	R1	7672	1496	–	93.09	0.16	0.20	75.68	0.16	0.20	76.66
Q4	R1	334	53	–	3.30	2.03	2.37	63.67	0.22	0.29	81.89
RN1	<None>	8216	8216	–	98.38	0.18	0.25	118.02	–	–	–

Fig. (5): Flow Cytometric analysis of a case of CLL, MDM2 positive and p53 negative.



Region	Gate	Ungated	Count	Count/ml	% Gated	GMn-x	Mean-x	CV-x%	GMn-y	Mean-y	CV-y%
R1	<None>	1909	1909	–	60.34	61.66	63.14	22.47	7.00	8.33	58.25
Q1	R1	30	7	–	0.37	0.23	0.27	61.37	41.14	281.58	93.96
Q2	R1	179	13	–	0.68	11.96	94.95	286.25	25.79	315.52	147.73
Q3	R1	922	559	–	29.28	0.30	0.36	60.79	0.23	0.26	55.18
Q4	R1	2033	1330	–	69.67	4.51	6.31	386.06	0.18	0.20	46.50
RN1	R1	2963	1891	–	99.06	0.20	0.22	54.17	–	–	–

Fig. (6): Flow Cytometric analysis of a case of CLL, which is p53 positive.



Region	Gate	Ungated	Count	Count/ml	% Gated	GMn-x	Mean-x	CV-x%	GMn-y	Mean-y	CV-y%
R1	<None>	3411	3411	–	79.68	93.89	94.21	8.23	5.41	5.88	39.46
Q1	R1	0	0	–	0.00	–	–	–	–	–	–
Q2	R1	52	6	–	0.18	10.78	24.31	135.03	2.30	3.14	84.75
Q3	R1	557	255	–	7.48	0.38	0.47	56.61	0.17	0.19	55.22
Q4	R1	3672	3150	–	92.35	2.76	2.99	39.76	0.20	0.24	60.46

Fig. (7): Flow Cytometric analysis of a case of CLL, which is BCL2 positive.

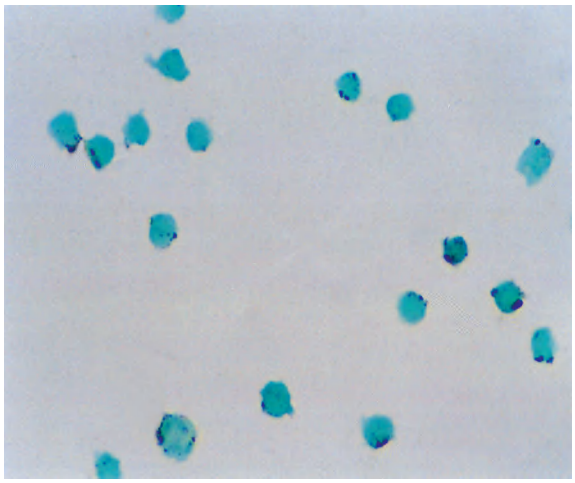


Fig. (8): p21 Positive nuclear expression by immunocytochemistry.

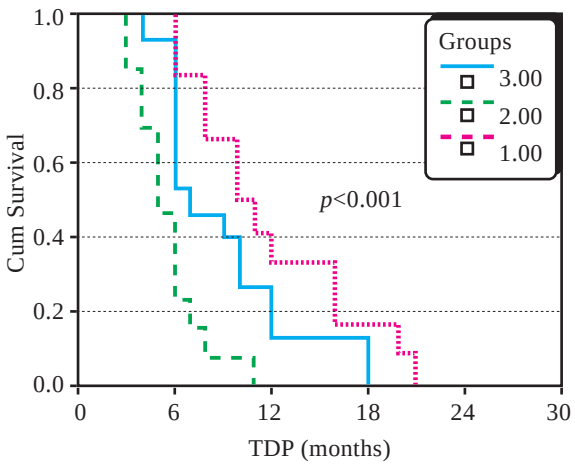


Fig. (9): Time to disease progression of the studied group according to p53 response to chemotherapy.

Table (4): Correlation with overall survival.

	(*r*)	*p* value
P53	–0.425	0.006
MDM2	–0.61	0.07
P21	0.325	0.041
BCL2	–0.09	0.57
CD34	0.116	0.4

r = Pearson correlation coefficient.

DISCUSSION

B-cell chronic lymphocytic leukemia (B-CLL) is characterized by the accumulation of long-lived CD5 (+) B lymphocytes. Several drugs currently used in the therapy of B-CLL act, at least partially, through activation of the P53 pathway. DNA-damaging agents increase p53 levels by posttranslational stabilization and induce p53-dependent cell death **[18]**. Importantly, B-CLL cells from patients with p53 mutations or deletions are associated with drug resistance and short survival **[19]**. As TP53 mutations occur in only 10% to 15% of patients with CLL **[12]**, it is possible that p53 dysfunction occurs in the disease through alternative mechanisms. MDM2 over expression has been reported as an alternative cause of p53 dysfunction **[20]**. MDM2 was suggested to abrogate the transactivating and growth inhibitory functions of the wild type p53 in tumor cells expressing this gene by binding to the acidic activation domain of p53 **[21]**.

In this study, p53/p21 response after chemotherapy was estimated in 40 CLL cases to detect the patient group having p53 dysfunction. MDM2 expression was measured as a trial to correlate its over expression to p53 dysfunction and decreased response to chemotherapy observed in those patients.

Of the 40 CLL studied cases, p53 and p21 measured after chemotherapy, showed a higher mean percentage expression than that of the normal control. These differences were statistically significant. This is in agreement with previous studies, which reported that chemotherapy of patients with chronic lymphocytic leukemia induces a p53-dependent gene expression response **[2]**. p53 accumulates in response to DNA damage and coordinates the cellular response to such damage by cell cycle arrest (by transcriptional activation of p21) or inducing apoptosis (by repressing BCL2) **[10]**.

However, in this study BCL2 was not repressed and was significantly higher than the control group. In accordance to our results, other researchers have found that, in response to DNA damaging effect, p53 regulates the expression of p21, but not BAX or BCL2 **[13]**.

A negative correlation was found between percentage expression of BCL-2 and CD38, with a significant correlation value. In contrast, other researchers suggested that CD38 confers to CLL cells a more malignant cellular phenotype. They explained that CD38 antigen has an important role as a modulator of intracellular signals and that cross-linking of CD38 up-regulates BCL-2 and inhibits apoptosis **[16]**.

MDM2 measured either by; percentage expression or MFI of the studied group was higher than that in the normal control. Several previous articles have reported that MDM2 protein is over expressed in a variety of neoplasms, including leukemia and lymphoma **[22,23]**.

Several mechanisms have been proposed for how MDM2 decreases the therapeutic benefits of cytotoxic drugs. The most obvious explanation is the role that MDM2 plays in p53 degradation. Because p53 is up regulated by DNA damaging agents, including chemotherapy and radiotherapy, the level of MDM2 is increased as a result of its role in feedback control. As a result, p53 degradation increases resulting in no subsequent increase in p21 so, preventing cell cycle arrest. MDM2 may also have a direct inhibitory effect on p21 **[24]**. In accordance, in this study a negative correlation was found between p21 and MDM2 percentage expression with a significant correlation value.

Another possible role for MDM2 in decreasing response to chemotherapy is by increasing expression of the multi drug resistance gene **[25]**. Thus there are many possible p53 dependants and independent mechanisms of action for the MDM2 mediated resistance to radiation therapy and chemotherapy. In this study we focused on MDM2 effect on p53 and its related molecules.

The study group was further subdivided according to p53 functional status into three subgroups, This categorization was suggested previously that impaired up regulation of p21 in response to DNA damaging effect defines a state of p53 dysfunction, while level of p53 itself determine the type of dysfunction Thus in type A defect, mostly associated with p53 mutation, p53 levels are increased, reflecting the prolonged half life of mutant p53 as compared with the wild type protein **[13]**.

In contrast, in the type B defect (suspected to be caused by MDM2 over expression), p53

levels were not increased in response to DNA damaging effect **[13]**. P53 percentage expression was highest in group 2 followed by group 1. Lowest expression was detected in group 3. The difference between groups was statistically significant.

In type A p53 dysfunction, the high percentage of p53 expression was suggested to be caused by p53 mutation. P53 mutation typically prolong the half-life of the protein, therefore increasing its level. This abnormally prolonged half-life enables the detection of p53 expression by flowcytometer or immunohistochemistry using anti p53 monoclonal antibody. However, even when activated, mutant p53 protein cannot regulate gene expression because of its inability to bind to specific DNA sequence **[11]**. This explains the low level of p21 observed in this group of patients.

The high p53 percentage expression detected in normal p53 response was explained in earlier studies. In quiescent cells, levels of p53 protein are low owing to its short half life. After DNA damage, the half-life of p53 becomes prolonged **[6]** and the protein accumulates in the nucleus **[7]**.

However, p53 level was higher in type A, compared to that of normal response group, suggesting a more stabilizing effect of p53 mutation on p53 protein level. This is in agreement with previous reports, which suggested that, the high p53 protein detected should be considered as a marker of p53 gene mutation **[5,26]**.

P21 showed a mean percentage expression in type A and in type B dysfunction group significantly lower than that of normal response group ($p<0.001$). This is in agreement with what was previously documented. The expression and function of p21 after DNA damage appear to be strictly dependent on the presence of functional wild type p53 **[27]**. So, the decrease in p21 level in both type A and type B dysfunction groups confirms their categorization as having p53 dysfunction.

Level of MDM2% expression or MFI was highest in patients with type B p53 dysfunction (suggested to be caused by MDM2 over expression) followed by those with p53 normal response. The lowest expression was detected in patients with type A p53 dysfunction (suggested to be caused by p53 mutation). This is in accordance of previous reports, which stated that an excess of MDM2 protein could abrogate transcriptional activation by wild type p53 **[28]**.

The difference of mean percentage CD38 expression between normal response, type A and type B dysfunction groups was statistically not significant. Our results suggest no correlation between CD38 expression and p53 functional status. This is in accordance with what was reported earlier **[16]**.

Also no significant difference was found on comparing the 3 groups regarding BCL2 percentage expression. Previous reports have suggested that p53 was regulating the expression of p21, but not BCL2 **[13]**.

A positive correlation was found between LDH level and p53% expression with a significant correlation value. Also, a negative correlation was found between p53% expression and over all survival with significant correlation value.

P53% expression showed no significant correlation with the other known prognostic factors such as age, Hb concentration or platelet count. Previous studies have reported significant direct correlations between the percentage of p53 positive staining cells and other CLL aggressive features including ß2-microglobulin, lower hemoglobin level and increased age **[29]**.

Among patients with advanced stages of the disease (stage III and IV), 47.6% were of type A p53 dysfunction, 42.9% were of type B p53 dysfunction and only 9.5% were of normal p53 response group. This difference was statistically significant. These data are consistent with that reported by others **[30,31]**.

In our study, Patients with normal p53 response tend to have a higher complete response rate and less treatment failure with statistically significant longer time to disease progression (mean 12 months ±5.1 SD) than either those with type A (mean 5.8 months ±2.3 SD; $p=0.001$) or type B (mean 9 months ±4.4SD; $p=0.08$) p53 dysfunction. In accordance, previous studies, had reported that MDM2 overexpression influences the cellular response to cytotoxic/DNA damaging agents and as negative regulator of p53 is related to decreased response

to both chemotherapy and radiation therapy and increased risk for relapse **[29,31]**. Others had reported that MDM2 expression become markedly reduced or absent during remission **[32]**. On comparing the TDP between type A and type B dysfunction, it was shorter in type A than in type B. In accordance, Francis et al., 2003 **[16]**, reported that positive p53 percentage expression was strongly associated with p53 gene mutation and progressive disease.

A higher percentage of dead patients was detected in group "2" (54.5%) and group "3" (45.5%) compared to normal response group (0%) (p=0.02). On comparing OS of patients with type A versus that of type B dysfunction, no statistically significant difference was obtained. However, a high significant decrease of overall survival was observed on comparing type A with normal response group (p=0.001). In agreement with our results, other investigators reported that positive expression of p53 protein as measured by immunohistochemistry was strongly associated with p53 gene mutation, refractoriness to therapy and reduced survival **[29]**.

On comparing OS of type B dysfunction to that of normal response group, no statistically significant difference was obtained (p=0.3). However, a negative correlation was found between MDM-2% expression and over all survival, where r=–0.61 and p=0.07 with borderline significant correlation value. Previous studies reported that on separating patients according to their p53 functional status, those with demonstrable p53 dysfunctional status had a much shorter disease specific survival. On subdividing the p53 dysfunctional cases, patients with the type A p53 defect, had a significantly shorter survival than patients with type B defect **[16]**.

From this study we conclude that:

- P53 dysfunction groups had a decreased response rate, a more advanced stage of the disease and less TDP than that of p53 with normal function group. This goes with the established importance of the p53 pathway in maintaining genomic integrity and mediating the action of certain cytotoxic agents including purine analogues.
- A decrease in overall survival was observed in groups with p53 dysfunction. This suggests that p53 function test is a powerful predictor of outcome in CLL.
- On comparing the two methods used for evaluating MDM2 expression, both percentage expression and MFI revealed a significant increase of MDM2 in group 3. However, using percentage as cut off point is subjective. So the use of both MFI in association with percentage positivity can be a better predictor of disease progression and outcome of the disease than percentage alone.
- Type B p53 dysfunction group (with high MDM2 expression level) showed a higher percentage of patients in advanced stage of the disease, decreased response rate and decreased TDP. So MDM2 may be used as a marker for advanced stage. Cut off values of 0.09% and 1.16 by MFI was suggested in this study.

Recommendations:

- Considering results of this study and the established oncogenic potential of over expressed MDM2 proteins, a possible role of MDM2 proteins in promotion of CLL disease remains to be further evaluated.
- Drugs that target MDM2-p53 interaction could provide a novel therapeutic strategy for CLL should be investigated for clinical applications in the treatment of CLL.

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Journal of the Egyptian Society of Haematology & Research, Vol. 2, No. 2, September: 87-94, 2006

The Repertoire of Cytokines Produced by Human Leukocyte Subsets

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ABSTRACT

We defined the cytokine repertoire of leukocyte subsets after activation with phorbol myristate acetate (PMA) and ionomycin (IONO) or lipopolysaccaride (LPS). We found that granulocytes produced only IL-8. IL-3, 11-13, IL-15, IL-18, TNFß and FLT3 were not produced by any subset. B cells exhibited no intracellular cytokines nor did T cells and NK cells when stimulated by LPS.

When stimulated by PMA + IONO, T cells and NK cells did not produce IL-4, IL-6 or IL-10, but produced INFγ, TNFα and MDP-1ß. T cell subsets also produced IL-la, IL-lα, IL-2, IL-8, IL-12, GM-CSF, G-CSF and M-CSF, except the CD4-CD8- subset, which did not produce IL-1α. NK cells produced the same cytokines except IL-2. Monocytes constitutively produced IL-8, but when stimulated by PMA + IONO, they produced IL-lα, IL-lß, IL-6, IL-8, IL-12, TNFα, M-CSF and MIP-lß. LPS induced additional production of IL-10 and G-CSF and enhanced the production of IL-6 by monocytes.

Key Words: *Activation - Characterization - Flow cytometry.*

Abbreviations used:

PB : Peripheral blood.
TH1 : T-helper cell type 1.
TH2 : Helper cell type 2.
IL-X : Interleukin X.
TNF : Tumor necrosis factor.
PMA : Phorbal-13-myristate acetate.
IONO : Ionomycin.
LPS : Lypopolysaccharide.
FSC : Forward scatter.
SSC : Side scatter.
FITC : Fluoresce in isothiocyanate.
PE : Phycoerythrin.
PC-PE : Cyanin 5.
PcP : Peridinin chlorophyll protein.
APC : Allophycocyanin.
PBS : Phosphate buffered saline.
PAB : PBS supplemented with 0.5% bovine serum albumin (BSA) and 0.1% sodium azide.

INTRODUCTION

Cytokines and chemokines are the molecules that provide communication among cells. A single cytokine is but one word, and it is the repertoire of them all when simultaneously presented to the target cell that results in the function elicited. The cytokines come from many different cell subsets, each of which is itself a cytokine target. Within this milieu occur the functions of life.

While there is a wealth of data on the characterization of the cytokines themselves extensively summarized **[1]**, nearly all the information about cellular production is from cell lines, predominantly murine, and to a lesser extent, normal murine hematopoietic cells. In human, most of our understanding about cytokines has focused on the TH1 and TH2 responses, and the characterization of the cells that produce the TH1 cytokines, IL-2, IL-4 and INFγ, or the TH2 cytokines) **[2-4]**. More recently, production of cytokines by dendritic cells has been evaluated because of their recognized importance in antigen presentation **[5-8]**. Furthermore, most of the research in our current understanding of their action has been on a very limited number of cytokines for the chosen subset for study.

There are currently over 30 cytokines and 11 chemokines that have been associated with human leukocyte subsets **[1]**. No comprehensive study was found that evaluated the identity of human leukocyte subsets from healthy donors that produce these cytokines. Defining all the conditions under which they are produced would certainly be an enormous task and is currently being addressed by many investigators. We hypothesize that the repertoire of cytokine pro-

duction by resting human leukocyte subsets and those stimulated with PMA + IONO, as an example of a general stimulus, and LPS, as an example of an important specific activation molecule, will differ by both Subset and stimulus. Results clearly show that not all cytokines are produced by leukocyte subsets. Our hypothesis was confirmed, as the results also show the repertoire of cytokines that are produced is the result of the kind of stimulus presented to the responding cells. They also show that there is a great deal of heterogeneity in the frequency of cells within a subset that produce any given cytokine.

MATERIAL AND METHODS

A total of 11 peripheral blood (PB) samples from healthy adult subjects were analyzed under a protocol approved by the RPCI Institutional Review Board. All specimens were obtained as excess material used for other clinical tests. All samples were collected in heparinized green top vacationers (Becton Dickinson, San Jose, CA) as anticoagulant (10 units/ml).

Two different activation regimens were used. In the first, PMA at 1.0µg/ml (Sigma, St. Louis, MO) and IONO at 50µg/ml, from streptomycines conglobatus, (Sigma) were used. In the second, LPS at 100ng/ml from Escherichia Coli Serotype 026-B6 (Sigma) was used. These were all final concentrations.

Cytokine production was analyzed after six hours of incubation of PB supplemented with an equal volume of RPMI 1640 (GIBCO Life Technology, Grand Island, NY) in the presence of 10µg/ml Brefeldin A (Sigma). The incubation was performed at 37°C in a 5% CO_2 humid atmosphere. Three conditions were evaluated. First, no activation was provided so basal endogenous cytokine levels could be evaluated. Second, cells were incubated after adding PMA and IONO in a final volume of 1ml. Third, 1ul of LPS (Sigma) was added also in a final volume of 1ml. Cells were at a final concentration of 10^6/ml. The medium also contained 15% fetal bovine serum.

Immediately after the incubation period, both stimulated and unstipulated samples were transferred to an ice bath. To block Fc receptors, 34ul of a 3mg/ml solution of normal mouse IgG (Caltag/Burlingame, CA) was added to each tube and incubated on ice for 10 minutes.

Samples were then liquated into 20 tubes of 100ul each and stained with appropriate combination of monoclonal antibodies to membrane markers, as previously described in detail **[9]**. The combinations and amount added to tubes was as follows: 5ul CD3-FITC (Becton Dickinson), 5ul CD56-PE (Beckman-Coulter, Hialeah, FL) and 5ul CD33-PC (Immunotech-Beckman-Coulter, Hialeah, FL). The second and third combinations were evaluated on only eight donors: CD3-FITC CD8-PE (Caltag) CD4-PcP (BD) CD41-FITC (Caltag) (CD3CD56CD33)-PE CD19-PC (Immunotech-Beckman Coulter). We purchase our reagents in bulk at higher concentration than when they are delivered in 100 test vials. All reagents are titered prior to use to verify the saturating concentration as described in detail elsewhere **[5]**.

After gentle mixing, cells were incubated for 15 minutes on ice. Erythrocytes were lysed using three ml of pre-warmed (25°C) lysing reagent (8.26gm NH_4C1 + 1.00gm KCO_3 + 0.037gm tetra Na-EDTA in one liter distilled water) into each tube, inverted twice and centrifuged at 1500 x G for three minutes at room temperature. After discarding the supernatant and re-suspending the cells in residual buffer the cells were fixed in 200ul of 2% ultra-pure formaldehyde (Polysciences, Malvern, PA). Three ml of PAB (PBS + 0.5% BSA + 0.1% NaN_3) was added to each tube and centrifuged at 1500 x G for three minutes at room temperature. After discarding the supernatant, the cells were permeabilized with 100 ul of Permeabilization reagent (Caltag) followed immediately with five ul of appropriate biotinylated antibody to the appropriate cytokine. The cytokine antibodies used in this study were IL-lα, IL-1ß, IL-2, IL-3, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, IL-15, IL-18, INFγ, TNFα, TNFß, GM-CSF, G-CSF, M-CSF, MIP-1ß and FLT3. They were all from R&D Systems except IL-4, which was from (BD PharMingen, San Diego, CA). After incubation for 30 minutes at room temperature, 3ml of PAB was added to each tube for 15 minutes. This incubation step provides time for unbound antibody to diffuse out of the cell. They were then centrifuged at 1500 x G for three minutes at room temperature; decanted and blotted; the cells were permeabilized again with 100ul of Permeabilization reagent followed immediately with 5ul of APC streptavidin (Caltag) and incubated for 30 minutes at room tem-

perature. Three ml of PAB was added for 15 minutes, the cells were centrifuge as above, the supernatant discarded and the cells fixed in 250ul of 2% ultra-pure formaldehyde.

Data was acquired using a FACSCalibur flow cytometer. The instrument performance was verified daily using microspheres. PB stained with CD45-FITC, CD4-PE, CD8-PC or CD45- APC as single color reagents in separate tubes. A tube creating cells co-stained with CD45-APC, CD4-PE and CD8-PC was also prepared. After verifying instrument timing according to instructions supplied by the manufacturer, microspheres and stained cells were acquired. (They all must appear in a specific region for the instrument to be verified). These important evaluation processes have been described in more detail elsewhere **[9]**. Color compensation was performed using the single color reagent stained cells and verified using the co-stained cells, as previously described in detail **[11,12]**. Twenty thousand ungated events were acquired on every sample.

Data was analyzed using WinList (Verity Software House, Topsham, ME). The gating strategies are described in the results. Data from WinList was transferred to EXCEL where the mean and standard deviation were computed.

RESULTS

We initially screened three donors for endogenous production, and for production after PMA + IONO or LPS stimulation. IL-lα, IL-1ß, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, INFγ, TNFα, GM-CSF, G-CSF, M-CSF and MIP-1ß were the 14 cytokines found to be expressed in >2% of cells within at least one subset and these were further evaluated in eight additional healthy donors.

The leukocyte subsets, shown in Fig. (1), were resolved into monocytes (CD33 high), granulocytes (CD33 1ow), T cells (CD3+), Helper T cells (CD4+), Suppressor T cells (CD8+), cytotoxic T cells (CD3+ CD56+), NK cells (CD3- CD56+), B cells (CD19+) and platelets (CD41+). The frequency of each subset for 11 healthy donors used in this study is shown in Table (1). The values are consistent with our clinical reference ranges. The strategy for resolving each leukocyte subset is shown in Fig. (1). The forward scatter (FSC) versus side scatter (SSC) bivariate histogram, shown in A, was not used for gating. The first antibody combination, which contains CD3-FITC, CD56-PE and CD33-PC) is shown in Fig. (1B-C), and it can be divided into CD33 high fluorescent cells that are monocytes and CD33 low fluorescent cells that are predominantly granulocytes. Dendritic cells were less than 1% of these cells and were not studied. After gating on the CD33 negative population, cytotoxic T cells and NK cells are resolved as shown in Fig. (1C). The combination CD3-FITC CD4-PE and CD8-PC, shown in Figure ID, after gating on CD3+ cells, produced the four T cell subsets based on CD4 and CD8 expression. The combination CD41-FITC, (CD3, CD56, CD33)-PE and CD19-PC was used to resolve platelets and B cells. This was accomplished by producing the Boolean gate: NOT (CD3 or CD56 or CD33)-PE and gating the bivariate histogram (CD41 versus CD19), as shown in Fig. (1E).

PMA + IONO had an extremely toxic effect on granulocytes and not all CD33 1ow cells were found to be granulocytes. The kinetics of granulocyte killing is shown in Fig. (2). By five hours only about 5% resistant cells (CD33dim) were found. We identified this resistant subset by immunophenotyping and cell sorting (for morphological assessment), The resistant cells were found to be highly granular monocytes (data not shown). The CD33 1ow subset of monocytes found in the blood may be newly released and less differentiated monocytes from bone marrow that also exhibit a lower expression of CD33. These cells are negative or express CD14 at a lower intensity and can be completely resolved as monocytes because their CD4 expression is identical to that found on mature monocytes. (Data not shown).

Since cytokine expression by granulocytes stimulated with PMA + IONO could not be evaluated; only LPS stimulation was studied. IL-8 was the only cytokine produced by a significant fraction (40%) of granulocytes after LPS. No endogenous cytokine was produced by granulocytes.

Cytokine production by monocytes is shown in Fig. (3). Unstimulated monocytes produced only IL-8 and no other cytokines. For PMA + IONO, monocytes could be divided into CD33 high and CD33 1ow subsets. IL-lα and ß, IL-

6, IL-8, TNFα, M-CSF and MIP-1ß, were produced by both CD33 high and low monocytes. The CD33 high monocyte subset had a greater frequency producing IL-lα and ß, IL-8, TNFα, M-CSF and MIP-1ß while the CD33 low subset produced more IL-6 as well as some IL-4 and IL-12. Some of this production could be associated with dendritic cells that were not specifically resolved. LPS induced the highest frequency of cytokine positive monocytes and they did not produce IL-2, IL-4, INFα, GM-CSF and M-CSF.

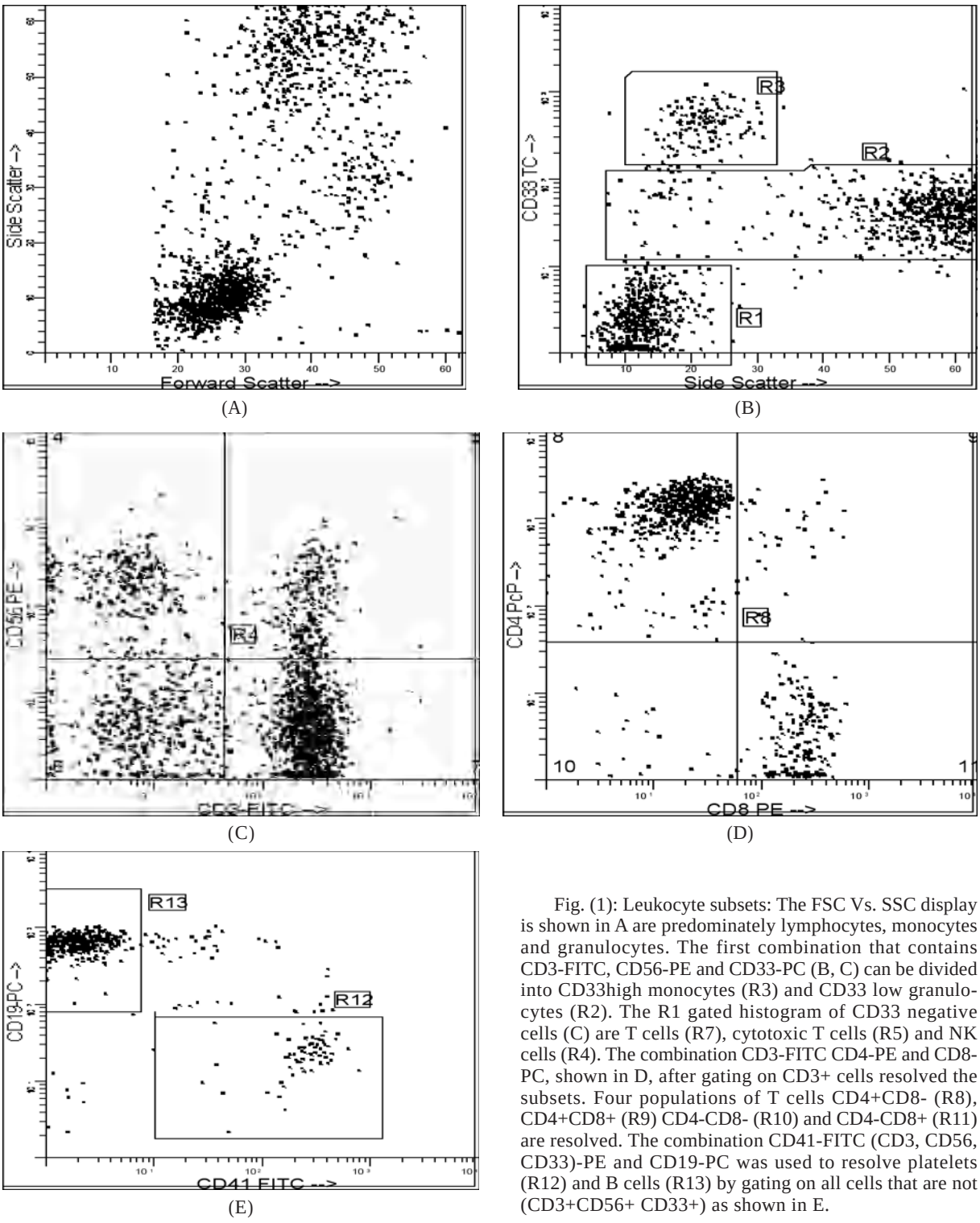

Fig. (1): Leukocyte subsets: The FSC Vs. SSC display is shown in A are predominately lymphocytes, monocytes and granulocytes. The first combination that contains CD3-FITC, CD56-PE and CD33-PC (B, C) can be divided into CD33high monocytes (R3) and CD33 low granulocytes (R2). The R1 gated histogram of CD33 negative cells (C) are T cells (R7), cytotoxic T cells (R5) and NK cells (R4). The combination CD3-FITC CD4-PE and CD8-PC, shown in D, after gating on CD3+ cells resolved the subsets. Four populations of T cells CD4+CD8- (R8), CD4+CD8+ (R9) CD4-CD8- (R10) and CD4-CD8+ (R11) are resolved. The combination CD41-FITC (CD3, CD56, CD33)-PE and CD19-PC was used to resolve platelets (R12) and B cells (R13) by gating on all cells that are not (CD3+CD56+ CD33+) as shown in E.

Table (1): Leukocyte subset differential for healthy donors.

Control	Percent of total cells	Absolute count	Range (Mean ± 2 Std)
CD33 high	5.6	336	264-408
CD33 1ow	50.8	3048	2160-3936
T cells (CD3+)	20.9	1254	1026-1482
CD4+CD8-	13.8	828	588-1068
CD8+CD4-	5.6	336	180-492
CD4+CD8+	0.51	31	22-40
CD4-CD8-	0.71	43	20-66
CD56+	1.5	90	6-174
NK cells (CD3-CD56+)	3.1	186	42-330
B cells (CD19+)	2.9	174	90-258
Platelets (CD41+ D45-)	1.7	102	6-204

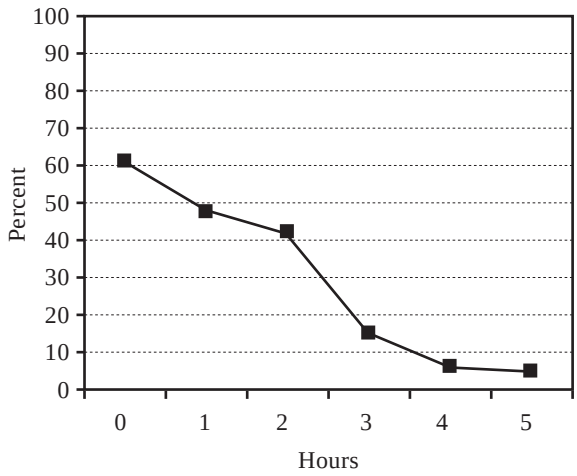

Fig. (2): The kinetics of granulocyte killing by PMA. Blood was incubated for five hours with PMA + IONO. At one-hour intervals an aliquot was removed and the percentage of CD33 1ow cells remaining was determined.

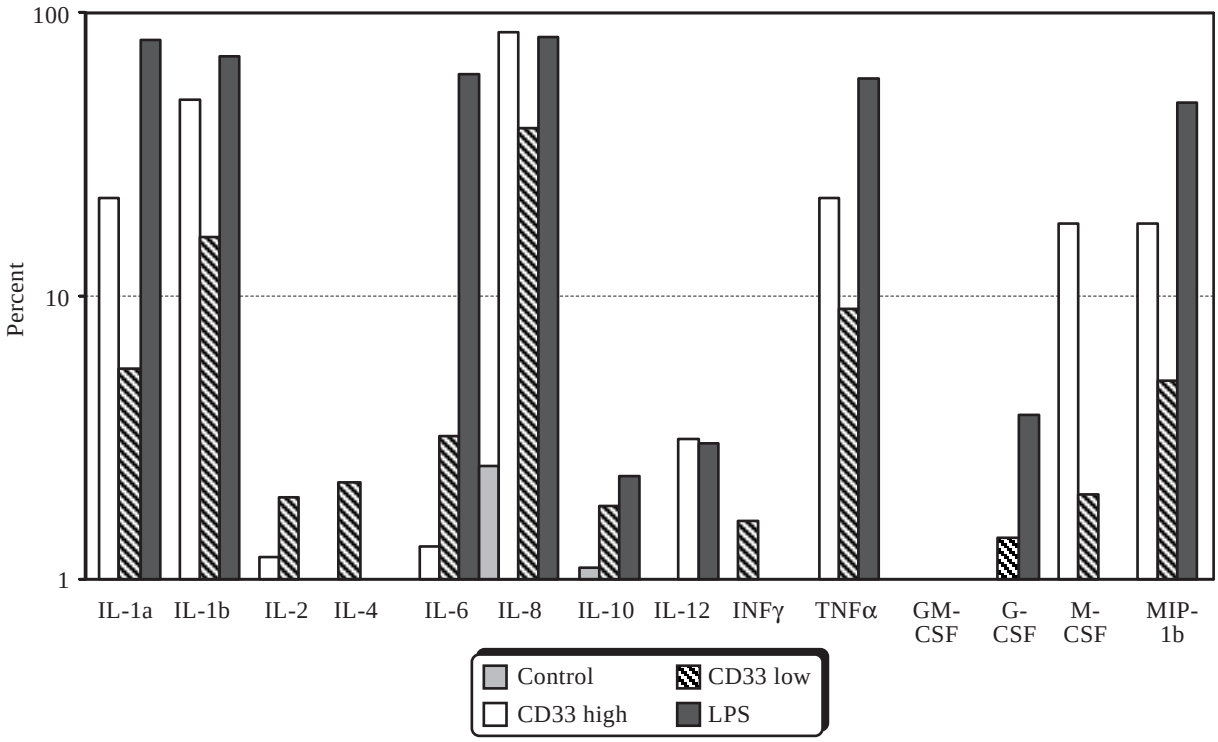

Fig. (3): Cytokine Production by Monocytes. The percentage of monocytes that produce cytokines constitutively (control) and after PMA + IONO or LPS stimulation for six hours is shown. Monocytes stimulated with PMA + IONO could be further resolved into CD33 high and CD33 low populations.

CD3+ T cells can be divided into five subsets. CD4+CD8-, CD4+CD8+, CD4-CD8-, CD4-CD8+ and CD56+ (Figure 1). The latter is also a subset of CD8+ T cells and is the large granular cytotoxic T cell fraction. The CD3-CD56+ cells are NK cells. As shown in Fig. (4), PMA + IONO was the most potent stimulus for inducing cytokine production by T cells. Using an arbitrary threshold of 2% positive cells for cytokine production, all the cytokines were evaluated except IL-3, IL-4, IL-6, and IL-10 and were produced by at least one subset of

T cells. The CD4+CD8+ subsets produced IL-lα, IL-l*ß*, IL-2, IL-8, IL-12, INFγ, TNFα, GM-CSF, G-CSF, M-GSF and MIP-l*ß*. This immature subset represented only 0.51% of cells. The CD4-CD8- subset represented 0.71% of cells and they are mainly delta gamma TCR expressing T cells and they produced IL-1*ß*, IL-2, IL-8, INFγ, TNFα, M-CSF and MIP-1*ß*. The CD4+CD8- and CD4-CD8+subsets produced IL-2, IL-8, INFγ and TNFα. The CD4+CD8- cells produced GM-CSF, while MIP-l*ß* was produced by the CD4-CD8+ subset. The T cell subset (CD3+CD4-CD8+CD56+) produced IL-2, INFγ, TNFα, GM-CSF and MIP-1*ß*. Except for IL-2, NK cells (CD3-CD56+) also produced these cytokines. No lymphocytes were found to produce detectable cytokines if they were not stimulated or if they were stimulated with LPS. Neither B cells nor platelets produced any cytokines with any regimen used in this study.

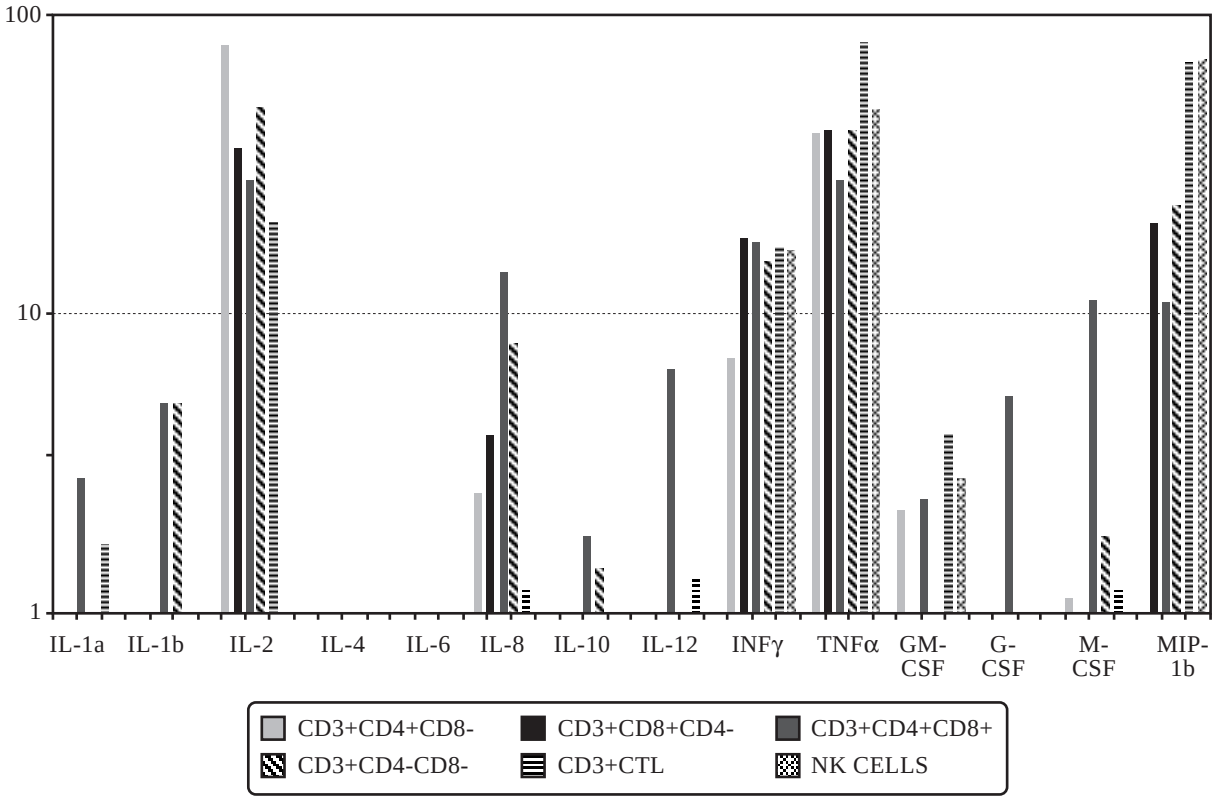

Fig. (4): Cytokine Production by Lymphocytes. Blood was incubated six hours with PMA + IONO. The frequency of positive lymphocytes for intracellular cytokines is shown for all subsets that produced cytokines. The four major subsets of T cells as well as the CD8+ cytotoxic T cell subset (CTL) and NK cells are shown. B cells did not produce any measurable cytokines.

DISCUSSION

Our study represents the most comprehensive evaluation of cytokine production reported so far by leukocytes from healthy human donors, We compared our results to similar reports **[5,13-20]**, and with a few exceptions our results mirror those of others. Our study design, however, differs from others in that intracellular cytokine production was measured by phenotypically resolved subsets of all major leukocyte populations. Baran, et al. **[13]** reported production of IL-4 and TNF*ß* by CD4 and CD8 T cells stimulated with PMA + IONO that differ somewhat from our Studies. Their placement of the marker for positive cells is considerably different from ours on the univariate histogram even though it was objectively set using an isotype control. This is the most likely reason for the difference and underscores the subjectivity of marker placement for determining positive and negative cells and the effect it can have on results when so few cells are actually positive. There appears to be a misprint in the report by Krampera, et

al. **[14]** in the results of TNFα and TNF*ß*. Their percentage positive CD4 and CD8 T ceils are reversed in the text from those reported in Table (1). Otherwise, their results are in agreement with those reported here.

Bueno, et al. **[5]** found 55±13% positive monocytes expressed IL-12. We did not find IL-12 expression by monocytes activated by LPS alone. This difference is likely due to the addition of TNFα to the stimulation protocol, demonstrating the profound influence of cytokines in the activation process. They and others have also shown that dendritic cells, which are present at a very low frequency (less than 1% in our specimens) and were not resolved by us, are a dominant source of this cytokine. While performed in separate studies, it is certainly likely that combinations of activators can produce profound differences in the repertoire of cytokines produced by any leukocyte subset. These are the words cells communicate with.

Because the frequency of cells producing most cytokines is low, and in most cases less than 5% of cells resolved by the antibody combination, it is clear that the antibodies we used to subset some populations are much too broad. Thus, those cells producing any given cytokine in low frequency are themselves a subset within the subset that might be resolvable using combinations of more specific differentiation markers. Dendritic cells are a prime example of the importance of this approach. We are focusing on the development of more specific differentiation markers associated with the subset of cells that produces the cytokine to better resolve them from those that do not.

When cytokines are combined with other agents the leukocyte response is likely to be specific for the combination. Because of the huge number of possible combinations, it will be a huge task to decode the language of cells. We believe it will be necessary to create a centralized database, similar to that found for the genome project. This database will then become the dictionary of words used by cells.

Supported by grants CA16056, NIHCA 602006 from NIH and the New York State Department of Health. The authors wish to thank Earl Timm, Jr., Sigrid J. Stewart, and Marilyn Price for their assistance.

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Journal of the Egyptian Society of Haematology & Research, Vol. 2, No. 2, September: 95-102, 2006

Viral Infections: Is it a Possible Etiological Factor of Pediatric Acute Lymphoblastic Leukemia?

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ABSTRACT

Introduction: Pediatric acute lymphoblastic leukemia (ALL) is considered the commonest malignancy affecting children. There is some supportive evidence for an infectious etiology to ALL.

Objective: To evaluate the possible etiological relation between some viral infections (Epstein Barr virus, Cytomegalovirus, Herpes Simplex virus-1, Hepatitis B, C and G viruses) and pediatric ALL.

Patients and Methods: Thirty newly diagnosed ALL patients, with a negative history of blood transfusion, at the National Cancer Institute (NCI), Cairo University were included. Their mean age was 3.6±1.2 years. They were subjected to complete medical evaluation and investigations including complete blood picture and bone marrow aspiration with or without biopsy. Polymerase Chain Reaction (PCR) for Epstein Barr virus (EBV), Cytomegalovirus (CMV), Herpes Simplex virus-1 (HSV-1), Hepatitis B (HBV), C (HCV) and G (HGV) viruses was done for all patients.

Results: PCR results showed seropositivity of 40% for CMV, 36.7% for HSV-1, 10% for each of EBV and HBV, 23.3% for each of HCV and HGV and 6.7% for combined HBV and HCV.

Conclusion: The results of our study are more towards the possibility of viral infections having a role in the etiology of pediatric ALL. Further studies involving larger sample of population and including other viruses are recommended to investigate the viral etiology theory.

Key Words: *Pediatric ALL - Viruses - Infectious etiology.*

INTRODUCTION

Childhood leukemia is a biologically and clinically diverse disease and probably arises via several etiological pathways **[1]**. Only a few cases can be explained by known risk factors, such as ionizing radiation, cancer chemotherapy or Down's syndrome. The etiology of childhood leukemia remains uncertain, but recent research has provided new clues. Both genetic susceptibility and environmental exposures are likely to be involved.

It is possible that both prenatal and postnatal environmental exposures may play a crucial role in triggering the onset of leukemia. The process leading to the onset of childhood leukemia is likely to involve at least two events **[2,3]**. Whilst the first event may be either germ line (although this appears to be rare in childhood leukemia) or somatic because of endogenous or environmental factors, the final 'critical' event may always involve an environmental factor. Both events would lead to cellular genetic changes and/or the proliferation of premalignant clones. Infections have been considered a prime candidate for such environmental etiological agents that promote the onset of leukemia. Epidemiological evidence suggests that infection is involved either by stimulating an inappropriate immune response or in the form of a classical transforming agent **[4]**. Several observations contribute to the theory that a transmissible agent is potentially involved in the oncogenic process of childhood leukemia. First, the peak incidence of childhood leukemia and that of common childhood infections both occur among children 2-5 years of age, the age group least likely to possess sophisticated immune systems **[5]**. Second, a viral etiology has been shown for some animal and human cancers (e.g. EBV for Burkitt lymphoma) **[1]**. Third, evidence exists of an apparent seasonal variation in the onset of childhood leukemia. Statistically significant seasonal variation for ALL with a peak in the

summer **[6]**, in the autumn-winter **[7]** and in the early spring among 1- to 6-year-old children **[8]** have been described.

Several viruses have been incriminated in the causation of ALL in childhood. Viral particles have been isolated in cultures of mononuclear cells from patients with ALL **[9]**.

The aim of our study is to evaluate the possible etiological relation between some viral infections (EBV, CMV, HSV-1, HBV, HCV and HGV) and pediatric ALL.

PATIENTS AND METHODS

Patients:

Thirty patients with ALL diagnosed and managed at NCI, Cairo University were randomly selected to participate in this study. All patients were newly diagnosed by means of complete blood picture and bone marrow aspiration with or without biopsy. They all had a negative history of blood transfusion. Patients with ALL associated with other possible known etiology e.g. Down syndrome were excluded.

Detection of active viral infection was done using Polymerase Chain Reaction (PCR) for EBV, CMV, HSV-1, HBV, HCV and HGV.

Methods:

Clinical evaluation of the patients included full history taking and thorough physical examination. Laboratory investigations included complete blood picture, bone marrow aspiration with or without biopsy and PCR for EBV, CMV, HSV-1, HBV, HCV and HGV.

Collection of serum samples:

Five milliliters of peripheral venous blood were collected from patients by venipuncture for PCR assessment. Samples were centrifuged to separate the sera. The latter were kept at a temperature of –80ºC till PCR assessment.

Molecular biology methods:

I- *RNA Extraction for detection of HCV and HGV-mRNAs:*

Total RNA was extracted from all sera using SV total RNA extraction kit provided from Promega Corporation, Madison, WI, USA. The amount of RNA was quantitated by reading the Optical Density (OD) at wave length of 260nm by using spectrophotometer.

A- *cDNA synthesis and PCR of HCV:*

The RT-PCR method here is the one step method. The kit was provided from Qiagen. The RT-PCR amplification protocol was formed of: Qiagen one step RT-PCR buffer (5x), dNTPs (10µM), primer (1CH, 10µM), primer (2CH, 10µM), Qiagen one RT-PCR enzyme mix and template RNA in a total volume of 50µL. The oligonucleotide primer sequences used for amplification of HCV were illustrated in Table (A)

The PCR cycling condition was as follows: one cycle of 50ºC for 30min, one cycle of 95ºC for 15min followed by 40 cycles of 95ºC for 1min, 45ºC for 1min and 72ºC for 1min. Then extension for 10min at 72ºC. To increase the sensitivity of the RT-PCR assay of HCV, 3µL of the first amplified product was subjected to nested PCR. The sequences of the nested oligonucleotide primers (4CH & 1TS) were illustrated in Table (A).

B- *cDNA synthesis and PCR of HGV:*

The RNA was reverse transcribed by using random primer, first strand buffer, dNTPs, RNase inhibitor and MMLV. The total volume of 50µL was introduced to PCR apparatus for one hour at 37ºC followed by 5min at 95ºC. The sequence of the primers used was illustrated in Table (A). The PCR mixture was performed in a total volume of 100µL reaction volume containing 5µL of the cDNA, buffer 2 (50mM KCl, 0.01% gelatin and 10mM Tris HCl, pH 8.3), $MgCl_2$ (2mM), dNTPs (100mM each), primer (101-sense), primer (285-antisense) and Taq polymerase (5u/µL). The PCR cycling condition was: 95ºC for 5min followed by 40 cycles of 94ºC for 1min, 55ºC for 1min and 72ºC for 1min.

II- *DNA Extraction for detection of HBV, HSV, CMV and EBV:*

DNA was extracted from plasma samples using the QIAamp® DNA minikit (Qiagen, USA) following the manufacturer's instructions. The extracted DNA was quantified and checked for purity using a spectrophotometer.

A- *PCR of HBV:*

The PCR mixture was performed in a total volume of 100µL reaction volume containing DNA, 10X buffer, $MgCl_2$, dNTPs, primer (T801-sense), primer (T935-antisense) and Taq polymerase (5u/µL). The primer sequences were

illustrated in Table (A). The PCR cycling condition was: 95ºC for 10min followed by 55 cycles of 94ºC for 20sec, 60ºC for 20sec and 72ºC for 30sec. The PCR products were detected by electrophoresis on 2% agarose gel stained with ethidium bromide and photographed under UV.

B- *PCR for CMV and HSV:*

The PCR mixture was performed in a total volume of 50µL reaction volume containing DNA, 10X buffer, $MgCl_2$, dNTPs, specific primers and Taq polymerase (5u/µL). The primer sequences were illustrated in Table (A). The PCR cycling condition was: 95ºC for 5min followed by 35 cycles of 94ºC for 60sec, 60ºC for 60sec for CMV and 57 for HSV and 72ºC for 60sec.

C- *PCR for EBV:*

A nested-PCR method was used to detect viral DNA of EBV-1 and EBV-2. The primers had demonstrated high specificity and no cross-reactivity with the human genome. The sequences of EBV outer and inner primers were illustrated in Table (A). The first round PCR reaction was carried out in a volume of 40µl total mixture that included 5µl of the template, 1xPCR buffer (pH 8.3) and 1.25U Taq DNA polymerase, 0.2mmol/L of each dNTP, 1.5mmol/L $MgCl_2$, and 50pmol/L of the EBV outer primers. PCR amplification included an initial denaturation step at 94°C for 5min followed by 30 cycles of denaturation steps at 94°C for 1min, primer annealing at 60°C for 1min and an extension step at 72°C for 1min, and then a final extension step at 72°C for 7min. In a new tube, a second round of amplification was performed using 2µl of the first round PCR product, 50pmol of the inner primers for EBV, and PCR buffer, Taq DNA polymerase, dNTP and $MgCl_2$ in concentrations described above. The program for the second PCR was 35 cycles of denaturation step at 94°C for 1min, an annealing step at 55°C for 1min and an extension step at 72°C for 1.5min, and then a final extension step at 72°C for 7min.

III- *Agarose gel electrophoresis:*

Ten µL of the PCR product was separated electrophoretically in a 1.5% agarose gel stained with ethidium bromide and observed under UV light. The expected length of the PCR product was as illustrated in Table (A). Negative and positive controls were included in every run. In parallel, a DNA marker was separated.

Data management and statistical analysis:

The data was coded and entered on an IBM compatible personal computer using the statistical package SPSS ver. 9.0. The mean ± standard deviations were calculated for the numerical data.

Table (A).

	Oligonucleotide sequences	Product size
1CH	5'GGTGCACGGTCTACGAG ACCTC3'	
2CH	5'AACTACTGTCTTCACGC AGAA3'	289bp
4CH	5'ATGGCGTTAGTATGAGT G3'	
1TS	5'GCGACCCAACACTACTC GGCT3'	187bp
101-sense	5'CGGCCAAAAGGTGGTG GATG3'	
285-antisense	5'CGACGAGCCTGATGTTG GG3'	185bp
T801-sense	5'GCTACGTCACTAACCAC GTG3'	
T935-antisense	5'CTACGGTGTGTAAACTC ACC3'	200bp
F (CMV)	5'TTGCAGGCCACGAACA ACGT3'	
R (CMV)	5'GTCTACGGATTGCTGAC GCT3'	305bp
F (EBV) outer	5'-AGG GAT GCC TGG ACA CAA GA-3'	
R (EBV) outer	5'-TGG TGC TGC TGG TGG TGG CAA-3'	432bp
F (EBV) inner	5'-TCT TGA TAG GGA TCC GCT AGG ATA-3'	
R (EBV) inner	5'-ACC GTG GTT CTG GAC TAT CTG GAT	160bp
F (HSV)	C-3'	
R (HSV)	5'-CGGAATTCCGTCATCT CA CGGGGACAC-3'	324bp
	5'-CGGGATCCCGACGGT ATC GTCGTAAA-3'	

RESULTS

Among the thirty studied patients, 16 were males (53%) and 14 were females (47%) with male to female ratio of 1.14:1. Their mean age was 3.6±1.2 years. The PCR results for the studied patients are shown in Table (1) and Fig. (1).

Pallor was the commonest sign observed in all patients. Rash was evident in 18 (60%) of our patients. Cervical lymphadenopathy was present in 26 patients (86.7%), hepatomegaly in 22 patients (73.3%) and splenomegaly in 23 patients (76.7%).

The mean hemoglobin of studied patients was 7.42±1.95gm/L (ranging from 2.6 to 11gm/L). The total leucocytic count was increased in 24 patients (80%) with a mean of 26.17±16254/cmm (with a range of 1050 to 71000/cmm). Their mean platelet count was 42866±34129/cmm. Diagnostic blast cells in both peripheral blood and bone marrow were evident in all patients.

Table (1): PCR results among studied patients (n=30).

PCR positive	No.	Percentage
CMV	12	40
HSV-1	11	36.7
EBV	3	10
HBV	3	10
HCV	7	23.3
HGV	7	23.3
Mixed HBV & HCV	2	6.7

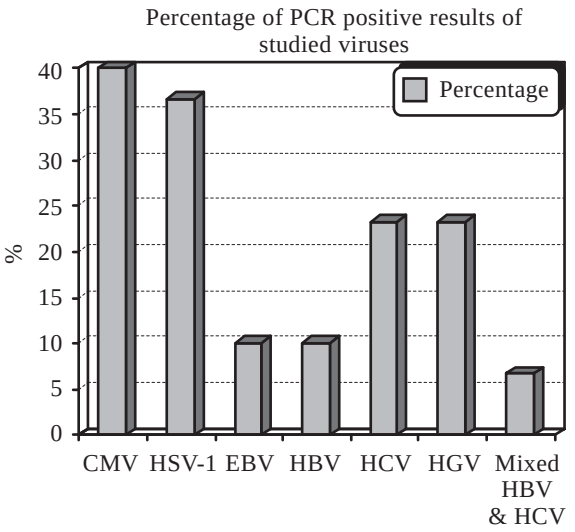

Fig. (1): Percentage of PCR positive results of studied viruses.

DISCUSSION

Leukemia is the commonest form of cancer in children accounting for around a third of all childhood cancer **[10]**. ALL accounts for approximately three-quarters of all pediatric leukemia diagnoses **[11]**.

There are three current hypotheses concerning infectious mechanisms in the etiology of childhood leukemia: exposure in utero or around the time of birth, delayed exposure beyond the first year of life to common infections and unusual population mixing. No specific virus has been definitively linked with childhood leukemia and there is no evidence to date of viral genomic inclusions within leukemic cells **[12]**. This study was carried out to investigate the possible etiological relation between some viral infections (EBV, CMV, HSV-1, HBV, HCV and HGV) and pediatric ALL.

This study included 30 (16 males and 14 females) newly diagnosed ALL patients with a mean age of 3.6±1.2 years.

The seropositivity of screened viruses in our study carries the possibility of viral etiology of ALL, which agrees with Greaves' hypothesis **[3]**, who suggested that the most common form of childhood ALL, common ALL, which is responsible for the age peak, may be due to two separate genetic events **[3,13]**. The first event is thought to be a spontaneous mutation in a B cell precursor and occurs in utero. This transformed B cell precursor clone will proliferate when exposed to a later antigenic challenge. The second stage is influenced by external agents, which results in an expansion of the transformed B cell clone into clinically overt ALL. Furthermore, delay in the normal pattern of exposure of the immune system to infection might lead to an increase of the number of susceptible preleukemic cells and thus the chance of the second critical mutation occurring, leading to overt leukemia **[3]**. It was also suggested that no specific infectious agent is involved but reduced antigenic challenge in infancy can lead to increased proliferation of a preleukemic clone when a later infection occurs.

The evidence of viral infection among our studied patients also goes with another hypothesis which suggests that common ALL may be a rare response to an unidentified mild or sub

clinical infection, the transmission of which is facilitated when large numbers of people come together, particularly from a variety of origins **[14,15]**. Another model, called the aberrant response model, suggested that a substantial proportion of childhood ALL cases arise as a rare host response to certain patterns of exposure to common infectious agents **[16]**. A specific, but yet unknown transmissible agent is causally associated with childhood ALL. For children diagnosed in the childhood peak, primary infection may occur shortly before diagnosis, while for other ages, attention has focused on gestational/neonatal exposure leading to persistent infection. There is also evidence that population density is correlated with childhood ALL risk, which may be indicative of a viral involvement **[17]**. There is some supportive evidence for an infectious etiology provided by the findings of space-time clustering and seasonal variation **[12]**. Genetically determined individual response to infection may be critical in the proliferation of preleukemic clones as evidenced by the human leukocyte antigen class II polymorphic variant association with precursor B-cell and T-cell ALL **[18]**. Similarly, three studies showed a statistically significant increased risk associated with childhood exposure to some infections **[19,20,21]**. However, some studies showed a statistically significant protective effect **[22,23,24]**. One study showed a statistically significant protective effect of infection with roseola (fever plus rash) against ALL when exposure occurred during the first year of life, but a statistically significant increased risk of developing ALL for those with tonsillitis 3-12 months before diagnosis **[21]**. Other authors supported that early common infections may play a protective role in the etiology of childhood leukemia, although this effect was not more marked for ALL **[25]**.

For infection-related hypothesis, the agent must be common in childhood, should also infect adults and should be able to establish persistent infection. There have been limited studies to date trying to define the organism(s) to which abnormal immune responses might lead to the conversion of a preleukemic clone into overt leukemia **[12]**. Several viruses were blamed in the causation of ALL including cytomegalovirus **[26]**, herpes virus **[23]** and varicella zoster **[27]**.

Our results showed CMV seropositivity of 40% among all patients. This agrees with the statement that CMV is one of the viruses incriminated in the causation of childhood ALL by arresting maturation of lymphocytes and megakaryocytes in bone marrow, thus causing proliferation of their immature forms due to dysregulation of cycline E gene expression in human infected cells **[28]**. In another study, it was found that 75% of B-ALL samples of malignant cells at diagnosis were positive for CMV using real time quantitative PCR, and CMV loads were significantly higher in B-ALL samples than in multiple myeloma, B-chronic lymphocytic leukemia or acute myeloid leukemia samples **[29]**. In 2003, a case report was described with CMV infection inducing thrombocytopenia and B-lymphocytes progenitor-precursor cells **[30]**. On the other hand, some authors investigated the presence of prenatal CMV infection in children who later developed ALL and their results showed that prenatal CMV infection does not seem to be associated with later development of childhood ALL **[31]**.

Among our patients, HSV-1 seropositivity was 36.7%. In a study on 68 newly diagnosed pediatric patients with ALL, significantly high percentage of HSV-1 and 2 IgM or reactivated infection was found among leukemic children, 17/68 (25%) compared with normal control 0% and the prevalence of HSV-1 and 2 IgG increased from 18/33 (54%) in children <5 years to 11/13 (77%) in children >10 years, and reactivation of HSV-1 and 2 increased with increasing age from 1/33 (3%) in children <5 years to 4/13 (30%) in children >10 year **[32]**. On the other hand, screening for herpes virus genomes in common ALL showed that, in spite of being present at low levels, detection rates and levels were similar in leukemic and control panels; so it was concluded that a herpes virus is unlikely to be etiologically involved as a transforming agent in common ALL **[33]**. Otherwise, the literature was deficient as regard the etiological relation of HSV-1 and ALL.

In our study, EBV seropositivity was found in 10% of all patients. This agrees with a study that showed that children under 6 years with acute leukemia were more likely to be EBV seropositive than age-matched controls **[34]**. However, another study found that, for children aged 0-4 years, leukemia was inversely associated with EBV seropositivity **[20]**.

The relation of EBV and infectious mononucleosis, pharyngeal carcinoma and Burkitt's lymphoma is well known **[35]**. The higher immunologic response against EBV suggests that such agent could play a role in the onset of ALL in children, if not as a direct etiological factor. Some studies have reported a significantly increased risk for childhood leukemia associated with maternal infection during pregnancy, specifically, EBV infection **[36]**. ALL, a rare complication of viral infection, was reported to follow EBV infection, where infection of T-cells at an early stage of differentiation may lead to failure of normal T-cell repertoire development with subsequent autoimmunity or malignancy **[37]**.

HCV infection is a common health problem in Egypt with a high prevalence rate. A seropositivity of 12% in normal children was reported and it was stated that a relatively higher prevalence of HCV antibody seropositivity was found in healthy Egyptian children compared to reports from other countries **[38]**. Another study showed that the prevalence of HCV antibodies increased from 2.7% in those <20 years of age to more than 40% in males aged 40-54 years in rural areas of Egypt **[39]**. HGV is a single stranded RNA virus which belongs to the flavivirus family. Its genome structure resembles that of HCV, but its transmission is largely independent of HBV and HCV. No association was found between HGV viremia and hepatitis, or with co-infection with either HBV or HCV **[40]**. A relatively high prevalence of GBV-C/HGV-RNA was reported among different Egyptian groups compared to international figures **[41]**. Moreover, despite of compulsory hepatitis B vaccination in Egypt, one study showed that HBV infection occurred in 6.8% of vaccinated children **[42]**.

High incidence of HBV and HCV infection was noticed amongst patients with lymphoproliferative disorders with increased carrier rate **[43]**. HGV shares genetic and biological features with HCV, thus it might also be involved in lymphomagenesis **[44]**. Hypoplastic anemia, a precursor of lymphoblastic leukemia, was reported as sequel of Non-A, Non-B viral hepatitis infection **[45]**. Our results showed seropositivity of 23.3% for each of HCV and HGV. We also found that 10% and 6.7% of our patients had HBV and combined HBV and HCV respectively. HCV was reported in 7.6% of ALL patients and it was suggested that HCV infection may be associated, not only with B-Non Hodgkin lymphoma, but also with some other lymphoid and myeloid malignancies **[46]**.

All our studied patients were diagnosed and managed at the NCI, which is a governmental free of charge referral center for most rural and urban areas with low socioeconomic standard in Egypt. Thus, the patients' socioeconomic standard varied from low to middle class. Some authors indicated that the risk of common ALL increases by higher socioeconomic status, isolation, and other community characteristics suggestive of abnormal patterns of infection during infancy **[1]**. Children in developed countries are up to 4 times more likely to get leukemia than those living in developing countries and the risks are higher for rich children **[47]**. The marked childhood peak in resource-rich countries and the increased incidence of childhood peak in ALL ages 2-6 years predominantly with precursor B cell ALL is supportive of the concept that reduced early infection may play a role **[12]**. It was suggested that socioeconomic factors, associated with community characteristics rather than individual lifestyle, are related to the risk of childhood leukemia and that these factors act early in life **[48]**.

Our results showed that pallor was the commonest sign, being evident in all patients. This agrees with most studies that report pallor as a basic sign of ALL **[49]**.

The role of viral infections as an etiological factor for childhood ALL is still debatable, but the results of our study are more towards this possibility. Further studies involving larger sample of population and including other viruses are recommended to investigate the viral etiology theory. Vaccinations, if available, as means of prophylaxis against these infections as well as close follow up of patients for evaluation of the fate of these viral infections are recommended.

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Journal of the Egyptian Society of Haematology & Research, Vol. 2, No. 2, September: 103-107, 2006

Epistaxis in Thalassemia: Study of the Hemorrhagic Profile

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ABSTRACT

Introduction: Significant alterations in the hemostatic system already exist in polytransfused children with beta thalassemia that make it a high risk condition for both hemorrhagic manifestations and future development of thromboembolic events. The pathophysiologic defects may result from platelets abnormalities, inherent red cell defects, coagulation inhibitors deficiency or additional acquired abnormalities like cardiac or liver dysfunction and hormonal deficiencies.

Objective: To assess the hemostatic defect underlying epistaxis in thalassemic children and to compare results with matched controls having epistaxis as the presenting symptom but not due to a hematological disorder.

Patients and Methods: Twenty one thalassemic patients (19 thalassemia major & 2 thalassemia intermedia) attending the hematology clinic of the New Children's Hospital, Cairo University were included in the study together with ten age and sex matched controls. All patients were subjected to full clinical examination, complete blood count, serum ferritin, alanine transaminase (ALT), aspartate transaminase (AST), total and direct bilirubin, alkaline phosphatase, prothrombin time (PT), prothrombin concentration (PC) and activated partial thromboplastin time (aPTT).

Results: A highly significant decrease was observed in platelet aggregation to ADP and ristocetin in patients than control (p=0.000, 0.000 respectively). PC and PT were significantly affected in patients than control (p=0.000, 0.001 respectively). PTT was non significantly prolonged in patients. AST and ALT were increased in patients with only significant increase in ALT (p=0.000). Platelet aggregation was increased in splenectomized versus non-splenectomized thalassemics.

Conclusion: Bleeding tendency in thalassemia can be attributed to a defect in platelet aggregation. Hepatic dysfunction associated with the disease can be a contributing factor.

Key Words: *Epistaxis - Thalassemia - Hemorrhagic profile.*

INTRODUCTION

Standards of care for thalassemic patients have improved in recent years, resulting in almost doubling of the average life expectancy. As a consequence, additional previously undescribed complications are now being recognized. In particular, profound hemostatic changes which can present either as thrombotic or hemorrhagic events are being observed in patients with ß-thalassemia major and intermedia.

Many thalassemic patients suffer from bleeding tendency, notably epistaxis and easy bruising, possibly attributed to defective platelet aggregation in response to ADP, collagen and ristocetin **[1]**.

Minor abnormalities in the coagulation mechanism of these patients were reported as prolonged prothrombin time and reduction in the plasma levels of clotting factors I, II, V, VI, IX and XI.

Reduced levels of prothrombin in different age groups of thalassemic were reported suggesting that this anomaly is related to the thalassemia rather than to hepatic dysfunction due to hemosiderosis which is a rare occurrence in children **[2]**.

Spontaneous intracranial hemorrhage is one of the cerebrovascular complications in ß thalassemia major which is most probably multifactorial in origin, the predisposing factors included recent blood transfusion, prolonged prothrombin time and partial thromboplastin time as well as decreased platelets **[3]**.

We conducted this study to assess the hemostatic defect underlying epistaxis in thalassemia children and to compare results with matched control suffering from epistaxis without any hematological disorder.

PATIENTS AND METHODS

Patients:

Twenty one thalassemic patients (12 males and 9 females) attending the Hematology Clinic of the New Children's Hospital, Cairo University were included in this study together with ten age and sex matched control.

The patients were composed of 19 ß - thalassemia major (TM) and 2 thalassemia intermedia (TI) with a mean age of 11.0±4.5 years.

Ten patients (47%) were splenectomized and 11 (52.4%) had splenomegaly. All patients were presenting with epistaxis as a frequent symptom and were assessed for the underlying cause of bleeding together with 10 age and sex matched control.

All patients were on regular 3-4 weeks blood transfusion therapy of 10-15ml/kg except the 2 thalassemia intermedia. Eighteen patients were on chelation therapy (subcutaneous desferroxamine, 25-50mg/kg 5 days a week) but only 7 were compliant.

None of our patients were on anti-platelet therapy.

All patients and control were subjected to full history taking and thorough clinical examination and laboratory investigations.

- Complete blood count (CBC).
- Serum ferritin.
- Alanine transaminase (ALT), aspartate transaminase (AST), total and direct bilirubin and alkaline phosphatase.
- Prothrombin time (PT), prothrombin concentration (PC) and activated partial thromboplastin time (aPTT).
- Platelet aggregation was tested using adenosine 5-diphosphate (ADP) and ristocetin as in vitro aggregating agents.

Methods:

Blood samples were taken 3-4 weeks after blood transfusion and were sent for routine biochemical and hematological tests.

CBC including platelet count by electronic counters (Coulter, Advia 120).

Serum ferritin by microparticle immunoassay (IMX).

Platelet aggregation test was done using aggregometer by method of Born **[4]**.

The patients and control were tested using platelet rich plasma (PRP) which was obtained by adding one-tenth volume of 3.2% trisodium citrate to collected blood. Aggregation test was done by addition of aggregating agents (ADP, Ristocetin sulphate).

The results were quantitated by measuring the percentage fall in the optical density of PRP, 3 minutes after the addition of the aggregating agent. Normal range for ADP and ristocetin is 50-90%.

Statistical analysis:

All data were summarized as mean ± standard deviation (SD) for numerical data and as percentage and ratio for categorical data.

Appropriate statistical tests of significance (unpaired student *t*-test) was used to compare results in different groups.

RESULTS

A significant decrease was found in platelet aggregation to ADP and ristocetin in patients than control (p=0.000, 0.000 respectively) (Table 1 & Fig. 1). PC and PT were significantly affected in cases than control (p=0.000, 0.001 respectively). PTT was prolonged in patients but with no statistical significance. Platelet count, AST and ALT were also increased in patients than control with only ALT of significance (p=0.000) (Table 1).

Further splitting of the patients was done based on the splenic status. Platelet count was significantly increased in splenectomized patients (p=0.008) versus non-splenectomized while platelet aggregation markers showed a non-significant increase in splenectomized patients (Table 2).

Table (1): Clinical and laboratory data of patients and control.

	Patients (n=21) Mean ± SD	Control (n=10) Mean ± SD	*p*-value
Age	11.0±4.5	8.4±2.7	
Gender	12 M, 9 F	5 M 5 F	
Type of thalassemia:	19 TM, 2 TI		
Splenic	10 splenectomized		
Status	11 splenomegaly		
Hb (g/dl)	6.8±1.4	11.3±1	0.000
Hct (%)	21.1±4.4	33.4±2.7	0.000
Platelet (X 10^9/L)	393.34±296.6	302.1±100	NS
Plt. Aggreg:			
ADP (%)	28.3±27.5	88.6±12.13	0.000
Ris (%)	43.9±28.4	91.1±9.70	0.000
PC (%)	72.7±17.2	98.0±3.39	0.000
PT (Sec)	15.29±3.0	12.6±0.2	0.001
PTT (Sec)	42.5±10.54	38.0±17.0	NS
AST (IU/L)	102.57±90.1	52.35±40.4	NS
ALT (IU/L)	114.47±79.4	27.0±24.2	0.000

M : Male.
F : Female.
TM : Thalassemia major.
TI : Thalassemia intermedia
Hb : Hemoglobin.
Ht : Hematocrite
ADP : Adenosine diphosphate.
Ris : Ristocetin.
PC : Prothrombin concentration.
PT : Prothrombin time.
PTT : Partial thromboplastin time.
ALT : Alanine transaminase.
AST : Aspartate transaminase.
p.value <0.05 = Significant.
>0.05 = Insignificant.

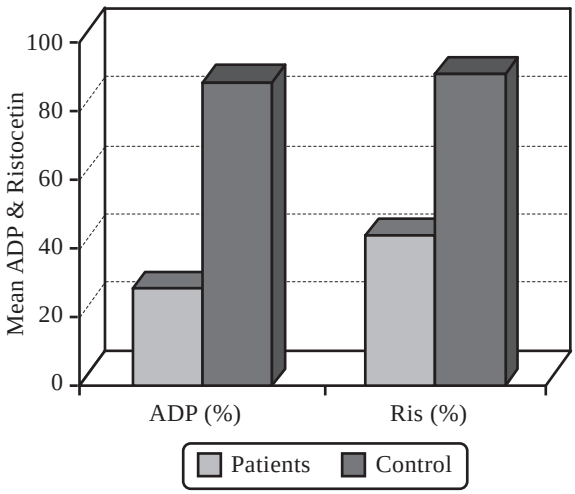

Fig. (1): ADP and Ristocetin levels in patients and control.

Table (2): Platelet changes in patients, splenectomized Vs non-splenectomized.

	Splenectomized (n=10)	Non-splenectomized (n=11)	*p*-value
Platelet (X 10^9/L)	583.4±304	197.7±115.7	0.008
Plt. Aggreg:			
ADP (%)	34.3±34.1	23.9±22.3	NS
Ris (%)	47.7±31.4	39.4±27.8	NS

DISCUSSION

The currently used therapeutic strategies in ß-thalassemia major and intermedia have prolonged the survival for many patients. This longer survival has been accompanied by the appearance of a number of previously underscribed complications, in particular hemostatic derangements.

A previous study evaluating the hemostatic changes in a group of 50 thalassemics reported 32% of cases having minor bleeding tendency and one with ecchymotic patch while none of the patients had thrombo-embolic episodes **[5]**.

Many thalassemics experience epistaxis as well as easy bruising. To study hemorrhagic events in our patients we assessed platelet aggregation to ADP and ristocetin in thalassemic children presenting with epistaxis and compared them to their matched control.

Our patients showed significant Platelets hypoaggregation with ADP and ristocetin. It was reported that there are several platelet abnormalities in thalassemia as impaired aggregation, increased circulating aggregates and shortened platelet survival **[2]**. Diminished plate-

let aggregation response to ADP, epinephrine, collagen and ristocetin may be due to an intrinsic defect of thalassemic platelets e.g., membrane abnormality which can be due to the release of some substances by hemolysed red cells inducing a platelet defect directly or at the membrane level **[6]**. On resuspension of these platelets in normal plasma, their diminished aggregation didn't correct.

Impaired aggregation can also be attributed to the presence of plasmatic isoantibodies resulting from previous blood transfusion **[7]**.

Mild hemorrhagic tendency in the form of bruising and epistaxis was observed in a group of ß-thalassemia patients. A consistent platelet anomaly manifested by diminished platelet aggregation to ADP, collagen and ristocetin was found and could be responsible in part for the hemorrhagic phenomena **[1]**.

Some studies showed that not only hypoaggregation can occur in thalassemics but also hyper-or normal aggregation **[8]**. This is in contrast to our results where only 9% showed normal aggregation and none showed hyperaggregation.

In our study, splenectomized patients had increased platelet count and better aggregation with ADP and ristocetin than those with intact spleen which agrees with the results of Opartkiattikul and Colleagues, 1992) **[9]**.

Thrombocytosis, increased platelet aggregation and decreased natural coagulation inhibitors (protein C and antithrombin III) in splenectomized patients may be significant in thrombotic complications in such cases while defective platelet aggregation and prothrombin activity in non-splenectomized thalassemics may give rise to hemorrhagic tendencies **[10]**. In splenectomized thalassemics, platelet aggregation to ADP, ristocetin, adrenalin and collagen showed better results than non-splenectomized cases. Splenectomy is recommended to improve preexisting hemostatic defects especially with regards to platelet function **[6]**.

In addition to increased platelet number in splenectomized patients, chronic platelet activation is present in ß-thalassmia major and intermedia. This may explain the weak response of thalassmic platelets to aggregation agonists as the activated platelets become refractory to additional stimulation **[11]**. The presence of morphological platelet abnormalities in splenectomized patients with ß-thalassmia may also contribute to an enhanced risk of vascular complications **[12]**.

Our results showed that PT and PTT were prolonged in patients than control which agrees with the results of a study reporting a 33.3% and 40.7% prolongation of PT and PTT in 30% of a group of poorly chelated thalassemia major patients exhibiting bleeding manifestations. **[13]**. ALT and PT showed significant elevation in patients than control (p=0.00, 0.001 respectively). Furthermore our patients were iron overloaded with a mean serum ferritin of 2855.9± 1906.2ng/ml which correlated positively with liver function **[14]**. Therefore, epistaxis in our patients may be partly related to hepatic dysfunction.

Despite that none of our patients suffered from thromboembolic problems, splenectomized patients are at high risk of developing thrombosis due to the existence of a low grade consumptive coagulopathy **[15]**.

Thalassemics have a chronic hypercoagulable state with increased incidence of thromboembolic episodes. The pathophysiologic defects include inherent red cell defects, platelet abnormalities, deficiency of coagulation inhibitors and additional acquired abnormalities like cardiac and liver dysfunction and hormonal deficiencies **[16]**.

Venous thrombosis is more prevalent in B-thalassemia intermedia patients who are not receiving regular transfusions and who have undergone splenectomy. Thalassemia intermedia occurred 4.38 times more frequently in thalassemia intermedia than thalassemia major with more arterial events occurring in thalassemia major **[17]**.

Thalassemia Intermedia patients have high plasma levels of coagulation and fibrinolysis activation. Furthermore, thalassemic red cells and erythroid precursors from splenectomized patients had an enhanced capacity to generate thrombin.

The addition of prophylactic antithrombotic therapy has only recently been suggested for high-risk patients with ß-TI who are exposed

to transient thrombotic risk factors. Thalassemia major patients who had developed an acute thrombotic event should be considered for prolonged antithrombotic therapy **[18]**.

In conclusion, bleeding tendency in our thalassemic patients can be attributed to a defect in platelet hemostasis namely platelet hypoaggregation. Hepatic dysfunction associated with the disease can be a contributing factor as well.

Vitamin K supplementation to compensate for hepatic synthesis defects can be of benefit.

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Journal of the Egyptian Society of Haematology & Research, Vol. 2, No. 2, September: 1 09-117, 2006

Apoptosis in the CD34$^+$ Cells and Glycophorine A$^+$ Cells in Thalassemic Patients

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ABSTRACT

ß-thalassemia major is characterized by ineffective erythropoiesis leading to severe anemia and extensive erythroid expansion. The ineffective erythropoiesis is in part due to accelerated apoptosis of the thalassemic erythroid precursors. In a trial to identify the stage of differentiation at which apoptosis occurs, we have investigated the expression and function of Fas, Bcl_2 and Bcl_{XL} on CD34$^+$ cells and glycophorin A$^+$ cells by flow cytometry and serum and BM plasma levels of tumor necrosis factor-alpha (TNF-α) were estimated by ELISA in 15 patients with ß-thalassemia major compared to 10 healthy controls. There was a decrease in the percentage of CD34$^+$ cells, with increased apoptosis in these cells, but not in glycophorin A$^+$ cells in patients. Upregulation of Fas expression occurred in CD34$^+$ cells. However, that did not explain molecular basis for apoptosis in ß-thalassemia patients. There was an increase in the level of TNF-α in patients than controls in both BM plasma (p<0.001) and serum (p=0.037). This suggested that TNF-α may have significant role in Fas upregulation on CD34$^+$ cells. Changes in expression of apoptotic antagonists, suggested that Bcl_{XL} may not play a significant role in regulation of apoptosis in progenitor cells in ß-thalassemia patients also. The present results suggest that apoptosis in ß-thalassemia major is more in the early erythroid precursors than in the more mature cells. An alternative mechanism for apoptosis other than the Fas-induced one is assumed.

Key Words: *Apoptosis – CD34 – Glycophorine A – ß-thalassemia – TNF-α.*

INTRODUCTION

ß-thalassemia is an inherited disorder that arises from mutations in the ß-globin gene that reduces or totally abolish synthesis of ß-globin chain with subsequent development of red cell hypochromia, microcytosis and anemia **[1]**. Several studies have suggested increased apoptosis as a mechanism of ineffective erythropoiesis in the erythroid lineage in the bone marrow (BM) of thalassemia patients **[2-6]**. Despite that many of the molecular abnormalities that led to the disease have been revealed, the underlying mechanisms of increased apoptosis have not yet been clarified **[1]**. There was considerable variation in measurement of apoptosis in each specific form of thalassemia, the variation was at least partially dependent on the extent of erythroid expansion in patient **[2]**. One interpretation of this finding is that under extreme erythropoietic drive leading to greater erythroid expansion, the erythroid precursors are more likely to make errors that turn on apoptotic programs. Morphologic analysis of marrow in severe forms of ß-thalassemia showed a decrease in late orthochromatic normoblasts **[3]**. But in vitro studies have led to the suggestion that apoptosis primarily occurs at the polychromatophilic normoblasts stage **[4]**. Salasaa and Zoumbos **[7]** showed that increased productions of tumor necrosis factor-alpha (TNF-α) in ß-thalassemia, which usually characterize the acute response to infectious agents, have a negative effect on erythropoiesis. Other study showed that addition of TNF-α induced increase fas expression in normal CD34$^+$ cells **[8]**.

Defining the mechanism of increased apoptosis in ß-thalassemia cells is important for understanding the pathophysiology of the disease, as well as for devising new therapeutic strategies.

This work aimed to study the role of Fas and anti-apoptotic proteins (Bcl_2 and Bcl_{XL}) in $CD34^+$ cells and glycophorin A^+ cells in b-thalassaemia major in trial to detect the stage at which apoptosis can occur and the possible role of (TNF-α).

PATIENTS AND METHODS

Patients:

This study was carried on 15 children with *ß*-thalassemia major, 11 boys and 4 girls, 10 of them had been splenectomized, their ages ranged from 6 to 16 years with mean of 9.13±2.7. They were referred to the out patient clinic of Pediatrics Department, Sohag University Hospital for follow-up or blood transfusion. It also included ten apparently healthy controls after having an informed written consent in accordance with Sohag University Hospital ethical committee guide lines. All were subjected to thorough history taking, clinical and laboratory evaluation.

Methods:

1- *Collection of samples:*

- Peripheral blood samples were taken for ELISA assay of (TNF-α).
- BM samples (10ml) collected in preservative free heparin (10ul/ml).

2- *Mononuclear cell separation:*

BM samples were centrifuged at 800rpm for 10min. The supernatant was collected for ELISA assay (TNF-α). The BM sediment was diluted 1:1 in phosphate buffered saline (PBS), pH.7.4 (Biosource international, Camarillo, California, USA). Diluted BM was centrifuged at 1500rpm for 25min on Ficoll-Hypaque (Biochrome AG, USA) and the BM mononuclear cells (BMMCs) were washed twice in PBS supplemented with 1% fetal calf serum (FCS) (Sigma) and 0.05% sodium azide (Winlab, UK).

3- *ELISA assays:*

The TNF-α kit is a solid phase enzyme amplified sensitivity immuno assay (EASIA) performed on microtiter plate (Biosourse International, Camarillo, California, USA). The assay is based on an oligoclonal system in which a blend of MoAbs directed against distinct epitopes of TNF-α are used. Human IFN-γ (hIFN-γ) kit is a solid phase sandwich ELISA (Biosourse International, USA).

4- *Flow cytometry studies:*

BMMCs were subjected to tripple staining followed by acquisition on a minimum of 50000 cells and analysis using FACSCaliber flow cytometry (Becton Dickinson) in South Egypt Cancer Institute, Assiut University. Anti $CD34^+$ monoclonal antibody (Clone 581, Pharmingen, BD, USA) was used as a marker for stem cells and early precursors and anti-glycophorin-A monoclonal antibody was used as a marker for late erythroid series. Every one of them was combined with surface death receptor (CD95, clone DX2, Pharmingen BD) and intracytoplasmic anti-apoptotic markers [Bcl_{XL} (clone 7B 2.5, Chemicon International, USA) and Bcl_2 (clone 3d-21100, Pharmingen BD)]. All were combined with fluorescence dye 7-amino actinomycin D (7AAD) (Pharmingen BD) to study the viability of the cells expressing those markers.

BMMCs were incubated with 40µl of a solution containing 2% human gamma globulin (Sigma), 1% FCS in PBS for 10min at 4ºC, 10µl of PE anti-CD34 or anti-glycophorin A antibody were added and incubated for 30min at 4ºC. For the negative control an isotype matched irrelevant mouse IgG conjugate with PE was used. Cells were washed twice in PBS then 5µl of 7-AAD was added, mixed well and incubated for 10 min at 4oc protected from light then washed twice and resuspended in 50µl of PBS. 10µl of FITC anti-Fas were added and incubated for 20min at 4ºC.

For intracytoplasmic staining, 250µl of cytofix/cytoperm solution was added and incubated for 20min at 4ºC then washed twice in 1ml and resuspended in 50µl of perm/wash buffer then 5µl of FITC Bcl_{XL}, were added in one tube and 10µl of FITC Bcl_2, in another tube, both were incubated for 20min at 4ºC, washed twice and resuspended in 50µl PBS then 0.5ml of parafomaldhyde (Sigma) was added and kept at 4ºC protected from light until analyzed by FCM.

BMMCs were defined on a scatter diagram combining forward and right angle light scatter (FSC & SSC respectively). Region (R1) was drawing to exclude debris. BMMCs were expressed on a scatter diagram combining SSC with antiglycophorin A fluorescence and a region (R2) was drawn around positive population for antiglycophorin A. To quantify viability of

glycophorin A positive cells within R2, region were drawn satisfying 7-AAD-negative (viable), dim (apoptotic) and bright (dead). Glycophorin A positive cells within R1 and R2 were further expressed on a histogram of anti CD95, anti-Bcl_2 and anti-Bcl_{XL} fluorescence. BMMCs were expressed also on a scatter diagram combining SSC with CD34, a region (R8) was drawn around a clear cut population, having low SSC and high CD34 fluorescence. Analysis for $CD34^+$ cells were done as for Glycophorin A^+ one (Fig. 1).

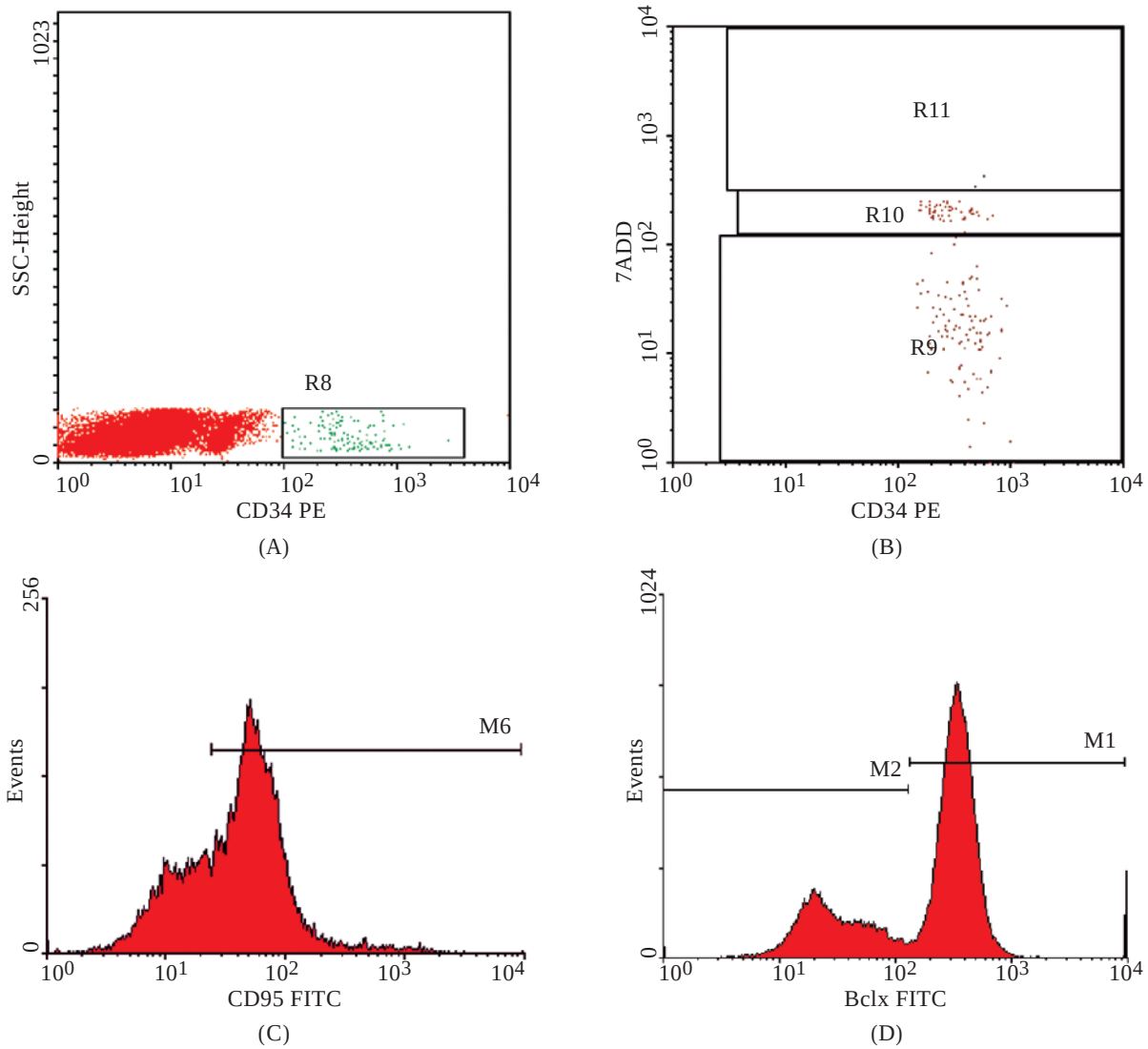

Fig. (1): (A) Dot plot of SSC versus anti-CD34 showing a distinct population of $CD34^+$ cells (R8). (B) Dot plot of FSC of $CD34^+$ cells versus 7AAD fluorescence, divided into viable cells negative for 7AAD (R9), apoptotic cells dim for 7AAD (R10) and dead cells bright for 7AAD (R11). (C) Histogram of CD95 expression of $CD34^+$. (D) Histogram of Bcl_{XL} expression of $CD34^+$ cells divided by median fluorescence into Bcl_{XL}^{hi} (M1) and Bcl_{XL}^{lo} (M2).

RESULTS

There was a decrease in the percentage of the $CD34^+$ cells in ß-thalassemia patients when compared with normal controls, significantly in viable ones (p=0.06) (Table 1).

$CD95$ expression on $CD34^+$ BMMCs and their viability in both patients and controls:

To investigate whether the decreased in $CD34^+$ percentage may be caused by increased apoptosis, we determined the expression of Fas

antigen on CD34+ cells and their viability (Table 2).

Serum and BM plasma levels of TNF- α:

The detection of the levels of TNF-α in both the serum and BM plasma respectively of both patients and normal controls resulted in that there was an increase in the levels of TNF-α in the serum and BM plasma of patients when compared with controls and this increase was statistically significant in the serum and highly significant in the plasma (Table 3).

There is no significant difference in the level of TNF-α between patients with spleenectomy and those without in both serum and BM plasma (serum, p=0.44; plasma, p=0.24).

Table (1): Percentage of CD34+ BMMCs and their viability in both patients and controls.

	CD34+ %	Viability of CD34+ cells %		
		Viable	Apoptotic	Dead
Patients	0.878±0.14	86.42±3.4	8.479±2.04	3.86±2.2
Controls	1.17±0.12	94.65±0.61	4.7±0.55	0.699±0.44
p value	0.166	0.06	0.154	0.27

Table (2): CD95 expression on CD34+ cells and their viability in patients and controls.

	CD95+ %	% of Viability of CD95+		
		Viable	Apoptotic	Dead
Patients	1.59±0.92	61.69±9.6	27.11±8.0	4.28±2.02
Controls	0.306±0.07	25.37±13.16	60.96±15.95	3.65±3.12
p value	0.272	0.03	0.04	0.8

Table (3): Levels of TNF-α in the serum and BM plasma of patients and controls.

	Serum TNF-α (Pg/ml)	BM plasma TNF-α (Pg/ml)
Patients	229.4±39.9	656.33±44.88
Controls	114.25±20.58	317.75±20.7
p value	0.037	<0.001

Comparative study between the levels of TNF-α in the serum and plasma:

Comparative study between the levels of TNF-α in the serum and plasma of both patients and controls resulted in that the level of TNF-α in the plasma was more than that of the serum of both patients and controls with highly significant statistical value (p<0.001).

Bcl_{XL} and Bcl_2 expression on CD34+ BMMCs and their viability:

Regarding the anti-apoptotic markers, we found that all the CD34+ cells were expressed BclXL and Bcl2, the median of their expression was used to divide CD34+ cells into CD34+/Bcl_{XL}^{hi} and CD34+/Bcl_{XL}^{lo}. The percentage of expression of Bcl_{XL}^{hi} and Bcl_2^{hi} on CD34+ cells and the percentage of apoptosis in them are shown in Tables (4,5).

Glycophorin-A positive BMMCs and their viability:

The glycophorin A+ cells in patients were more viable when compared with their normal controls (patients: 71.47%±2.85; controls: 47.46%±5.98; p=0.005).

Percentage of CD95 expression on glycophorin A+ and their viability:

The glycophorin A+ cells have shown a reduction in the expression of CD95 in patients and the glycophorin A+/CD95+ were less apoptotic in patients (Table 6).

Table (4): Bcl_{XL} expression on $CD34^+$ cells and their viability in patients and controls.

	Bcl_{XL}^{hi} %	Viability of Bcl_{XL}^{hi} %			Bcl_{XL}^{1o} %	% of the Viability of Bcl_{XL}^{1o}		
		Viable	Apoptotic	Dead		Viable	Apoptotic	Dead
Patients	14.17±3.07	75.79±5.1	22±4.05	2.37±0.67	82.98±3.8	94.69±0.96	7.12±1.9	0.037±0.008
Controls	12.15±3.47	69.42±6.03	27.69±16.1	2.26±1.01	87.85±3.47	94.99±0.66	9.26±4.66	0.02±0.01
p value	0.67	0.365	0.39	0.92	0.38	0.82	0.63	0.25

Table (5): Bcl-2 expression on $CD34^+$ cells and their viability in patients and controls.

	Bcl_2^{hi} %	Viability of Bcl-2^{hi} %			Bcl_2^{1o} %	% of the Viability of Bcl-2^{1o}		
		Viable	Apoptotic	Dead		Viable	Apoptotic	Dead
Patients	8.25±1.82	78.58±7.4	10.43±3.02	10.13±6.9	96.16±0.84	96.16±0.84	8.89±0.84	6.8±0.017
Controls	1.56±0.56	55.75±11.0	33.08±12.6	9.26±4.92	97.45±0.74	97.08±0.47	2.9±0.41	0.025±0.007
p value	0.008	0.087	0.049	0.93	0.298	0.42	0.33	0.064

Table (6): Percentage of $CD95^+$/glycophorin-A^+ and their viability in patients and controls.

	$CD95^+$ %	Viability of $CD95^+$ %		
		Viable	Apoptotic	Dead
Patients	10.15±1.8	77.79±2.1	17.42±1.96	4.02±0.73
Controls	26.43±3.95	33.56±4.89	51.35±4.5	13.42±1.09
p value	<0.001	<0.001	<0.001	<0.001

Bcl_{XL} and Bcl_2 expression in glycophorin A^+ cells and their viability:

As $CD34^+$ cells, all glycophorin A^+ cells were expressed Bcl_{XL} and Bcl_2, their expression was divided into high and low as described previous. The percentage of Bcl_{XL}^{hi} and Bcl_2^{hi} expression on glycophorin A^+ BMMCs and the percentage of apoptotic cells are shown in Tables (7,8).

Table (7): Percentage of Bcl_{XL} expression of glycophorin A^+ cells and their viability in patients and controls.

	Bcl_{XL}^{hi} %	Viability of Bcl_{XL}^{hi} %			Bcl_{XL}^{1o} %	Viability of Bcl_{XL}^{1o} %		
		Viable	Apoptotic	Dead		Viable	Apoptotic	Dead
Patients	45.39±4.66	65.81±6.04	13.7±1.6	26±4.99	53.21±4.7	87.15±1.7	11.68±1.5	0.16±0.17
Controls	52.7±4.7	34.8±8.05	20.6±2.31	49.37±6.25	47.06±4.7	92.08±0.94	6.36±0.79	0.035±0.027
p value	0.229	0.005	0.02	0.008	0.389	0.04	0.014	0.035

Table (8): Percentage of Bcl_2 expression on glycophorin A^+ BMMCs and their viability in patients and controls.

	Bcl_2^{hi} %	Viability of Bcl_2^{hi} %			Bcl_2^{1o} %	Viability of Bcl_2^{1o} %		
		Viable	Apoptotic	Dead		Viable	Apoptotic	Dead
Patients	9.35±1.26	3.0±0.75	19.5±4.02	71±5.36	90.36±1.3	92.73±1.0	6.67±0.91	0.21±0.07
Controls	19.14±2.3	1.17±0.46	19.43±6.6	78.34±6.7	81.85±6.79	96.25±0.46	3.21±0.5	0.04±0.008
p value	0.001	0.08	0.98	0.45	0.003	0.012	0.009	0.066

Correlation studies:

There was highly significant correlation between apoptotic cells and age of the patients (duration of the disease) Fig. (2). However, there was no relation between apoptotic cells and sex, splenectomy, anemia and Hb F.

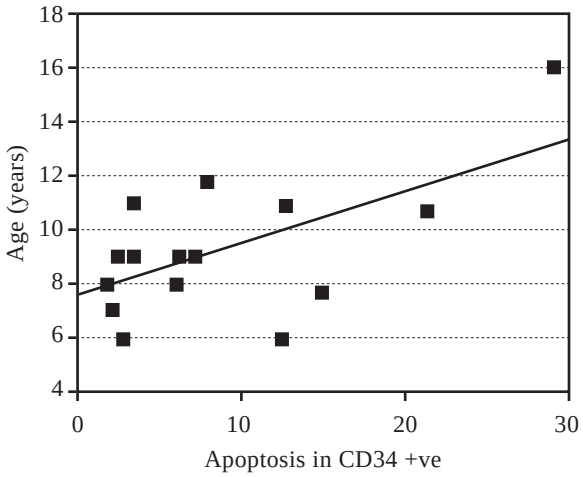

Fig. (2): Correlation between apoptosis in CD34$^+$ cells and age (r=0.633, p=0.01).

There is a positive correlation between the expression of CD95$^+$ and both $Bcl_{XL}{}^{hi}$ and $Bcl_2{}^{hi}$ on CD34$^+$ of patients (r=0.53; p=0.04 and r=0.67; p=0.03 respectively) but there is no correlation among controls (r=–0.117, p=0.74 and r=0.46, p>0.08 respectively). With no correlation between the expression of $Bcl_{XL}{}^{hi}$ and $Bcl_2{}^{hi}$ on CD34$^+$ cells of both patients and controls (patients, r=–0.08; p>0.75: Controls, r=–0.063, p=0.86).

As regard to glycophorin A$^+$ cells, there is no correlation between the expression of both CD95$^+$ and $Bcl_{XL}{}^{hi}$ in both patients and their normal controls (patients, r=–0.41; p=0.12: Controls, r=–0.06, p=0.87). There is no correlation between the expression of $Bcl_{XL}{}^{hi}$ and $Bcl_2{}^{hi}$ in both patients and controls (patients, r=–0.11; p=0.69: Controls, r=–0.5, p=0.14). Conversely there is a positive correlation between CD95$^+$ and $Bcl_2{}^{hi}$ in both patients and controls and it was statistically significant (patients, r=0.5; p=0.05: Controls, r=0.77, p=0.009).

There is no correlation between TNF-α in the serum and BM plasma of patients with CD95$^+$, $Bcl_{XL}{}^{hi}$ and $Bcl_2{}^{hi}$ in both CD34$^+$ and glycophorin A$^+$ cells.

DISCUSSION

ß-thalassemia major is characterized by ineffective erythropoiesis leading to severe anemia and extensive erythroid expansion. Pathophysiology of ß-thalassemia has been the subject of several extensive reviews **[1,9]**.

Ferokinetic studies showed that 60-80% of erythroid precursors die in the marrow or extra medullary sites. However, study of marrow aspirate does not reveal huge numbers of dead and dying erythroid precursors. Angelucci et al. **[10]** explored this apparent discrepancy with the hypothesis that enhanced phagocytosis of thalassemic erythroid precursors was a likely explanation. They concluded that enhanced apoptosis is certainly responsible for part of the increased phagocytosis of thalassemic erythroid precursors. So the ineffective erythropoiesis is in part due to accelerated apoptosis of the thalassemic erythroid precursors; however, the extent of apoptosis is surprisingly variable **[3]**.

In this work, there was a decrease in the percentage of CD34$^+$ cells in ß-thalassemia patients when compared with the normal controls, in which an increase in apoptosis of BM progenitor cells has been observed. This apoptosis was significantly correlated with age of patients which influence the duration of the disease. There are no similar previous studies to compare except the finding of Mathias et al. **[4]** who reported that erythroid cultures initiated from ß-thalassemia major BM CD34$^+$ cells expanded 10 to 20 fold less than from normal BM. They explained that there were less viable cells. But we can suggest that may be due to apoptosis in CD34$^+$ subsets. The studies of Philpott et al. **[11]**, Killick et al. **[12]** and Ismail et al. **[13]** reported that the percentage of CD34$^+$ cells was significantly reduced compared with controls. However, those studies were done on patients with aplastic anemia in which an increase in apoptosis of BM progenitor cells has been observed.

The patients' CD34$^+$ cells upregulate fas antigen but this upregulation was statistically insignificant compared with controls. As Krammer's study **[14]** showed that the expression and function of fas in hematopoietic cells directly correlate with the rate of proliferation. This suggests that this upregulation has relation to increased erythroid expansion in patients with

ß-thalassemia and may be influenced by the haematopoietic microenvironment **[15]**.

In this study we found that there is an increase in the level of TNF-α in patients than the controls in both BM plasma and serum and this increase was statistically significant in the serum and highly significant in the BM plasma. This increase may explain the up regulation of fas on $CD34^+$ cells in this study, especially that this increase was more in the plasma than serum ($p<0.001$), although no correlation was observed. Shetty et al., study **[8]** concluded that the addition of TNF-α induced increase fas expression in normal $CD34^+$ cells.

Also in Meliconi et al. **[16]**; Lombardi et al. **[17]** and Chuncharunce et al. **[18]** studies ß-thalassemia patients had high serum TNF-α concentration compared with controls. These were not related also to sex, age (duration of disease), number of blood transfusion or splenectomy.

Wanachiwanawin et al. **[19]** found that serum level of TNF-α above the normal range in 13% of ß-thalassemia major and suggested that elevated serum level could contribute to complications of the disease, such as cachexia and thromboembolic phenomena.

Also Kyriakou et al. **[20]** found that the level of TNF-α in the serum of patients with ß-thalassemia were higher than ß-thalassaemic carrier and normal controls and suggested that these may be related to the vascular complications in these patients and might be useful markers for the follow-up of the vascular disease. This confirmed by Butthep et al., study **[21]** in which increase number of circulating endothelial cells (CEC) was demonstrated in a and ß-thalassemic patients, and ß-thalassemia (both splenectomized and non splenectomized) had higher number of CECs than a thalassemia.

$CD34^+/CD95^+$ were more apoptotic than $CD34^+/CD95^+$ in patients and controls. This might explain molecular basis for exacerbation of apoptosis in marrow cells of ß-thalassemia major. However, we have noticed that $CD34^+/Fas^-$ cells showed apoptosis, and the comparison of apoptosis in $CD34^+/CD95^+$ between patients and controls showed increase of percentage of apoptotic cells in controls than in patients, suggesting an alternative mechanism for apoptosis in addition to fas induced one.

We investigated whether changes in expression of the apoptotic antagonist Bcl_{XL} and Bcl_2 could have a role in the increased apoptosis of stem cells in ß-thalassemia patients and normal controls. We found that all $CD34^+$ cells expressed Bcl_{XL} and Bcl_2 in bimodal distribution of high and low expression in both patients and controls.

Both Bcl_2 and Bcl_{XL} function as suppressors of programmed cell death on growth factor withdrawal in cytokine-dependent hematopoietic cells lines. Peters et al. **[22]** analyzed the expression of Bcl_2 and its related proteins in hematopoietic precursors and progenitors from adult mobilized peripheral blood, cord blood and adult BM. They showed that variable levels of Bcl_2 were expressed by the most primitive hematopoietic cells as well as the most mature ones.

The expression of $Bcl_2{}^{lo}$ & $Bcl_2{}^{hi}$ and $Bcl_{XL}{}^{lo}$ & $Bcl_{XL}{}^{hi}$ within the $CD34^+$ population is unlikely to be related to the permeabilization technique that we adapted for the measurement of intracellular proteins. The fixative and permeabilizing agents we used permeabilizes cells without altering their scatter features and their membrane or cytoplasmic staining.

Our results agree with the result of Peters et al. **[22]** and Ismail et al. **[13]**, but contrast with the result of Park et al. **[23]** who reported that most primitive BM haemopoietic cells ($CD34^+/Lin^-/CD38^-$) express Bcl_{XL} but not Bcl_2. However, it should be noted that the later used immunocytochemistry to detect Bcl_{XL} & Bcl_2 expression in contrast to the flow cytometry used in this study and those of Peters et al. **[22]** and Ismail et al. **[13]**.

Maurillo et al. **[24]** study suggested that in an early phase progenitor cells are protected from apoptosis mostly by Bcl_X subsequently by Bcl_2 which in turn, is lost with differentiation.

The percentage of high expression was higher in patients than controls, it was significant in Bcl_2 but insignificant in Bcl_{XL}. Although there was a positive correlation between the expression of $CD95^+$ and both $Bcl_{XL}{}^{hi}$ and Bcl_2 on $CD34^+$ in patients, with no correlation

between the expression of Bcl_{XL}^{hi} and $Bcl2^{hi}$. These results suggest that upregulation of fas affects the upregulation of antiapoptotic members of Bcl_2 family. Unfortunately, we could not find similar previous studies to compare with except that of Maurillo et al. **[24]** which done on normal hematopoietic precursors not thalassemic patients. They observed that Fas and Bcl_2 in early progenitor cells are not inversely balanced. They suggested that these proteins expressed independently of one another, as we found in our controls. That may be as a consequence of positive and negative stimuli induced by cytokines and adhesion molecules.

This result implies that the regulation of fas antigen in the CD34$^+$ cells in ß-thalassemia patients may be influenced more by antiapoptotic effect of Bcl_2 than Bcl_{XL}. As well as previous studies have shown that fas-induced apoptosis may or may not be inhibited by expression of Bcl_{XL} or Bcl_2 depending on the cell type **[13,25-29]**.

The glycophorin A$^+$ cells from the patients were less apoptotic in this study than the controls but without statistical significance and this suggests that apoptosis in ß-thalassemia major is more in the early erythroid precursors than in the more mature cells, where the percentage of apoptosis in glycophorin A$^+$/CD95$^+$ were more in controls than patients. This agrees with the result of De Maria **[30]** who reported that Fas is rapidly upregulated in early erythroblasts and expressed at high levels through terminal maturation. However, Fas cross linking was effective only in the less mature erythroblasts, particularly at basophilic level, where it induced apoptosis antagonized by high levels of erythropoietin. Also, in the most recent study for Liu et al. **[31]** which was done in the mouse spleen, an erythropoietic reserve organ, early erythroblasts were present at lower frequencies and were undergoing higher rates of apoptosis than equivalent cells in BM. A high proportion of splenic early erythroblasts coexpressed the death receptor Fas, and FasL. Fas-positive early erythroblasts were significantly more likely to coexpress annexin V than equivalent, Fas-negative cells, suggesting that Fas mediates early erythroblast apoptosis in vivo. They examined several mouse models of erythropoietic stress, including ß-thalassemia, but didn't compare them with normal.

On comparison of apoptosis in glycophorin A$^+$/Bcl_{XL}^{hi} and $Bcl2^{hi}$ between patients and controls. We have shown that Bcl_{XL} and Bcl_2 expression don't influence the effect of Fas, as we found that although there was a decrease in the percentage of apoptosis in the glycophorin A$^+$/Bcl_{XL}^{hi} cells in the patients than the controls with statistical significance. There was no correlation between CD95 and Bcl_{XL}^{hi}, and the percentage of apoptosis in glycophorin A$^+$/Bcl_2^{hi} cells was similar in patients and controls although there was correlation between CD95$^+$ and Bcl_2^{hi}.

In conclusion: Apoptosis occurs in CD34$^+$ more than mature cells. Although, Fas antigen is upregulated on CD34$^+$ cells, its role in the pathophysiology of apoptosis in ß-thalassemia is still unclear, indicating that many factors positively or negatively interfere with the Fas-mediated pathway. TNF-α may have role in that and may influence the susceptibility to the precursor cells to Fas mediated killing. Furthermore Bcl_{XL} may not play a significant role in regulation of apoptosis in hematopoietic progenitor cells in ß-thalassemia.

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Journal of the Egyptian Society of Haematology & Research, Vol. 2, No. 2, September: 119-124, 2006

Serum Matrix Metalloproteinase-9 and Transforming Growth Factor $ß_1$ in Patients with Acute Leukemia With and Without Extramedullary Involvement: A Comparative Study

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ABSTRACT

A novel functional relationship between matrix metalloproteinase-9 (MMP-9) and the multifunctional cytokine transforming growth factor ß (TGF-$ß_1$) in the control of tumor-associated tissue remodeling has been recently uncovered. The aim of this work was to estimate the serum levels of MMP-9 and TGF-$ß_1$ in patients with acute leukemia (AL) with and without extramedullary involvement (EMI). Twenty seven adult patients with newly diagnosed AL were enrolled: Group I; 10 patients with clinically evident EMI (mean age: 24.6±14.6 years) and Group II; 17 patients without EMI (mean age: 30.7±14.54 years). Ten healthy age-and sex-matched individuals were included as a control group (Group III).

In addition to routine diagnostic work-up and imaging studies, serum MMP-9 and TGF-$ß_1$ were estimated for all patients and controls using the ELISA technique.

The results showed that the mean values of serum MMP-9 and TGF-$ß_1$ were significantly higher in group I compared to group III. However, the mean serum MMP-9 did not differ significantly between group II and group III, although the mean serum TGF-$ß_1$ was significantly higher in group II compared to group III. In the mean time, group I showed significantly higher mean values of MMP-9 and TGF-$ß_1$ compared to group II. In conclusion, serum MMP-9 and/or TGF-$ß_1$ can be added as new biological markers that indicate the presence of EMI in adult patients with acute leukemia. However, the possible roles of these markers in the follow-up and in evaluating patients' response to therapy should be addressed in future studies.

Key Words: *Serum MMP-9 – TGF-$ß_1$ – AL.*

INTRODUCTION

A major hallmark of acute leukemia is the uncontrolled capacity of hematopoietic cells to proliferate and breakdown cell-stroma interactions leading to egress of immature blood cells from the bone marrow (BM) to the peripheral blood (PB) **[1]**. Extracellular matrix (ECM) degrading enzymes are required to breakdown these structural barriers. Extensive evidence indicates that several members of the matrix metalloproteinase (MMP) family of enzymes play a key role in this regard **[2]**.

MMP-9 (Gelatinase B), a member of MMP family, is capable of degrading type IV, V and VI collagens **[1]**. Thus, MMP-9 is assumed to play a key role during the metastasizing process through the disruption of basement membranes. Although the role of MMP-9 is widely studied in solid tumors, little is known about its role in hematological malignancies **[2]**. Moreover, little is known about the application of tissue inhibitors of MMPs (TIMMPs) in the treatment of leukemia **[3]**.

Transforming growth factor beta (TGF-ß) is a pleiotropic cytokine involved in a variety of biological processes in both normal and transformed cells. The TGF-ß signaling pathway is an essential regulator of cellular processes, including proliferation, differentiation, migration, and cell survival. During hematopoiesis, the TGF-ß signaling pathway is a potent negative regulator of proliferation while stimulating differentiation and apoptosis when appropriate **[4]**.

Deregulated TGF-ß signaling is known to be involved in a variety of human cancers including those of the colon, pancreas, breast and prostate. Recently, evidence demonstrating deregulated TGF-ß signaling in leukemogenesis,

has started to emerge **[5]** thus defining a tumor suppressor role for the TGF-ß pathway in human hematologic malignancies **[4,5]**. On the other hand, elevated levels of TGF-ß can promote myelofibrosis and the pathogenesis of some hematologic malignancies through their effects on the stroma and immune system **[4]**.

TGF-ß has three isoforms (TGF-$ß_1$, $ß_2$, and $ß_3$). The first recognized growth factor is TGF-$ß_1$. It belongs to a family of dimeric 25kDa polypeptides that are ubiquitously distributed in the tissues and synthesized by many different cells **[6]**.

A novel functional relationship between MMP-9 and the multifunctional cytokine TGF-ß in the control of tumor-associated tissue remodeling has been recently uncovered. The hyaluronan receptor CD44 provides a cell surface docking receptor for proteolytically active MMP-9. Cell surface localization of MMP-9 is an important factor in its ability to promote not only tumor invasion, but angiogenesis and growth as well. MMP-9 proteolytically cleaves latent TGF-ß thus providing a novel and potentially important mechanism for TGF-ß activation. It is tempting to speculate that latent TGF-ß activation may constitute a part of the mechanisms whereby MMP-9 activity induce or promote angiogenesis **[7]**.

The aim of this work was to estimate the levels of MMP-9 and TGF-$ß_1$ in the sera of patients with acute leukemia with and without extramedullary involvement. This could add new biological markers for this disease and possibly help to determine the patients who develop extramedullary infiltration.

PATIENTS AND METHODS

Twenty seven patients with newly diagnosed acute leukemia, 16 males and 11 females, were enrolled in the study. The patients were divided into two groups:

Group I : 10 patients with clinically evident extramedullary leukemic infiltration. Their ages ranged from 18 to 60 years with a mean age of 24.6±14.6 years.

Group II: 17 patients without extramedullary disease. Their ages ranged from 19 to 54 years with a mean of 30.7± 14.54 years.

In addition, 10 healthy age- and sex-matched individuals were included as a control group (group III). A written informed consent was obtained from all patients and controls before enrollment in the study. All patients were subjected to full history taking, thorough clinical examination and routine laboratory investigations including CBC, renal and liver function tests and serum uric acid. Imaging studies including CT scans and/or MRI were done whenever indicated.

The diagnosis of AML and ALL was made by standard morphology and cytochemistry of peripheral blood (PB) and bone marrow (BM) films according to the French-American-British (FAB) criteria **[8]** and the immunophenotyping using a comprehensive panel of monoclonal antibodies (mAbs) against myeloid and lymphoid associated antigens as proposed by the EGIL group **[9]**.

Immunophenotypic analysis: **[10]**

It was performed on fresh PB or BM samples taken at the time of diagnosis. Samples were analyzed using FACScan analyzer (Becton and Dickinson, San Jose, CA). Data were processed using Cell Quest Software (Becton and Dickinson, San Jose, CA).

A wide panel of mAbs was used. It included common leukocyte antigen CD45, myeloid markers; MPO, CD117, CD13, CD33, CD14, CD15, T cell markers; CD1, CD2, CD3, CD4, CD5, CD8, B cell markers; CD19, CD20, CD22, CD10, IgM, Kappa and Lambda light chains, stem cell marker CD34 and the erythroid marker glycophorin A. Double marker labeling was performed including proper isotype controls. All mAbs and isotype controls were supplied from Dako Cytomatiom (Denmark), and Immunotech (France).

A membrane surface marker is considered positive when over 20% of the gated population expressed it, and an intracellular marker (MPO and IgM) was considered positive when over 10% of the gated cells expressed it. In all experiments, a minimum of 10,000 cells were analyzed.

Determination of serum concentration of MMP-9: **[11]**

It employed the enzyme linked immunosorbent assay technique (ELISA). MMP-9 assay kit was purchased from quanti kine.

Standards and samples were pipetted into microwells precoated with anti MMP-9. Thus, any MMP-9 present in the standards or samples was bound by the immobilized antibody. After washing away unbound substances, an enzyme linked polyclonal antibody specific for MMP-9 was added to the wells. Following a wash to remove antibody-enzyme reagent, a substrate solution was added to the wells and a colour developed in proportion to the amount of total MMP-9 bound in the initial step. The colour development was stopped and the absorbance of each well was measured using a microplate reader set at 450nm. A standard curve was plotted from the seven MMP-9 standard dilutions and the concentration of each sample was determined.

Determination of serum concentration of TGF-ß1: **[11]**

It was done by enzyme linked immunosorbent assay (ELISA). TGF-$ß_1$ assay kit was purchased (from Bendermed systems).

Standards and samples were pipetted into microwells precoated with anti TGF-$ß_1$. Consequently, any TGF-$ß_1$ present in standards or samples was bound to the antibodies adsorbed to the wells; a Horse Radish Peroxidase (HRP) conjugated monoclonal anti TGF-$ß_1$ antibody was added and bound to TGF-$ß_1$ captured by the immobilized antibody.

After removal of the enzyme-antibody by aspiration and washing, a substrate solution reactive with HRP was added to the wells. A colour was formed in proportion to the amount of TGF-$ß_1$ present in the standards and samples. Finally, the reaction was terminated by addition of an acid and absorbance was measured using a microplate ELISA reader set at 450nm. A standard curve was plotted from seven TGF-$ß_1$ standard dilutions and the concentration of each sample was determined.

Induction chemotherapy was instituted for all patients. For ALL patients; standard induction included prednisone, vincristine, anthracyclines and L-asparaginase **[12]**. For AML patients, the 7 and 3 protocol was given i.e., doxorubicin 45mg/m^2/day for 3 days and cytosine arabinoside 100-200mg/m^2/day for 7 days **[13]**.

Patients were considered in complete remission (CR) when they have a morphologically normal BM containing <5% blasts and no Auer rods; absence of extramedullary leukemia and normalization of neutrophils ($\geq 1.5x10^9$/L) and platelet counts ($>100x10^9$L). These criteria should be maintained for at least 4 weeks or until initiation of intensification therapy if earlier than 4 weeks. Partial remission (PR) was defined by 5 to 25% BM blasts. Patients was considered refractory when their BM contained ≥25% blasts **[12,13]**. All patients were followed-up for at least 6 months to assess their response to chemotherapy.

Statistical analysis was done using SPSS package. Data parameters were described in the form of mean ± standard deviation. For comparative studies, student '*t*' test was used for comparing the means of two continuous variables. The probability (*p* value) was considered significant when $p<0.05$ **[14]**.

RESULTS

The results of this study are presented in tables (1) through (3) and Figs. (1) through (3). Table (1) illustrates the clinical data in the two studied patients groups and their response to induction chemotherapy. Extrameduallary involvement was in the form of meningeal leukemia with CSF positive for blasts in 5 patients (4 patients had right facial palsy and one had bilateral facial palsy). One patient developed spinal compression and paraplegia, one had intracerebral mass (Chloroma), one had right proptosis, one had right kidney infiltration (diagnosed by renal biopsy) and one had testicular leukemia. No significant differences were found between groups I and II regarding the clinical data except for the response to induction chemotherapy which was significantly inferior in group I patients (with EMI) compared to group II (without EMI) (Pearson Chi-Square = 16.815, $p=<0.001$).

Table (2) shows the important hematological findings in the two studied patients groups and their statistical comparison. No significant difference was found between them except for the platelet count which was significantly lower in group I patients compared to group II.

The mean values of serum MMP-9 and TGF-$ß_1$ in the 3 studied groups are shown in Table (3), while Figs. (1,2,3) illustrate the statistical comparison as regards these two parameters

between group I and group III, group II and group III, and group I and group II, respectively.

The mean values of serum MMP-9 and TGF-$ß_1$ were significantly higher in group I compared to group III (p=0.002 and 0.004 respectively). However, the mean serum MMP-9 did not differ significantly between group II and group III, although the mean serum TGF-$ß_1$ was significantly higher in group II compared to group III (p=<0.001).

On comparing group I and group II, we found that the former showed significantly higher mean values of MMP-9 and TGF-$ß_1$ compared to the latter (p=<0.001 and 0.007 respectively).

Table (1): Clinical data in the two studied patients groups.

Parameter	Group I (n=10)		Group II (n=17)		p* value
	No.	%	No.	%	
Fever	7	70	8	47	0.424
Pallor	8	80	12	70.6	0.363
Hepatomegaly	5	50	7	41.1	0.656
Splenomegaly	7	70	7	41.1	0.236
Lymphadenopathy	4	40	6	35.3	1.000
Diagnosis:					
B-ALL	3	30	6	35.3	
T-ALL	1	10	3	17.6	0.777
AML	6	60	8	47	
Response to induction chemotherapy:					
CR	–	–	13	76.5	
PR	3	30	3	17.6	<0.001
Refractory	7	70	1	5.9	

Abbreviations:
CR = Complete remission. PR = Partial remission.
*Fisher's Exact Test and Person Chi-Square Test are used.

Table (2): Important hematological data in the two studied patients groups.

Parameter	Group I (Mean±SD)	Group II (Mean±SD)	t test	p value
Hb (g/dl)	7.03±1.27	7.14±1.96	–0.178	0.86
TLC ($x10^9$/L)	21.75±26.38	18.62±18.51	0.362	0.72
PLT ($x10^9$L)	35.4±30.78	71.29±59.6	–2.06	0.05*
BM blasts (%)	65.7±24.76	75.29±18.96	–1.134	0.27

Abbreviations:
Hb = Hemoglobin concentration. PLT = Platelet count.
TLC = Total leucocytic count. BM = Bone marrow.

Table (3): Mean values of serum MMP-9 and TGF-$ß_1$ in the three studied groups.

Parameter (Mean±SD)	Group I (n=10)	Group II (n=17)	Group III (n=15)
Serum MMP-9 (ng/ml)	2.63±0.99	0.55±0.39	1.08±0.91
Serum TGF-$ß_1$ (ng/ml)	137.96±104.74	23.58±11.79	8.6±2.17

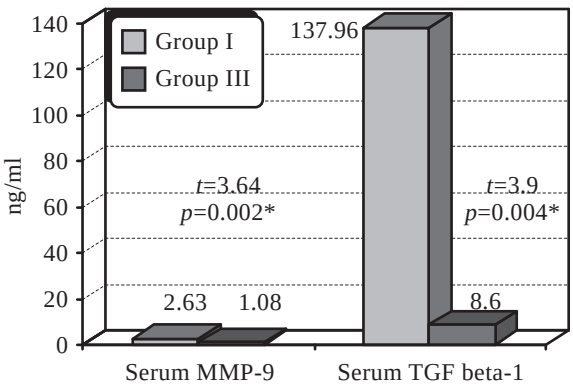

Fig. (1): Comparison between group I and group III as regards serum MMP-9 and TGF-$ß_1$.

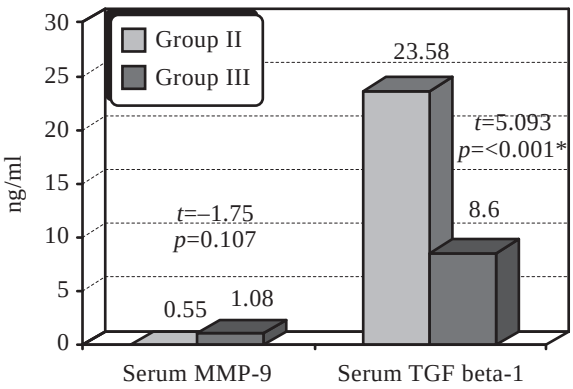

Fig. (2): Comparison between group II and group III as regards serum MMP-9 and TGF-$ß_1$.

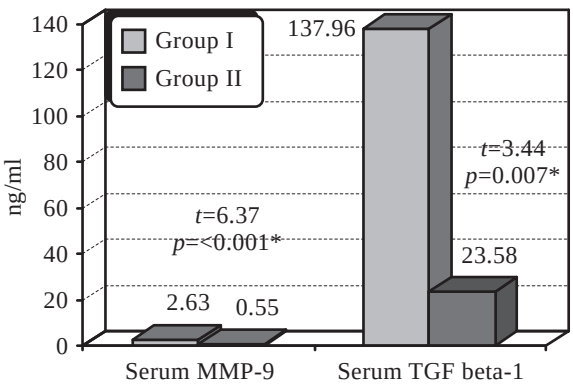

Fig. (3): Comparison between group I and group II as regards serum MMP-9 and TGF-$ß_1$.

DISCUSSION

The role of angiogenesis in solid tumors is well recognized, but its importance in hematological malignancies is less well understood. In leukemia, mainly the determination of microvascular density utilizing immunohistochemistry in the BM trephines and the measurement of soluble angiogenic factors have led to the recognition that angiogenesis may be important in leukemia as well **[15]**.

In the present study, leukemic patients without EMI exhibited lower (but not significant) serum level of MMP-9 compared to the controls. This is in accordance with Lin et al. (2002) **[1]** who reported a significantly lower MMP-9 level in the BM of patients with ALL or AML than in normal controls. Also, Ries et al. (1999) **[16]** reported that MMP-9 gene transcription level was lower in patients with AML and MDS than in healthy individuals. They explained this finding by the presence of MMP-9-releasing mononuclear phagocytes and lymphocytes in the BM-mononuclear cell fraction of healthy individuals compared to that of AML patients consisting predominantly of leukemic blasts. The same result has been more recently reported by Aref et al. (2003) **[17]**.

On the other hand, patients with acute leukemia and EMI had significantly higher level of MMP-9 compared to the controls and to patients without EMI. This result could be explained by the fact that MMP-9 which is involved in mobilization of normal hematopoietic cells from the BM to the PB, also plays a key role in tumor invasion by digestion of ECM. Extramedullary tissue involvement necessitates the excessive egress of leukemic cells from the BM into PB, followed by infiltration of various organs such as lymph nodes, liver, spleen, lungs, intestinal tract, skin or mucous membranes. This means that the cells have to cross, matrix barriers and penetrate blood vessel walls, depending on the catalytic modification of ECM and basement membrane. MMPs, including MMP-9 are capable of digesting almost all components of the ECM **[18]**.

In accordance with our results, Aref et al. (2003) **[17]** reported significantly higher MMP-9 levels in AML patients with EMI compared to those without. They suggested that MMP-9 is involved in the mobilization of leukemic cells. Blast cells purified from PB of AML patients with EMI have been documented to continuously release MMP-9 **[19]**.

In a very recent study, Yang et al. (2006) **[20]** found significant positive correlation between the expression of MMP-9 and vascular endothelial growth factor (VEGF) m-RNA or protein levels in AML patients. Moreover, significant higher expression was noted in patients with EMI. They suggested that VEGF and MMP-9 may participate in the extramedullary leukemic invasion in AML patients.

Kuittinen et al. (2001) **[21]** found significant positive correlation between MMP-9 expression and EMI in adult ALL, but not in pediatric patients indicating basic biological differences between adult and childhood ALL.

As regards TGF-$ß_1$, we observed significantly higher serum levels in group I and group II patients, when each group was compared with the controls. Also, group I exhibited significantly higher TGF-$ß_1$, level than group II. Our results are in agreement with Albitar (2001) **[21]** who reported increased levels of various angiogenic factors including TGF-*ß* in patients with AML and MDS. Moreover, these findings support the recent speculation that elevated TGF-*ß* is involved in the pathogenesis of some hematologic malignancies including leukemias **[4,5]**.

This seems to be in contrast to the results reported by Al-Mowalled et al. (2006) **[15]** who found no significant difference between TGF-$ß_1$ levels in children with ALL compared to controls. However, this could be explained by the fact that the majority of the cases included in the present study were adults and there are basic biological differences between adult and childhood ALL as proposed by Kuittinen et al. (2001) **[22]**.

In the present study, the response to induction chemotherapy was significantly inferior in patients with EMI compared to those without (p=<0.001). Complete remission was achieved in 13 out of 17 patients without EMI while none of those with EMI achieved CR. The former group exhibited significantly lower serum levels of MMP-9 and TGF-$ß_1$ compared to the latter. These data could delineate a strong association between serum MMP-9 and TGF-$ß_1$ and response of patients with acute leukemia

to induction chromotherapy. Similar results were reported by Lin et al. (2002) **[1]** and Aref et al. (2003) **[17]** who found that MMP-9 levels were significantly lower in AML patients who achieved CR compared to those who did not.

It can be concluded from the present study that estimation of MMP-9 and/or TGF-$ß_1$ in the sera of adults' patients with acute leukemia can be used as new biological markers that indicate the presence of extramedullary involvement. However, the possible roles of these markers in the follow-up and in evaluating patients' response to therapy should be addressed in future studies.

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Journal of the Egyptian Society of Haematology & Research, Vol. 2, No. 2, September: 1 25-134, 2006

HOX11L2 Expression in Egyptian Pediatric T-Cell Acute Lymphoblastic Leukemia

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ABSTRACT

Background and Purpose: A cryptic translocation t(5;14) (q35;q32) brings HOX11L2 on chromosome 5 under the influence of CTIP2 on chromosome 14 which is highly expressed during T lymphoid differentiation. Studies indicate that t(5;14) and/or HOX11L2 ectopic expression is restricted to T-lineage ALL and is more frequent in children (about 20-25%) than in adults and can represent a frequent specific genetic alteration in childhood T ALL. The deregulation of HOX11 with or without the presence of t(10;14) (q24;q11) or t(7;10) (q35;q24) has been described in 4% to 10% of children with T-ALL respectively and lead to HOX11 gene activation. The aim of this work is to study the frequency of HOX11L2 and HOX11 expression in Egyptian pediatric T-ALL cases and its clinical relevance.

Patients and Methods: Sixty five pediatric patients with newly diagnosed T-ALL, who presented to the National Cancer Institute, Cairo-Egypt, during the period between 1st of January 2004 and end of February 2005, were immunophenotyped by flowcytometry and tested by molecular analysis (RT-PCR) for HOX11L2 expression. Cases positive for HOX11L2 expression were retested whenever bone marrow was done during follow-up. HOX11 expression was tested in 58 cases. As controls, 20 children with B-lineage ALL and 20 with AML were also examined.

Results: No expression of HOX11L2 was detected in B-ALL or AML samples. T-ALL cases were mostly compartment II [32/65, (49.2%)], 26.2% in compartment I and 24.6% in compartment III. Out of the 65 T-ALL cases, 8 (12.3%) expressed HOX11L2 (3 in compartment I, 2 in compartment II and 3 in compartment III). Positive cases had a median age of 10.5 years and 5/8 cases (62.5%) had total leukocytic counts $>100x10^9$/l. During follow-up, positive cases in complete remission showed no HOX11L2 expression while the relapsing case showed HOX11L2 expression 2 months before hematological relapse. The expression of HOX11 was observed in 2 of 58 (3.4%) T-ALL samples tested. None of the HOX11L2 positive cases tested (7/8) expressed HOX11.

Conclusion: Our T-ALL cases showed a lower incidence of HOX11L2 expression than recorded in western countries but HOX11 expression is comparable. HOX11L2 expression may be used as a marker for minimal residual disease detection, however, further study on a larger scale is recommended.

Key Words: *T ALL - HOX11 - HOX11L2.*

INTRODUCTION

Acute leukaemias are the most common form of cancer in children, accounting for approximately one-third (30%) of all juvenile neoplasms (age <16 years). The majority of these cases are classified as acute lymphoblastic leukaemia (ALL) with about 15% and 26% of these being of T-cell phenotype (T-ALL) in developed countries and Egypt respectively **[1,2]**. Despite continual improvements in treatment over many years, T ALL is still associated with a significant mortality, and relapsed ALL continues to contribute greatly to the overall morbidity and mortality of childhood cancer **[3-6]**. About 50% of T ALL has recognizable genetic abnormalities. Several of these abnormalities are specifically associated with T-ALL. The Genetic abnormalities can be divided into: deletions and translocations. The more common genetic defects in T-ALL are submicroscopic deletions of p16/INK4 in 40% to 80% **[7,8]** or SIL-TAL1 in 10% to 25% **[9-11]**. Most famous translocations are t(11;14), t(10;14) and t(1;14) **[12]**.

The most common recurring breakpoints are within the 14q11, 7q32-q36 and 7p15 bands, which contain the T-cell receptor (TCR) genes TCRα/δ, TCRβ and TCRγ, respectively **[3-6]**.

Molecular analysis of the chromosomal breakpoints has identified several T-cell oncogenes; most of them have been formally shown

to be tumorigenic **[13]**. Remarkably, the majority of T-cell oncogenes belong to a number of classic transcription factor families whose expression is most often intended for lineages other than T cells. The factors deregulated in T-ALL comprise the HOX11 homeobox genes, LMO1 and LMO2 which contain duplicated LIM zinc-finger motifs, and MYC, TAL1 (SCL), TAL2 and LYL1 which all encode helix-loop-helix proteins. Studies on gene expression profiles in T-ALL confirmed the activation of these transcription factors to be a hallmark in these leukaemias **[14]**.

Recognized T-ALL oncogenic pathways include transcriptional deregulation by juxtapositioning to one of the TCR loci, resulting in transcriptional deregulation of genes such as HOX11/TLX1, LMO2, LMO1, LYL1, and TAL1/SCL, each of which is present in less than 10% of cases **[12]**.

The HOX11 and the closely related HOX11L2 genes are called orphan homeobox genes because they are located outside the 4 mammalian HOX clusters. They were both identified at recurrent chromosomal breakpoints in T-ALL **[15,16]**.

HOX11 is not expressed in healthy T cells **[17]**. Translocation t(10;14) is a nonrandom alteration observed in both T-ALL and T-cell lymphoblastic lymphomas **[18]**. It leads to high HOX11 expression. There is some evidence that HOX11 may play an important role in leukemogenesis **[17]**. The deregulation of HOX11 in the t(10;14) (q24;q11) or t(7;10) (q35;q24) has been described in 4% to 10% of children with T-ALL respectively and lead to HOX11 gene activation by bringing HOX11 coding sequence under the transcriptional control of regulatory sequence of the TCR gene **[6,17,19]**. HOX11 over expression has been demonstrated in absence of 10q24 rearrangement. HOX11 expression in leukaemic blasts conferred a prognostic advantage **[1,12]**.

Cryptic translocations were also recognized by fluorescence insitu hybridization (FISH), with the most common being the t(5;14) (q35;q32), leading to over expression of HOX11L2/TLX3 in 25% to 30% of pediatric T-ALL **[14-17,20,21]**. This translocation leads to the ectopic expression of HOX11L2 possibly by bringing it under the influence of regulatory elements of CTIP2, a gene highly expressed during T-lymphoid differentiation. It seems to be restricted to T-lineage ALL and is more frequent in children than in adults **[17]**. Another rare translocation observed in T-ALL, t(5;14) (q33;q11), involves the T-cell receptor (TCRα/δ) gene locus on chromosome 14. Its breakpoint on chromosome 5 is located 2kb upstream of the HOX11L2 coding sequence **[22]**. In this respect it could lead to HOX11L2 transcription deregulation **[17]**.

The aim of this study was to prospectively evaluate the frequency and the clinical relevance (possible role in follow-up and the prognostic value) of HOX11 and HOX11L2 expression in childhood T-ALL.

PATIENTS AND METHODS

Patients:

Patients were children with T-ALL presenting to the pediatric oncology department at NCI, Cairo University, from beginning of January 2004 to end of February 2005. Sixty five patients were diagnosed as T-ALL during that time, included 48 males and 17 females with age ranging from 0.5-17 years with a median of 10 years. Bone marrow (BM) or peripheral blood (PB) samples were obtained at the time of diagnosis and during cytologic remission or at relapse whenever possible. Diagnosis was based on standard morphologic, cytochemical parameters of leukemic cells and on the expression of T-cell antigens and the absence of B-cell and myeloid antigens. Clinical data of patients are summarized in Table (1). Samples from children with B-ALL (20 patients), and acute myeloblastic leukemia (AML, 20 patients) diagnosed during the same period of time were also tested.

Written informed consent was obtained from the patients' parents and the protocol was approved by the Institution Research Board.

Immunophenotyping:

Immunophenotypic analysis was performed on peripheral blood or bone marrow samples taken at the time of diagnosis. It was assessed by multicolor flow cytometry (Coulter Epics XL, Hialeh). A wide panel of FITC (fluorescin) or PE (phycoerythrin) conjugated monoclonal antibodies (MoAbs) was used. The panel used is listed in Table (2). Double and Triple marker

labeling was performed, including proper isotype controls. All MoAbs and isotypic controls were supplied from Beckman Coulter and Dako Cytomation (Denmark).

Detection of surface markers by direct staining:

The whole blood staining method was performed. Ten µl labeled Mo Ab was added to 100µl whole blood (or BM), incubated in the dark for 20 minutes, hemolyzed (Optilyse® Nowash Lysing solutions, A11894, Beckman Coulter) and washed by PBS then analyzed.

Detection of intracellular markers (CD3 and CD22):

One-hundred µl of whole blood was lysed using lysis solution (Becton & Dicknson) for 10 minutes. Cells were washed once and resuspended in 1ml PBS. A mixture of 500µl 4% paraformaldehyde as fixative, 500µl PBS and 5µl tween 20 as detergent was added to the cells and incubated for 10min. The cells were washed and 10µl MoAb was added and incubated for 30min at 4°C. Cells were washed, suspended in 500µl PBS and analyzed **[23]**.

Any antigen was considered positive when ≥20% of blast cells were stained above the negative control except for CD34 where ≥10% was considered positive.

RNA methods:

Total RNA was extracted from patient samples using Total RNA isolation kit, Purescript, Gentra Minneapolis USA according to the manufacturer's instructions. Whole blood and/or BM was collected on EDTA and processed immediately. The red blood cells (RBCs) in 300ul blood/BM were lysed by RBCs lysis solution, then the cell pellet lysed by cell lysis solution. The protein and DNA were precipitated by protein-DNA precipitation solution. The supernatant containing RNA was collected on 100% isopropanol and centrifuged to precipitate RNA which was then washed by ethanol. RNA pellet was left to dry then dissolved in 20ul RNA hydration solution. RNA was stored at -80ºC until used.

RNA was reverse transcribed from 1µg total RNA in a final volume of 20µL containing reverse transcription-polymerase chain reaction (RT-PCR) buffer (1mM each dNTP, 3mM $MgCl_2$, 75mM KCl, 50mM Tris-HCl pH 8.3), 10U RNAsin (Promega, Madison, WI), 100mM dithiothreitol, 100U Superscript II (Gibco-BRL, Cergy Pontoise, France), and 25µM random hexamers. One hundred nanograms cDNA equivalent of RNA was analyzed in each PCR experiment. PCR was carried out in a final volume of 50µL with 0.5U AmpliGold polymerase (PE Applied Biosystems, Foster City, CA), 200µM of each dATP, dCTP, dGTP and dUTP, 25pmol each primer and 2.5mM $MgCl_2$. Cycle parameters were set for 10 minutes at 95°C and for 15 seconds at 95°C, 40 seconds at 60°C and 40 seconds at 72°C for 35 cycles. PCR products were run on 2% agarose gel at 120 Volt for 20 minutes. Cases were considered positive when a single 244-nucleotide fragment was observed.

Primers **[16]**: Screening for HOX11L2 and HOX11 expression was carried out by Standard RT-PCR using the following primers:

- HOX11L2 2Fo: GCGCATCGGCCACCCCTACCAGA;
- HOX11L2 3Rw: CCGCTCCGCCTCCCGCTCCTC;
- HOX11-712Fo: CTGGCCAAGGCGCTCAAAATG; and
- HOX11-810Rw: GGCCTCCCGTTCCTCCGCAGTC.

Positive control for HOX11L2:

T ALL cases with 100% blasts in peripheral blood were tested for HOX11L2, 2 cases were positive and showed the single 244-nucleotide fragment. They were relapsed cases and hence were not included in the study and either was used as a positive control for every run.

Treatment:

Patients were treated according to the Egyptian NCI treatment protocol modified from the total therapy study XIII-B of St. Jude Children's Research Hospital (SJCRH) **[24]**. It included 6 weeks of induction, 2 weeks of consolidation, and 120 weeks of continuation therapy.

Induction therapy consisted of dexamethasone (Dex), vincrstine (VCR), daunorubicin, asparaginase, etoposide (VP-16), aracytin (Ara-C), in addition to triple intrathecal (IT) therapy for CNS prophylaxis. Consolidation therapy included 2 courses of high dose methotrexate (HDMTX), 6-mercaptopurine (6-MP) and triple IT therapy. Continuation therapy consisted of

extended triple IT therapy and 15 cycles of 8-week course of VP-16+cycophosphamide (CTX), 6-MP+MTX, MTX+Ara-C, dexamethasone (Dex)+VCR, VP-16+Ara-C, 6-MP + HD-MTX, VP-16+Ara-C, Dex+VCR. A reinduction phase for 6 weeks was given starting on week 16 of continuation therapy and consisted of VCR, daunorubicin, asparaginase, Dex, HD-MTX, 6-MP and triple IT therapy. HDMTX was replaced by MTX IV or IM after week 53. VP-16 was replaced with oral 6-MP for 7 days after week 54 to minimize late drug effect.

Triple IT was given every 8 weeks during continuation therapy and discontinued after week 53 (first year of continuation treatment). Only patients with CNS leukemia at diagnosis and those with higher risk for CNS relapse (WBC count of 100 x 10^9/L or more) received IT therapy every 4 weeks during continuation therapy, followed by cranial irradiation at week 56 of continuation therapy. Cranial irradiation was given during weeks 56 to 59 to patients with high-risk for CNS relapse (18Gy) or CNS leukemia at diagnosis (24Gy).

Clinical evaluation and follow-up:

Bone marrow aspiration was done to evaluate response to chemotherapy at day 14 of induction therapy (early response) and day 43 (status post induction). Evaluable cases included 51 T-ALL. Cases who died before treatment or their data were not available were excluded.

Evaluable patients were followed up to evaluate disease status for a period ranging from 9-33 months with median observation period of 14 months.

Complete remission (CR) was defined as a normocellular BM containing less than 5% blast cells and showing evidence of normal maturation of other marrow elements.

Statistical analysis:

Patient data were tabulated and processed using SPSS for Windows **[25]**. Qualitative data are expressed as frequency and percentage, quantitative data as mean ± standard deviation and median. The Student *t* test and the chi-square test were used for comparative analysis. For 2 x 2 contingency tables, the Fisher exact test was used. Differences were considered significant at a *p* value of ≤0.05 and highly significant at a *p* value of ≤0.01 **[25]**.

Life-table estimates were calculated using the Kaplan-Meier method, and the standard error of the life-table estimates was calculated with the Greenwood formula. Patients without adverse events were censored on the date of the last reported contact. The differences between curves were tested for statistical significance using the log rank test.

RESULTS

HOX11 gene family expression analysis:

RT-PCR was performed to detect HOX11L2 and HOX11 expression in 105 pediatric ALL cases, a cohort of 65 pediatric patients with T-ALL, 20 with B-ALL and 20 with AML. No specific fragment could be amplified from B-ALL, or AML samples, whereas a single 244-nucleotide fragment was observed in 8 of 65 (12.3%) T-ALL samples when tested for HOX11L2 expression. The expression of HOX11 was observed in 2 of 58 (3.4%) T-ALL samples tested, also detected as a single 244-nucleotide fragment. None of the HOX11L2 positive cases tested (7/8) expressed HOX11 as well. Patients' description is shown in Tables 3 and 4. PCR products of some HOX11L2 positive cases are presented in Fig. (1).

T-ALL cases were mostly compartment II (T intermediate, Ti) [32/65, (49.2%)], 26.2% in compartment I (T early, Te) and 24.6% in compartment III.(T late, Tl) Phenotype was heterogeneous for HOX11L2 (3 in compartment I, 2 in compartment II and 3 in compartment III).

Clinical outcome and follow-up:

Statistical analysis showed no statistically significant correlation between the HOX11L2 expression and age (median 10.5 years and 9 years for positive and negative cases respectively, p=0.38), gender (males 62.5% and 73.8% respectively, p=0.5) and total leucocytic count (TLC) (median 122.5x10^9/l and 121x10^9/l respectively, p=0.44). As for immunophenotyping the only statistically significant correlation (p=0.05) was seen between HOX11L2 expression and CD8. CD8 was expressed on 2/8 HOX11L2 positive cases irrespective of their maturation compartment, (1/2 T intermediate and 1/3 T late). Also it was expressed on. 1/3 T early but was 19% and in absence of surface CD3 the case was typed T early. We only had 2 cases Tl cytotoxic (ctx) and one of them expressed HOX11L2.

Six out of 8 HOX11L2 positive and 45/57 HOX11L2 negative cases were evaluable. All 6 evaluable HOX11L2 positive cases had BM less than 5% blasts on day 14 of induction therapy. The remission rates were comparable in HOX11L2 positive and negative patients. Table (5) shows status post induction of evaluable patients.

Event free survival (EFS) (Fig. 2) estimated at 3-years was 60%±22% for HOX11L2 positive and 70.6%±7.3% for HOX11L2 negative (p=0.59, log rank).

HOX11L2 as a marker for minimal residual disease (MRD) monitoring:

Because HOX11L2 expression is absent or barely detected in normal hematopoietic tissues **[12,16]**, HOX11L2 expression was tested in bone marrow samples from 3/8 cases during follow-up (Table 3). BM samples were collected in complete haematologic remission. Samples collected from patients 3 and 5 during CR at 1, 2 and 8 months after diagnosis showed no band at the expected site of HOX11L2. Three Samples were analyzed from patient 8. A sample at the time of lymph node relapse which occurred 9 months after diagnosis and although bone marrow was in CR (3% blasts) it showed a band at 244 base pairs (bp), the expected size of HOX11L2. A bone marrow sample was analyzed 11 months after diagnosis. It showed bone marrow relapse (blasts 57%) and HOX11L2 expression. HOX11L2 expression was detectable in the bone marrow 2 months before the frank bone marrow relapse. The patient received intensive therapy that included Fludarabin/ Aracytin/Doxorubicin and bone marrow blasts went down to less than 5% blasts with persistent cervical lymphadenopathy and splenomegaly (no samples were available for molecular testing) but a second medullary relapse (BM blasts 45%) occurred 5 months after the last sample analyzed (16 months after diagnosis) and was again positive for the HOX11L2 expression (Fig. 1).

We came across another case that showed no HOX11L2 expression at presentation but the expression was detected at relapse. The patient was a male, 15 years old, TLC 354x10^9/L and T early phenotype. He went into CR for a year, and then he had BM relapse with HOX11L2 expression.

Table (1): Characteristics of T ALL patients with HOX11L2 and HOX11 status.

	T ALL cases	HOX11L2 positive	HOX11 positive
No. cases	65	8/65 (12.3%)	2/58 (3.4%)
Gender:			
Male	48 (72.3%)	5	2
Female	17 (27.7%)	3	0
Age at diagnosis (years):			
Median	10	10. 5	
Range	5mth-17y	3-16 y	9 and 14y
Low risk age group (1-9.99 yrs)	31 (47.7%)	3	1
High risk age group (<1 and ≥10 yrs)	34 (52.3%)	5	1
TLC count (x10^9/l):			
Less than 50	20 (30.8%)	2	1
50-100	10 (15.4%)	1	0
>100	33 (50.8%)	5	1
Ipt of T lineage:			
Te	17 (26.2%)	3 (37.5%)	1
Ti	32 (49.2%)	2 (25%)	1
TL*	16 (24.6%)	3 (37.5%)	0
Th	8	1	0
Tctx	2	1	0

*Cases expressing surface CD3 were considered T late (TL) however some of them did not express CD4 or CD8 (6/16, 37.5%).
Ipt=Immunophenotype. Te=T early. Ti=T intermediate. Th=T helper. Tctx=T cytotoxic.

Table (2): Panel of monoclonal antibodies (MoAbs).

Monoclonal Ab	Clone	Source
Myeloid Markers:		
CD13	My7-PE	Coulter Hialeah, FL
CD14	RmO52 PE	Coulter Hialeah, FL
CD33	M9-PE	Coulter Hialeah, FL
Lymphatic Markers:		
B Lineage		
CD19	BL6-FITC	Immunotech Marseille, France
CD22	Sd10 PE	Immunotech Marseille, France
T Lineage		
CD1	BL6	Coulter Hialeah, FL
CD2	39C1.5 FITC	Coulter Hialeah, FL
CD3	UCHT1 FITC	Coulter Hialeah, FL
CD4	13B8.2-FITC	Immunotech Marseille, France
CD5	BL1A-PE	Coulter Hialeah, FL
CD7	3A FITC	Coulter Hialeah, FL
CD8	B9.11 - PE	Immunotech Marseille, France
NK		
CD16	3G8 FITC	Coulter Hialeah, FL
CD56	N901 (NKH-1) PE	Coulter Hialeah, FL
Others:		
CD45	Immu19.2-FITC	Coulter Hialeah, FL
HLA-Dr	B8.12.2 FITC	Immunotech Marseille, France
CD10	d5 FITC	Coulter Hialeah, FL
CD34	581	Immunotech Marseille, France
Isotypic Controls:		
IgG1 (Mouse)	FITC	DAKO/COULTER/DIACLONE
IgG1 (Mouse)	PE	DAKO/COULTER/DIACLONE
IgG2a (Mouse)	FITC	DAKO/COULTER/DIACLONE
IgG2a (Mouse)	PE	DAKO/COULTER/DIACLONE

Table (3): Characteristics of T ALL patients with HOX11L2 expression.

No.	Sex	Age at diagnosis (yrs)	WBC count ($x10^9$/l)	Ipt of T lineage	MRD +ve/no tested	BMd14	Induction Response	Status
1	M	11	200	T early	0/0	NE	NE	Non evaluable
2	M	8	115	T early	0/0	M1	Died	Died induction
3	F	15	6	T early	0/1	M1	CR	Alive, CR
4	F	10.5	3.8	T int	0/0	M1	CR	Alive, CR
5	F	16	293	T int	0/1	M1	CR	Alive, CR
6	M	7	85.6	TLh	0/0	M1	Died	Died induction
7	M	3	220	TLctx	0/0	NE	NE	Non evaluable
8	M	16	188	TL	3/3	M1	CR	Relapse

+ve = Positive.
BMd14 = Bone marrow day14.
CR = Complete remission.
M1 = <5% blasts in BM.
NE = Non evaluable.
M = Males.
F = Females.

Table (4): Characteristics of T ALL patients with HOX11 expression.

No.	Sex	Age at diagnosis (yrs)	WBC count ($x10^9$/l)	Ipt of T lineage	MRD	BMd14	Induction Response	Status
A	M	14	31	Te	0	M3	CR	Alive, CR
B	M	9	136	Ti	0	M1	CR	Alive, CR

Te = T early. CR = Complete remission. M1 = <5% blasts in BM. M = Males.
Ti = T intermediate. NE = Non evaluable. M3 = >25% blasts in BM. F = Females.

Table (5): Status post induction of evaluable patients.

Status post†induction	HOX112+ (n=2)	HOX11L2– (n=45)	*p* value
Complete†remission	4/6 (66.6%)	33/45 (73.3%)	
Remission†failure	0/6	5/45 (11.1%)	0.88
Died	2/6 (33.3%)	7/45 (15.6%)	

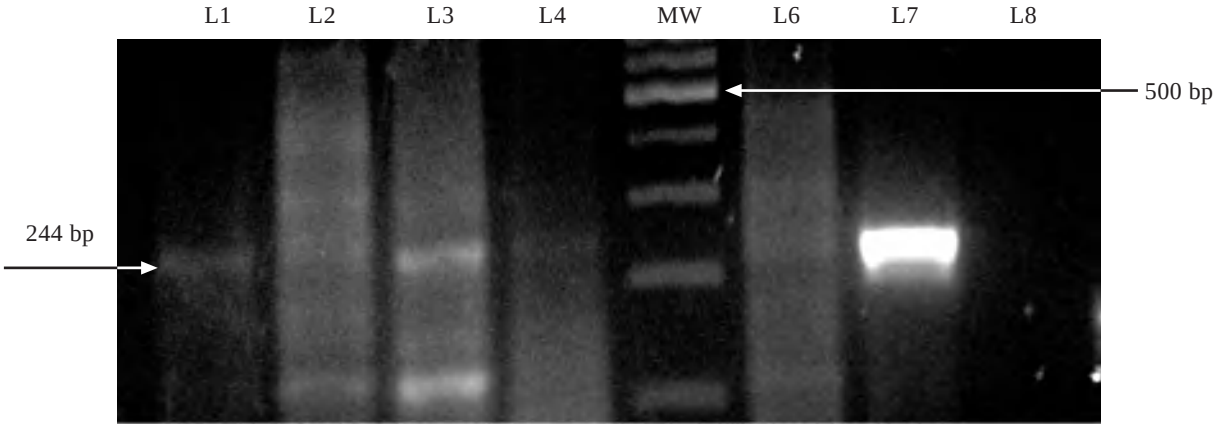

Fig. (1): PCR products of some cases tested for HOX11L2 expression, positive band 244bp.

L1: Follow-up sample of patient (8) 9 months after diagnosis.
L2 and L8: Are negative and template negative controls respectively
L3: BM relapse of patient (8) 2 months later, (11 months after diagnosis)
L4 and L6: Are follow-up BM samples for patients 3 and 5 in CR.
L5: 100bp MW marker
L7: Second BM relapse of patient (8) (16 months after diagnosis).

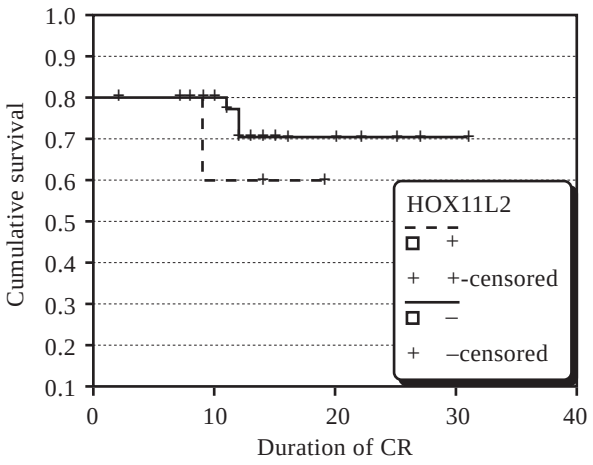

Fig. (2): EFS of studied patients.

DISCUSSION

T ALL comprises 26% of pediatric ALL in Egypt. In a study of T-cell compartments: 54%, 36% and 10% of patients had a phenotype of early (Te), intermediate (Ti) and late (Tl) T-cell maturation respectively (26). Early intrathymic compartment patients had 3-year probability of disease free survival (DFS) of 38% versus 62% and 100% for intermediate and late intrathymic compartments respectively **[2]**. In contrast, T-ALL cases in this study were mostly compartment II (49.2%), 26.2% in compartment I and 24.6% in compartment III.

T-ALL is infrequently characterized by chromosomal translocations, although several T-cell-specific genetic alterations have been described that correlate with different stages of thymocyte differentiation. Translocations t(11;14), t(10;14) and t(1;14) are the most common **[12]** with the most frequent recurring breakpoints within the 14q11, 7q32-q36 and 7p15 bands, which contain the T-cell receptor (TCR) genes TCRα/δ, TCRß and TCRγ, respectively **[3-6]**.

The cryptic translocation t(5;14) (q35;q32) seems to be restricted to T-ALL. Analysis of t(5;14) (q35;q32) by FISH and/or of its molecular consequence HOX11L2 ectopic expression has been reported to represent the most frequent genetic alteration (24%) found in childhood T-cell leukemias so far **[12,17]**. However in the pediatric T-ALL patients studied in this work the HOX11L2 expression was detected in only 12% of cases. It is note worthy that the age of patients seems to be an important point. It has been shown that HOX11L2 deregulation decreased with age **[12]**. In a study of 153 cases the median age was 8 years with an age range of the 6-9 years at diagnosis and an incidence 23% in the HOX11L2-positive cases **[17]**. The age of the cases in this study was older (median of 10 years) with an age range of 3-16 years at diagnosis in the HOX11L2-positive cases.

HOX11L2 expression was reported to have strong correlation with male gender in one study **[27]**. However in our series, no association was observed with gender which is in concordance with other series that showed no significant difference in gender predilection **[17]**.

HOX11L2 expression had no significant association with TLC count at diagnosis in this study as well as in other studies **[17,27]**. In a study all relapse events were observed in the group of patients with high TLC counts ($>50x10^9$/l) at diagnosis. Interestingly, all patients of this group who expressed HOX11L2 had relapses **[16]**. In our study, 6 patients with HOX11L2 had TLC count ($>50x10^9$/l), of which 2 were non evlauable, 2 died in induction, 1 haematologic relapse and one alive in CR.

Various studies reported that the phenotype was rather heterogeneous in the HOX11L2 group with two thirds (66%) having a cortical-T immunophenotype ($CD1a^+$) and 25% of the cases displaying a mature-T phenotype **[17]**. Some suggested that HOX11L2-expressing cases might define a T-ALL subgroup which shows a constant expression of CD1a and CD4 **[15,28]**. In this study the HOX11L2-expressing cases showed 2 cases (25%) with cortical-T immunophenotype and both ($CD1a^+$) and 3 cases (37.5%) mature-T phenotype with a statistical correlation with CD8 (p0.05). It is noteworthy to point out that there were 2 cases of T cytotoxic phenotype out of the 16 T late cases tested and one of them was HOX11L2-positive. However, CD8 expression was also found on a Te and a Ti HOX11L2-positive case.

Reports showed that the incidence of HOX11L2 decreased with age whereas that of HOX11 increased **[12]**. The impact on prognosis remains controversial; some reported HOX11L2 expression in pediatric T-ALL to be associated with poor prognosis **[16]**. Others did not confirm the unfavorable outcome **[17]**.

In a study including 28 T-ALL cases, 6 cases were HOX11L2 positive and 4 of those patients developed relapse and hence appeared to have poor prognosis **[16]**. However in the much larger series (153 cases) the clinical outcome of patients from the HOX11L2 group was similar to that of the patients who did not display this abnormality **[17]**. The ectopic expression of HOX11L2 is probably not associated with the poor prognosis previously reported by other studies **[16,17]**. In concordance, this study showed no significant correlation between HOX11L2 expression and the clinical outcome, however, the number of HOX11L2 group was not big enough to draw conclusions.

The case reported in this study that was originally HOX11L2 negative and then expressed it when relapsed raises again the question about relapse whether it is a simple recurrence of the same disease or a development in the disease appearing due to further genetic events.

Some studies observed high levels of HOX11 transcript in 13% (8/59) of pediatric patients with T-ALL **[14]**, while others reported it in only 7% (9/127) of cases **[17]**. Reports stressed on the association of the increased incidence of HOX11 expression with age **[12]**. However, the study that reported HOX11 expression to be in 7% of cases showed 2 peeks for the incidence

one at the age range 2-5 years and another above 10 years **[17]**. HOX11 over expression seems to be associated with a favorable outcome **[14,29]**.

The 2 HOX11 positive cases reported in this study were 9 and 14 years old and both maintained CR. Because of the rarity of this genetic alteration, all studies lacked the statistical power that would have allowed any definitive conclusion about its prognostic significance. Study of a larger number of cases is required to establish whether the seemingly better outcome associated with t(10;14) and/or HOX11 over expression is indeed real **[17]**.

The presence of occult disease in cancer patients after therapy is one of the major problems facing oncologists. Although 95% of pediatric T-cell acute lymphoblastic leukemia (T-ALL) patients have a complete therapeutic response to multiagent chemotherapy, half would relapse. These relapsing patients must have carried undetected MRD while in remission. The term MRD indicates the presence of leukemic cells below the detection level of conventional methods, i.e., $<10^{-2}$ leukemic cells **[30]**. Multivariate analysis showed that MRD information is an important prognostic factor at all follow-up time points taken during treatment and that this MRD information is independent of the classical clinical parameters at diagnosis such as age, gender, TLC, immunophenotype, chromosomal aberrations, and prednisone response. T-ALL is still heterogeneous in treatment response, but MRD information during treatment of these leukemia subtypes is more discriminative in predicting treatment outcome **[31]**.

Our data suggest that HOX11L2 expression might be considered a suitable marker for minimal residual disease follow-up. During follow up, positive cases in complete remission showed no HOX11L2 expression while the relapsing case showed HOX11L2 expression 2 months before frank hematological relapse. Similarly, Ballerini et al. (2002) reported that monitoring HOX11L2 expression could be useful to follow the clearance of leukemic cells during the early phases of treatment as one patient who maintained HOX11L2 expression had a relapse, whereas the other patient showed a quick drop in the expression of HOX11L2 and did not have a relapse for 10 months **[16]**.

Individualization of ALL treatment might further improve outcome and long-term quality of life. This may be achieved through MRD studies that allow the sensitive detection of leukemic cells undetectable by normal cytomorphologic examination, thereby providing accurate information about the in vivo efficacy of cytotoxic treatment **[31]**.

In Conclusion: Our T-ALL cases showed a comparable HOX11 expression as recorded in western countries. However, we recorded a lower incidence of HOX11L2 expression than that in western countries. HOX11L2 expression may be used as a marker for minimal residual disease detection, however, further study on a larger scale is recommended.

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Journal of the Egyptian Society of Haematology & Research, Vol. 2, No. 2, September: 135-143, 2006

Clinical Relevance of Death Receptors (TNFR1, TNFR2 and CD95) and the Chemokine Receptor CXCR4 Expression in Childhood Acute Leukaemia

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ABSTRACT

Introduction: Death receptors, such as TNF and FAS, play an important role in activating intracellular proteases leading to apoptosis. Some tumor cells such as T- & B-cell leukaemias, and acute myeloid leukemia (AML) over- and under-express FAS. These abnormal regulations of FAS suggest a significant association between tumor and host, namely tumor elimination by apoptosis and tumor survival or progression by escape from apoptosis. Recent studies have shown that CXCR4 is expressed on CD34+ haemopoietic progenitors and also variably on malignant CD34+ myeloid precursors (e.g. AML), as well as adult acute and chronic lymphoblastic leukaemias. A potential mechanism for trafficking of leukemia cells is the interaction of the chemokine receptor CXCR4 and its ligand SDF-1.

Aim of the Work: This study was conducted to evaluate the clinical relevance of expression of these molecules (TNFR1, TNFR2, CD95, and CXCR4) in acute leukaemia.

Material and Methods: We analyzed a cohort of 68 childhood acute leukemia patients (30 AML and 38 ALL) treated at Paediatric Oncology Department, National Cancer Institute, Cairo University, for the expression of the death receptors (FAS and TNFR1 & TNFR2) as well as the chemokine receptor CXCR4 by flow cytometry. We correlated the expression patterns with French-American-British (FAB) subclasses in AML and immunophenotypes of ALL and studied the relationship between expression levels of these molecules and survival.

Results: TNFR2 was expressed in 46.7% of AML and in 18.4% of ALL (p=0.012) while CD95 was expressed in 90% of AML and in 52.6% of ALL (p<0.001). The median expression of TNFR1 was observed to be higher in M4 & M5 than other AML FAB subtypes. The median expression of CD95 was 31 for T-ALL as compared to 12 for C-ALL, and 8 for pre-B ALL (p=0.04). In AML, no correlation between CD34 and CD95 expression was found, however, there was a significant association between extramedullary infiltration of malignant cells and expression of CXCR4 (p=0.05). No significant association was encountered between the studied parameters and response to induction chemotherapy in either ALL or AML. Survival analysis revealed that TNFR1 expression was significantly associated with both overall survival (OS) and disease-free survival (DFS) while expression of TNFR2 was significantly associated with OS in ALL.

We conclude that death receptors and CXCR4 can not be used to predict response to induction chemotherapy in paediatric acute leukaemia, however, TNFR-1 and -2 can predict survival. Since Fas expression is enhanced during maturation of the normal myeloid series and since we found no correlation between Fas expression and CD34 in AML, a possible interpretation is that Fas might be induced via several other pathways e.g. cytokines or cell to cell contact.

Key Words: *Death receptors - CXCR4 - Paediatric acute leukaemia.*

INTRODUCTION

Death receptors, such as TNF and FAS, play an important role in activating intracellular proteases leading to apoptosis. It has been demonstrated that T-cells can kill their target tumor cells using FAS and/or TNF receptors **[1,2]**. However it is less clear whether expression levels of death receptors may play a role in the susceptibility of the tumor to chemotherapy **[3,4]**.

Fas (APO-1/CD95) is a 45kd-membrane protein that belongs to the tumor necrosis factor (TNF)-nerve growth factor receptor family, a group of type 1 transmembrane receptors. Mutational analysis of Fas and the human TNF receptor (TNFR-1) proteins demonstrated that the cytoplasmic domains share a homologous

region necessary to induce the apoptotic signal. This conserved region of approximately 70 amino acids was, therefore, designated as the death domain (DD) **[5]**.

Based on the concept of activation-induced death in T-cells, the cytotoxicity of anti-cancer treatment using cytotoxic drugs or γ-irradiation has been studied with respect to involvement of CD95 receptor ligand interaction. In human T-cell lines, doxorubincin and other cytotoxic drugs used in chemotherapy of leukemias were found to induce CD95L expression **[4]**.

Some tumor cells such as T- & B-cell leukaemias, acute myeloid leukaemia (AML) over- and under-express FAS. These abnormal regulations of FAS suggest a significant association between tumor and host, namely tumor elimination by apoptosis and tumor survival or progression by escape from apoptosis. Indeed, down-modulation or abrogation of FAS on tumor cells such as hepatocellular carcinoma cells, cancer colon cells and chronic lymphocytic leukaemia cells whose Fas is constitutively expressed before transformation has been reported to be a selective advantage and to result in escape from Fas-mediated apoptosis. Fas also has been indicated as a surrogate marker of malignant behavior in T cell leukemia, implying that poor prognosis can be predicted based on the FAS expression levels **[6]**. In AML, some reports have suggested a correlation between FAS expression and complete remission rate and survival after chemotherapy treatment **[7,8]** although others have not confirmed these findings **[9,10]**.

Chemokines are chemotactic cytokines that coordinate development, differentiation, anatomic distribution and trafficking of leucoctyes during innate and adaptive immune reactions and appear to play an important role in tumour growth and expansion through autocrine or paracrine amplification mechanisms. The accumulation of malignant cells can indeed be favored by the production of chemokines by tumor cells themselves and/or surrounding nontumoral cells **[11]**. The stromal cell-derived factor 1 (SDF-1) plays an important role in leucocyte trafficking. It belongs to the CXC chemokine family, which is characterized by intervening residues within a conserved motif. In contrast to other members of the CXC chemokine family that are produced upon cytokine stimulation (e.g. increased interleukin 8 expression during inflammation), SDF-1 is constitutively produced by stromal cells. SDF-1 is not only restricted to the bone marrow, but also found in other tissues such as lymph nodes, liver, spleen, and brain. SDF-1 signals through a G protein-coupled receptor named CXCR4. Recent studies have shown that CXCR4 is expressed on CD34+ haemopoietic progenitors and also variably on malignant CD34+ myeloid precursors (e.g. AML), as well as adult acute and chronic lymphoblastic leukaemias **[12,13,14]**. A potential mechanism for trafficking of leukemia cells is the interaction of the chemokine receptor CXCR4 and its ligand SDF-1.

To evaluate the clinical relevance of expression of these molecules in paediatric acute leukemia, we analyzed a cohort of 68 childhood acute leukaemia patients presented to Paediatric Oncology Department, National Cancer Institute, Cairo University, for the expression of CXCR4 chemokine receptor as well as the death receptors FAS and TNFR1, and TNFR2. We correlated the expression patterns with French-American-British (FAB) subclasses in AML and immunophenotypes of ALL and studied the relationship between expression levels of these molecules and survival.

PATIENTS AND METHODS

Patients:

Sixty eight patients with newly diagnosed childhood acute leukaemia presented to the Paediatric Oncololgy Department, National Cancer Institute, Cairo University were included in the study following informed consent. Thirty patients were AML and 38 were ALL. The patients' characteristics are shown in Tables (1,2). Significant organ infiltration at initial diagnosis in children was defined by ultrasonografic extension of the liver edge ≥2cm below the right costal margin in the right midclacvicular line or the spleen edge ≥2cm below the left costal margin in the left midclacvicular line.

Methods:

The diagnosis of acute leukaemia was based on routine morphologic evaluation and cytochemical smears, as well as immunophenotyping according to the criteria of European Group of the Immunological Characterization of Leukemia (EGIL) **[15]**. B-cell acute lympho-

blastic leukemia (ALL) were subclassified into pro-B ALL, common ALL (n=15), pre-B ALL (n=9) and mature B ALL (n=1). T-cell ALL (n=13) were subclassified into early-T ALL, intermediate-T, and mature T ALL One case mature B ALL and one biphenotypic (B/T) were excluded for statistical reasons. Fresh bone marrow samples were obtained and processed using the whole blood lysing technique and two color staining methods using directly labeled monoclonal antibodies (MoAbs) against the myeloid and lymphoid-associated antigens. TNFR 1, TNFR2, CD95 and CXCR4 antigens were detected using fluorescin isothiocyanate (FITC) conjugated anti-TNFR1, TNFR2, CD95 and CXCR4, conjugated monoclonal antibodies (sc-12746, sc-12750, BD-555674, sc-12764#). FITC isotype matched mouse monoclonal antibodies were used as a negative control for expression analysis. Immunophenotype expression was measured by (FACVantage SE): Becton Dikinson, San Diego, USA) using the CellQuest software programe (Becton Dikinson). The blast region was gated by forward and right-angle light scatter parameters or CD45 antigen versus right-angle light scatter. The result of each antigen was expressed as percent positivity stained cells within the blast population. The antigen was considered positive when 20% or more cells in the blast region expressed the antigen **[16]**.

STATISTICAL METHODS:

Data management and analysis were performed using Statistical Analysis Systems. The graphs were done using Microsoft Word.

Numerical data were summarized using means and standard deviations. Categorical data were summarized as percentages. Comparisons between two groups with respect to numeric variables were done using the Mann-Witney nonparametric test, Kruskal-Wallis test was used for more than two groups. Comparisons between categorical variables were done by the chi square test or Fisher's exact for small sample size.

Overall survival was defined as the time from the date of diagnosis to the last date seen. Disease free survival was defined as the time from the end of treatment to the date of first relapse. Survival estimates were calculated using the Kaplan and Meier procedures. Comparisons of the survival time between the different groups were performed by the Log rank test **[17]**.

All *p*-values are two-sided. *p*-values less than or equal to 0.05 were considered significant.

RESULTS

Patients characteristics:

Clinical criteria of the studied AML and ALL patients are shown in Tables (1,2) respectively.

Expression of death receptors (TNFR1, TNFR2 and CD95):

TNFR2 was expressed in 14/30 (46.7%) and in 7/38 (18.4%) of AML and ALL respectively (p=0.012). On the other hand, CD95 was expressed in 27/30 (90%) of AML and in 20/38 (52.6%) of ALL (p<0.001) (Table 3).

In ALL, the median expression of TNFR1 & TNFR2 were not significantly different among ALL immunophenotypes (p=0.430 & 0.208 respectively). However, the median expression of CD95 was significantly higher in T-ALL than c-ALL or pre B-ALL (p=0.043). (Fig. 1, Table 4).

In AML, the median expression of TNFR1 in M4+M5 group was higher than in M3 or in M1+M2 groups. Such higher expression did not reach a statistical significance due to the small sample size. (Fig. 2, Table 5).

Expression of CXCR4 receptor:

CXCR4 expression did not differ between ALL and AML (p=0.4), among ALL immunophenotypes (p=0.779) or among AML FAB subtypes. (Tables 3,4,5).

Extramedullary infiltration and CXCR4 expression:

Patients were divided in two groups according to the presence or absence of splemomegaly and/or hepatomegaly at diagnosis.

In AML, there was a statistically significant association between CXCR4 expression and extramedullary involvement regardless of the FAB subtype (p=0.05). Patients with organ infiltration had a significantly higher expression of CXCR4 than those without organ infiltration, median 33.5 (9-82) and 13.5 (8-79) respectively.

Table (1): Clinical characteristics of AML patients at diagnosis (N=30).

Characteristic	
Male/female	1.5/1.0
Age years median (range)	8.8 (1.0-17.0)
WBC $X10^9$/L median (range)	16.4 (3.4-335.5)
HGB g/dl median (range)	7.3 (4.0-11.3)
PB blasts median (range)	57.0 (8.0-90.0)
BM blasts median (range)	71.5 (20.0-89.0)
Platelets $X10^9$/L median (range)	33.0 (5.0-84.0)
LDH I U/L median (range)	755 (335-2838)
FAB subtypes (No, %):	
M1 + M2	20 (66.7)
M3	4 (13.3)
M4 + M5	5 (16.7)
M6	1 (3.3)
Hepatomegaly (No, %)	9 (30)
Splenomegaly (No, %)	10 (33.3)
Lymphadenopathey (No, %)	6 (20)
CNS disease (No, %)	2 (6.6)
Response to induction chemotherapy (n, %):	
1- Complete remission	26 (86.7)
2- No response	4 (13.3)

Table (2): Clinical characteristics of ALL patients at diagnosis (N=38).

Characteristic	
Male/female	25/13
Age years median (range)	9 (1.0m-18.0)
WBC $X10^9$/L median (range)	26.6 (1.1-421.0)
HGB g/dl median (range)	7.7 (3.2-16.2)
PB blasts median (range)	34 (0.0-99.0)
BM blasts median (range)	87.0 (20.0-99.0)
Platelets $X10^9$/L median (range)	47.0 (10.0-329.0)
LDH I U/L median (range)	1074 (342-3848)
Immunophenotypes (No, %):	
B-ALL	25 (65.7)
T-ALL	13 (34.2)
Hepatomegaly (No, %)	18 (47.3)
Splenomegaly (No, %)	24 (63.1)
Lymphadenopathey (No, %)	17 (44.7)
CNS disease (No, %)	1 (2.6)
Response to induction chemotherapy (No, %):	
1- Complete remission	26 (68.4)
2- No response	12 (31.5)

Table (3): Expression of the studied markers on AML and ALL.

*Measurement	ALL N=38	AML N=30	*p* value
TNFR1	12 (31.6)	9 (30)	0.889
TNFR2	7 (18.4)	14 (46.7)	0.012
CD95	20 (52.6)	27 (90)	<0.001
CXCR4	18 (47.4)	17 (56.7)	0.446

* No (%).

Table (4): Expression levels of the studied markers among ALL immunophenotypes.

*Measurement	C-ALL N=15	Pre-B N=9	T-ALL N=13	*p* value
TNFR1	11 (0-82)	4 (0-32)	7 (0-30)	0.430
TNFR2	12 (0-72)	4 (1-34)	3 (0-18)	0.208
CD95	12 (1-79)	8 (2-41)	31 (2-82)	0.043
CXCR4	52 (1-87)	10 (2-67)	11 (1-96)	0.779

* Median (range).

Table (5): Expression of the studied markers among AML FAB subtypes.

*Measurement	M1+M2 N=20	M3 N=4	M4+M5 N=5
TNFR1	6.5 (1-34)	27 (3-39)	34 (2-82)
TNFR2	8.5 (1-68)	49 (2-77)	41 (1-77)
CD95	74 (10-92)	83 (36-93)	40 (11-70)
CXCR4	18 (8-82)	39 (9-55)	32 (8-79)

* Median (range).
p values not valid due to the small sample size.

In ALL, no statistically significant difference was found between the two groups (p=0.467).

Association between CD34 and the studied markers in AML:

TNFR1 was positive in 1/12 (8.3%) of the CD34 positive group as compared to 8/18 (44.4%) of the CD34 negative group with a significant difference between the two groups (p=0.049). On the other hand, expression of TNFR2, CD95, and CXCR4 was not different between CD34 positive and CD34 negative groups (p=0.072, 0.255, and 0.176 respectively). (Table 6).

Response to induction chemotherapy:

In AML, there was no significant association between TNFR1, TNFR2, CD95 and CXCR4 expression and response to induction chemotherapy (p=1.0 for all).

Similarly, in ALL, no significant association between TNFR1, TNFR2, CD95 and CXCR4

expression and response to induction chemotherapy was encountered (p=0.060, 0.075, 0.633, and 0.825 respectively).

Survival analysis:

Overall survival (OS):

One year OS in ALL was 100% for TNFR1 positive patients and 48% for TNFR1 negative patients with a significant difference between the two groups (p=0.016). The OS for TNFR2 was 100 versus 57% for patients with positive expression versus negative expression respectively with a significant difference between the two groups (p=0.042). (Table 7).

Disease free survival (DFS):

The median follow-up duration was 12 months ranging from 1.5 to 18 months. In ALL, DFS was 100% versus 59% for patients positively expressing TNFR1 versus those with negative expression with a significant difference between the two groups (p=0.005). (Table 8).

Table (6): Association between CD34 expression and the studied markers in AML.

*Measurement	CD34+ N=12	CD34– N=18	*p* value
TNFR1	1 (8.3)	8 (44.4)	0.049
TNFR2	3 (25.0)	11 (61.11)	0.072
CD95	12 (100)	15 (83.33)	0.255
CXCR4	5 (41.67)	12 (66.67)	0.176

* No (%).

Table (7): Impact of the studied markers on overall survival among ALL and AML.

	ALL			AML		
	Survival %	No.	*p* value	Survival %	No.	*p* value
TNFR1:						
Positive	100	12	0.016	88	9	0.808
Negative	48	26		83	21	
TNFR2:						
Positive	100	7	0.042	83	14	0.868
Negative	57	31		85	16	
CD95:						
Positive	73	20	0.246	86	27	0.270
Negative	61	18		66	3	
CXCR4:						
Positive	75	18	0.164	80	17	0.494
Negative	61	20		90	13	

Table (8): Impact of the studied markers on disease-free survival among ALL and AML.

	ALL			AML		
	Survival %	No.	*p* value	Survival %	No.	*p* value
TNFR1:						
Positive	100	11	0.005	83	6	0.837
Negative	59	15		78	14	
TNFR2:						
Positive	100	7	0.084	75	8	0.653
Negative	67	19		83	12	
CD95:						
Positive	84	13	0.176	82	17	0.428
Negative	61	13		66	3	
CXCR4:						
Positive	75	14	0.723	66	9	0.219
Negative	78	12		90	11	

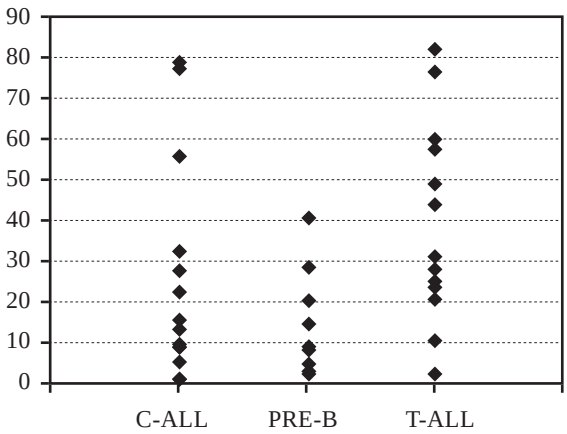

Fig. (1): Expression of CD95 among ALL immunophenotypes.

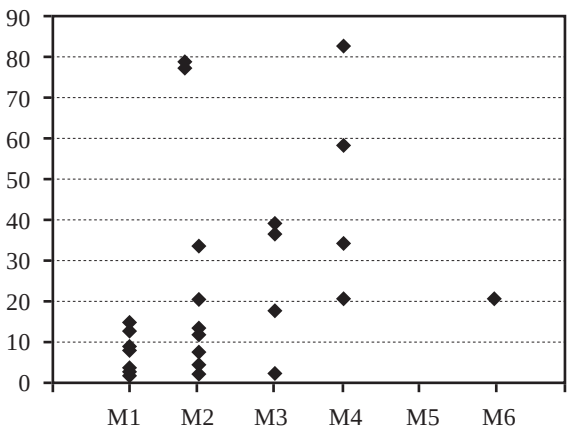

Fig. (2): Expression of TNFR1 among AML FAB subtypes.

DISCUSSION

Death receptors, such as TNF and FAS, play an important role in activating intracellular proteases leading to apoptosis. Some tumor cells such as T- & B-cell leukaemias, and acute myeloid leukemia (AML) over- and under-express FAS. These abnormal regulations of FAS suggest a significant association between tumor and host, namely tumor elimination by apoptosis and tumor survival or progression by escape from apoptosis. Recent studies have shown that CXCR4 is expressed on CD34+ haemopoietic progenitors and also variably in malignant CD34+ myeloid precursors (e.g. AML), adult acute and chronic lymphoblastic leukaemias. A potential mechanism for trafficking of leukemia cells is the interaction of the chemokine receptor CXCR4 and its ligand SDF-1 **[4]**.

To determine the clinical relevance of the expression of various death receptors (TNFR1, TNFR2, CD95) and the chemokine receptor CXCR4 on childhood AL cells, we performed a phenotypic analysis on leukaemia cells from 30 patients with AML and 38 patients with ALL. We also analyzed the relationship between expression levels of these molecules and OS as well as DFS rates.

In the current study, Fas was expressed with variable percentage (11-93%) in all AML cases and 90% were positive for FAS expression. Similarly, Iijima et al. and Munker et al. **[8,9]** found that Fas expression was quite variable in AML reflecting its heterogeneity.

In our AML cases, Fas was positive in 12 cases (100%) of the CD34+ group as compared to 15 cases (83.3%) of the CD34- group (p=0.2). Our result is similar to Iijima et al. **[8]** who found no correlation between Fas expression and CD34 expression. In normal haemopoesis, immature cells do not express a significant level of Fas. Fas expression becomes enhanced with the maturation pathway of myeloid series. Thus an alternative interpretation is that FAS might be induced via several pathways such as cytokines and cell-cell interactions. Previous studies have shown that INF γ and TNFα can induce FAS on normal and tumor cells **[8]**.

In our ALL, FAS expression was significantly lower (p<0.001) than in AML cases and this finding was supported by the previous study of Tsuruda et al. **[6]**. Despite this lower expression, CD95 was expressed in all cases of ALL with expression percentages varying between very low (1%) and high (89%). A comparison of B and T lineage ALL revealed a significantly higher CD95 expression in T-lineage ALL (p=0.04). Our results are similar to the previous studies **[6,18,19]**.

In this work, we found no association between Fas expression and response to induction chemotherapy in ALL. Similar data were also reported by Wuchter et al. **[19]**. The fact that, compared to AML, CD95 expression and function is constitutively low in ALL might explain the lack of its prognostic relevance in childhood ALL **[18]**. Also no significant association could be reached between CD95 expression and response to induction chemotherapy in AML. In contrast, CD95 expression was shown to correlate with response to induction chemotherapy in AML **[7,8]**.

Reports on FAS expression and prognosis are conflicting, showing no relationship **[9]** or a relationship with resistant disease **[7,8]** although no correlation between FAS expression and apoptosis could be proven. In the current study we did not find a significant relationship between CD95 expression and overall survival in AML or ALL. Our result may be supported by the previous observations that the expression of CD95 on the surface of leukaemic cells alone is not sufficient for CD95 induced apoptosis **[18]**. Although most of malignant cells expressed CD95 they failed to undergo CD95 mediated apoptosis showing low susceptibility to CD95 triggering.

Data regarding expression of TNFR1 and TNFR2 in AML are very scarce and no reports, so far, regarding their expression in ALL was found. In our work, TNFR1was significantly higher in AML than ALL (p=0.01) while TNFR2 was not different between both groups of AL (p=0.9). Both TNFR1 and TNFR2 expression was not significantly different among ALL immunophentotypes (p=0.4 & 0.2 respectively). Brouwer et al. **[16]** reported higher levels of TNFR1 and TNFR2 in AML-M4 & M5 FAB subtypes; our study only showed a higher median expression of TNFR1 in M4 & M5. Statistical analysis could not be done due to the small sample size. It is worth mentioning that, at the

individual case level, M1 showed the lowest values followed by M2. In ALL, we found a positive correlation between TNFR1 and TNFR2 expression and OS (p=0.016, and 0.04) respectively and a significant correlation between TNFR1 expression and DFS (p=0.005). TNFR1 may induce pro- and anti-apoptotic signals resulting in variable net effect **[20]**, whereas the role of TNFR2 is unknown. The relatively higher expression of death receptors in AML cells could imply that these cells are prone to apoptosis **[21]** but to date, there are no convincing data that show a clear correlation between high levels of FAS and TNFR and susceptibility to T, NK cell or chemotherapy mediated lysis **[4,22]**.

In our work, CXCR4 was expressed in all AML FAB subtypes with no observed difference between the groups. Our results are similar to the few available reports of Cignetti et al. and Mohle et al. **[11,12]** who found that all AML cases, regardless of the FAB subtype, express CXCR4. Although Brouwer et al. **[16]** found that FAB M4 and M5 show a significantly higher expression of CXCR4 than other FAB subtypes, our results could not support this finding. Expression of the chemokine receptor CXCR4 by the myeloid leukaemic cells suggests that such expression upon interaction with its ligand SDF-1 might mediate migration of leukaemic blasts across bone marrow endothelium.

In agreement with Mohle et al. **[13]**, we found a significant association between extramedullary organ infiltration of leukemia blasts and CXCR4 expression in AML. In our ALL, on the other hand, no significant association could be reached. This contrasts with the previous studies of Crazzaolara et al. **[23]** who found an association between extramedullary organ infiltration of leukemia blasts and CXCR4 expression. The reason for the discrepancy between the two studies could be attributed to small number of patients with mature B-ALL in our study (one patient) as it has been reported that the strongest expression of CXCR4 in ALL was found in mature B-cell ALL which is generally characterized by a high incidence of extramedullary bulky disease **[23]**.

In ALL patients CXCR4 expression was found in 47.3% of cases which was lower than the 91.9% observed by Crazzaolara et al. **[23]**. Their higher result could be due to the fact that they measured CXCR4 using mean fluorescence intensity (MFI) rather than percent expression as in our study thus allowing detection of low level of expression of the CXCR4 **[24]**.

The presumed normal counterparts of malignant acute lymphoblastic B-cells, have been studied for the expression and function of CXCR4 **[25,26]**. Expression of the chemokine receptor was found to be sinusoidal: Highest expression on pro-B cells, decreased as cells develop into immature B cells, and then increased again upon transition to mature B cell stage. In our study we found a similar expression pattern on lymphoblastic subpopulations: Highest expression in the c- ALL (median 52) with lower expression on pre-B ALL (median 10). Our results are supported by the finding of Crazzaolara et al. **[23]**.

In this work, no statistical difference in CXCR4 expression on lymphoblasts between B-and T-ALL (p=0.8). Similar observation was reported by Crazaolara et al. **[23]**.

In this study, no correlation was encountered between CXCR4 expression and either OS or DFS in ALL. Our result is not in agreement with Schneider et al. **[27]** who found that children with DFS under 24 months had a significantly higher expression of CXCR4 than children in first remission at 24 months. CXCR4 analysis in acute leukemia cells could be of value. However, its prognostic relevance requires further evaluation in large prospective studies of homogeneously treated patients.

In conclusion, TNFR2 and CD95 were significantly higher in AML than ALL while TNFR1 showed a higher median expression in M4 & M5 FAB subtypes. Since Fas expression is enhanced during maturation of the normal myeloid series and since we found no correlation between Fas expression and CD34 in AML, a possible interpretation is that Fas might be induced via several other pathways e.g. cytokines or cell to cell contact. There was a significant association betwee CXCR4 expression and extramedullary infiltration in AML. No correlation was found between expression of the studied molecules and response to induction chemotherapy in either ALL or AML. TNFR1 correlated significantly with both OS and DFS while TNFR2 correlated significantly with OS

in ALL. Expressopm of CXCR4 correlates with extramedullary infiltraion in AML. Death receptors and CXCR4 can not be used to predict response to induction chemotherapy in paediatric acute leukaemia, however, TNFR-1 and -2 can be used to predict survival in this group of patients.

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Journal of the Egyptian Society of Haematology & Research, Vol. 2, No. 2, September: 145-153, 2006

Genotype Phenotype Relationship in Gaucher's Disease in Egypt

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ABSTRACT

Background: Gaucher's disease is the most prevalent of the genetic lysosomal storage disorders. It is an autosomal recessive disease which was described by the French physician Philippe Gaucher in 1882. It is caused by a severe deficiency of glucocerebrosidase enzymatic activity with resultant accumulation of large quantities of glycolipid, glucocerebrosidase within the lysosomes of the phagocytic cells of the monocyte-macrophage system. Gaucher's disease is classified into three conventional types; Type I: Chronic non-neuropathic form which usually found in adults especially in Jewish population, Type II: Infantile neuropathic form which always appears by 6 months of age as a rapidly progressive neurological affection, and Type III: Juvenile sub-acute neuropathic with slowly progressive neurological disease that begins during childhood or adolescence. The aim of this study is to investigate the genotypes of Gaucher's disease, the most prevalent mutations in Egypt and to assess if a genotype-phenotype relationship could be elicited.

Patients and Methods: The present study included 20 Gaucher's disease documented patients; they were 10 males and 10 females. Their ages ranged from 3 to 43 years with of a mean of 10.15 and median of 6 years. All patients were subjected to clinical evaluation, revision of their filed clinical progress, radiological and laboratory data including full blood count, b-Glucocerebrosidase enzyme assay, liver enzymes bone marrow and splenic aspirate. In addition, we studied the most common GBA mutations using strip assay which is based on the reversed-hybridization principle The assay covered 8 of the most frequent GBA mutations: 84GG (452+G), IVS2+1 (484 G>A), N370S (1226 A>G), V394L (1297 G>T), L444p (1448 T>C), R496G (1604 G>A), and 2 recombinant alleles (RecNcil, RecTL).

Results: Neither the age of patients, age of onset of the disease nor the sex showed significant relation with homozygosity Vs double heterozygosity or the different genotype groups. The most common mutations found in this study were L444p/L444p (homozygous) and L444p/IVS2+1 (double heterozygous); 8 patients each (40%) followed by L444p/D409H (double heterozygous) in 3 patients (15%) whereas N370S/N370S (homozygous) was the least common mutation found in only 1 patient (5%). Allele frequency showed that L444p was found in 67.5% of studied chromosomes, IVS2+1 in 20%, D409H in 7.5%, whereas N370s was in only 5%. As for family history, 80% of cases had positive consanguinity, with 45% of patients parents being first cousins. All homozygous cases showed positive consanguinity (9 cases), whereas only 7 cases of 11 of the double heterozygous showed positive consanguinity. Also all patients with the genotypes L444p/L444p and N370S/N370S were from consanguineous parents; 8 of 8 and 1 of 1 respectively. On the other hand, the 3 patients with the genotype L444p/D409H had negative consanguinity, while the genotype L444p/IVS2+1 showed 7 of 8 cases with positive consanguinity and only 1 case with negative consanguinity.

Conclusion: The most common mutations found in our study were L444p/L444p homozygous and L444p/IVS2+1 double heterozygous. L444p was the most common allele. Analysis for the most common mutations was the method of choice for identification of Gaucher's disease carriers.

Both age of the patients and onset of the disease had no significant relation with either homozygosity Vs. double heterozygosity or the different genotype groups.

Gaucher's disease occured with equal frequency in males and females. Patients with homozygous gene mutations tended to have consanguineous parents.

Neurological manifestations, growth retardation and chest symptoms were the most common clinical conditions reported in studied cases. None of the previous conditions were significantly associated with certain genotype.

No correlation was detected between genotype or homozygosity Vs heterozygosity on one side and either age of patients, age of onset or clinical manifestations on the other side.

Key Words: *GD (Gaucher's disease) – GBA mutations – Reversed hybridization – Homozygous – Double heterozygous.*

INTRODUCTION AND AIM OF THE WORK

Gaucher's disease is the most prevalent of the genetic lysosomal storage disorders. It is an autosomal recessive disease which was described by the French Physician Philippe Gaucher in 1882. It is caused by a severe deficiency of glucocerebrosidase enzymatic activity with resultant accumulation of large quantities of glycolipid, glucocerebrosidase within the lysosomes of the phagocytic cells of the monocyte-macrophage system.

Gaucher's disease is classified into three conventional types in which clinical differentiation depends upon patients age and organs affected.

Type I: Chronic non-neuropathic form, usually found in adults especially in Jewish population. It is a chronic disease involving viscera and blood forming tissues. Most of these patients develop massive spleen enlargement, anemia and bleeding tendency. In addition, they may have bony pains and pathological fractures.

Type II: Infantile neuropathic form which always appears by 6 months of age. It is more rare than type I and does not have predominance in Jewish population. Neurological manifestations, spleenomegaly, pulmonary and bony affection are the most common presenting symptoms. Most patients die before 2 years of age.

Type III: Juvenile sub-acute neuropathic which includes a heterogenous group of patients with signs of chronic adult type combined with slowly progressive neurological disease that begins during childhood or adolescence **[1]** (Stone et al. 2000).

As there are limited data on the frequency of the different mutations in the Egyptian population, the aim of this study is to investigate the genotypes of Gaucher's disease, the most prevalent mutations in Egypt and to assess if a genotype-phenotype relationship could be elicited.

PATIENTS AND METHODS

Patients:

The present work included 20 Gaucher's disease patients diagnosed by enzyme assay of ß-glucosidase activity in peripheral leucocytes **[2]** (Wenger et al., 1978), who were registered and filed in hematology outpatient clinic of the New Children Pediatric Hospital (Cairo University), 10 were males and 10 were females. Their ages ranged from 3 to 43 years (with a mean of 10.15 and median of 6). All patients had been already diagnosed by clinical and laboratory investigations as Gaucher's disease patients.

Methods:

All studied cases were subjected to clinical evaluation, revision of their filed clinical progress, radiological and laboratory data as follows:

1- *Full medical history:*

Complete history was taken for age, sex, and family history including consanguinity, affected siblings and a similar condition in the family. The prenatal, past and present history of abdominal, respiratory, central nervous system, cardiovascular symptoms or any symptoms suggesting bleeding tendency, were also obtained. Assessment of both physical and mental development was performed for all patients.

2- *Full clinical examination:*

All patients were subjected to complete physical examination including assessment of weight, height and skull circumference to evaluate physical development. Vital signs including pulse, respiratory rate and temperature were determined. Systemic examination was done to every patient including abdominal examination, assessing any splenic or hepatic enlargement. Chest, cardiac and central nervous system examination was also performed.

3- *Investigations:*

A- *Preliminary investigations*:

1- CBC: to detect presence of anemia, thrombocytopenia or any other abnormalities (advia 120).
2- Liver functions which include prothrombin time, SGOT and SGPT.
3- Enzyme assay to detect levels of b-glucocerebrosidase, sphingomyelinase, as well as chitotriosidase enzyme.
4- Bone marrow aspiration to detect the presence of Gaucher's cells and/or the presence of any abnormalities of red and white series as well as megakaryocytes.

5- Splenic aspiration to detect the presence of Gaucher's cells and/or the presence of any other abnormalities.

6- Abdominal U.S to detect hepatomegally, spleenomegally and/or the presence of any other abnormalities.

B- *Specific investigations:*

Strip assay for the identification of glucocerebrosidase (GBA) gene mutations based on polymerase chain reaction (PCR) and reverse-hybridization.

Methodology:

The procedure includes three steps: **[1]** DNA isolation, **[2]** PCR amplification using biotinylated primers, **[3]** hybridization of amplification products to a test strip containing allele-specific oligonucleotide probes immobilized as an array of parallel lines. Bound biotinylated sequences are detected using streptavidin-alkaline phosphatase and color substrates.

The assay covers 8 common GBA mutations: 84GG [452+G], IVS2+1 [484 G>A], N370S [1226 A>G], V394L [1297 G>T], D409H [1342 G>C], L444P [1448 T>C], R463C [1504 C>T], R496H [1604 G>A], as well as 2 recombinant alleles derived from crossover between the GBA functional gene and pseudogene (rec Ncil, rec TL).

RESULTS

Family history of the studied patients revealed that 80% of cases had positive consanguinity, while 45% of patients' parents were first cousins. Eighty percent of cases had affected sibs, in 15% sibs were clinically free, while 5% did not have other sibs. History and clinical examination revealed that 11 (55%) of patients had delayed physical development, while only 2 (10%) showed mental retardation. Neurological manifestations were encountered in 6 (30%) cases; their types and frequency are shown in Table (1). Nine (45%) cases had bleeding tendency while only 1 (5%) case showed cardiac involvement.

Frequency of gene (homozygous Vs double heterozygous) among studied cases was 9 (45%) and 11 (55%) respectively.

There was no significant relation between neurological affection and the genotype, whether homozygous or double heterozygous. Also there was no relation between number of affected sibs and different genotype groups.

As regards the hematologic parameters, we did not find any significant relation between the different genotypes and the presence of anemia, leucopenia, thrombocytopenia or splenic infiltration with gaucher cells. Prothrombin concentration was low in 30% of our cases and GOT level was slightly elevated in 20% of cases. Both had no statistically significant relation with either gene homozygousity Vs double heterozygousity or the different genotype groups.

Table (1): Frequency and type of neurological affection among studied cases.

Neuro	Frequency	Percent
Free	14	70. %0
Hyperreflexia, hypotonia, +ve stepping reflex	1	5.0%
Occulomotor aprexia & squint	3	15.0%
Squint	2	10.0%
Total	20	100.0%

Table (2): Frequency of bone & chest affection among studied cases.

Bone affection	Frequency	Percent	Chest affection	Frequency	Percent
Bony pains, knock knee	1	5.0%	Dyspnea	1	5.0%
Recurrent bony crisis	1	5.0%	Recurrent chest infection	6	30.0%
Free	18	90.0%	Free	13	65.0%
Total	20	100.0%	Total	20	100.0%

Table (3): Frequency of cardiac affection and bleeding among studied cases.

Cardiac affection	Frequency	Percent	Bleeding	Frequency	Percent
Affected	1	5.0%	Positive	9	45.0%
Free	19	95.0%	Negative	11	55.0%
Total	20	100.0%	Total	20	100.0%

Table (4): Spleen and liver size among studied cases.

Spleen	Frequency	Percent	Liver	Frequency	Percent
Enlarged	5	25.0%	Enlarged	9	45.0%
Hugely enlarged	12	60.0%	Hugely enlarged	11	55.0%
Splenectomy	3	15.0%			
Total	20	100.0%	Total	20	100.0%

Table (5): Frequency of anemia, leucopenia and thrombocytopenia among studied cases.

Anemia	Frequency	Percent	Leucopenia	Frequency	Percent	Thrombocytopenia	Frequency	Percent
Absent	3	15.0%	Absent	12	60.0%	Absent	6	30.0%
present	17	85.0%	present	8	40.0%	present	14	70.0%
Total	20	100.0%	Total	20	100.0%	Total	20	100.0%

Table (6): Frequency of B.M and splenic aspirate infiltration by Gaucher's cells among studied cases.

B.M asp infiltration by gaucher's cells	Frequency	Percent	B.M asp infiltration by gaucher's cells	Frequency	Percent
No	4	20.0%	Yes	9	45.0%
Yes	11	55.0%			
Not performed	5	25.0%	Not performed	11	55.0%
Total	20	100.0%	Total	20	100.0%

Table (7): Frequency of gene mutation among studied cases.

	Frequency	Percent
L444p/D409H	3	15.0%
L444p/L444p	8	40.0%
L444p/IVSII+1	8	40.0%
N370s/N370s	1	5.0%
Total	20	100.0%

Table (8): Allele frequency among studied cases.

	Frequency	Percent
L444P	27	67.5%
D409H	3	7.5%
IVS2+1	8	20%
N370s	2	5%
Total	40	100%

Table (9): Relation of consanguinity to homozygousity Vs double heterozygousity.

GENE		Consanguinity			Total	*p* value
		Negative	Positive	Strong		
Homozygous	Count		6	3	9	
	percent		66.7%	33.3%	100.0%	<0.01
Double hetero	Count	4	1	6	11	(Highly significant)
	percent	36.4%	9.1%	54.5%	100.0%	
Total	Count	4	7	9	20	
	percent	20.0%	35.0%	45.0%	100.0%	

Table (10): Relation of consanguinity to homozygousity Vs double heterozygousity.

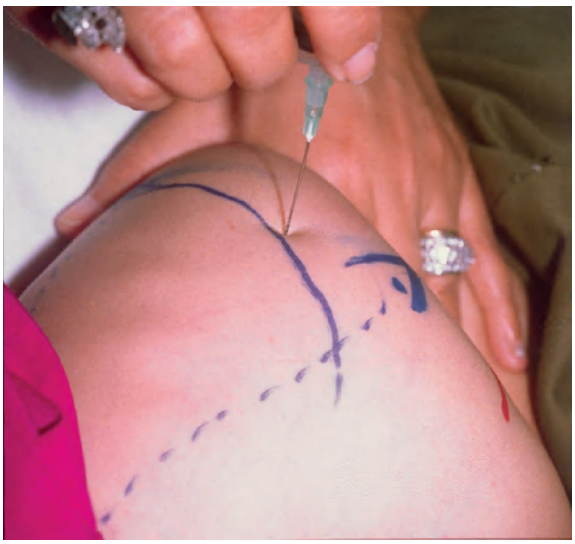
GENE		Present	First baby	No	Total	*p* value
Homozygous	Count	9			9	
	percent	100.0%			100.0%	0.061
Double Hetero	Count	7	1	3	11	(Borderline significace)
	percent	63.6%	9.1%	27.3%	100.0%	
Total	Count	16	1	3	20	
	percent	80.0%	5.0%	15.0%	100.0%	

Table (11): Relation of neurological affection to homozygous Vs double heterozygous.

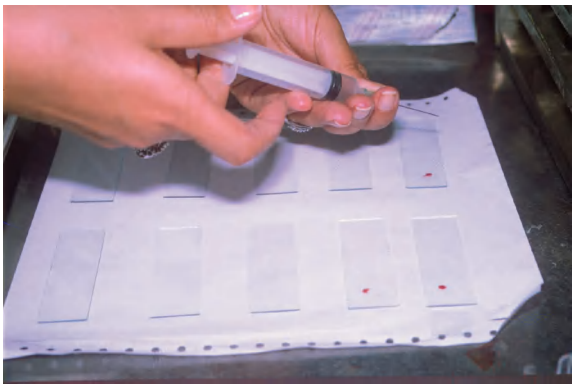
GENE		Absent	Present	Total	*p* value
Homo	Count	6	3	9	
	percent	77.7%	33.3%	100.0%	
Double hetero	Count	8	3	11	N.S
	percent	72.7%	27.3%	100.0%	
Total	Count	14	6	20	
	percent	70.0%	30.0%	100.0%	

Table (12): Relation of neurological affection to homozygous Vs double heterozygous.

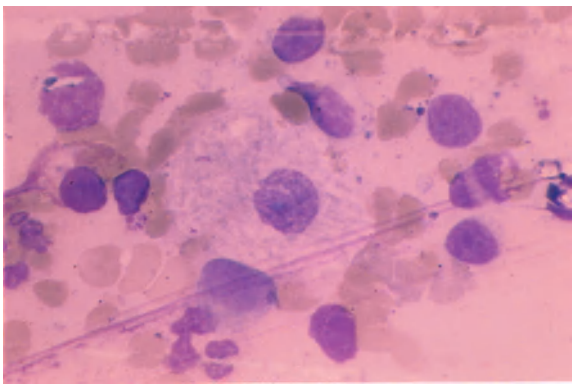
GENE		Free	Hyperreflexia, hypotonia, +ve stepping reflex	Occulomotor aprexia & squint	Squint	Total	*p* value
Homo	Count	6		1	2	9	
	percent	66.7%		11.1%	22.2%	100.0%	
Double hetero	Count	8	1	2		11	N.S
	percent	72.7%	9.1%	18.2%		100.0%	
Total	Count	14	1	3	2	20	
	percent	70.0%	5.0%	15.0%	10.0%	100.0%	



Figs. (1)



Figs. (2)



Figs. (3)

Figs. (1,2,3): Showing splenic aspirate infiltration by gaucher's cells

Fig (4): Strip showing L444P/L444P mutation.

Fig 5: Strip showing L444P/D409H mutation.

Fig 6: Strip showing N370S/N370S mutation.

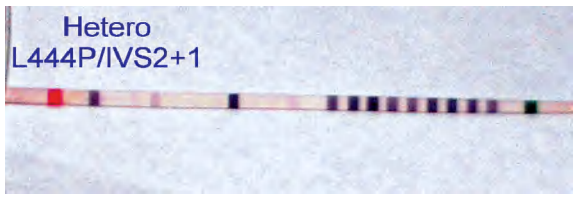


Fig 7: Strip showing L444P/IVS2+1 mutation.

DISCUSSION

In this work, 20 patients with documented Gaucher disease were investigated for their genotype. Correlation between genotype and phenotype was also evaluated.

The most common mutations encountered were L444p/L444p (homozygous) and L444p/IVS2+1 (double heterozygous), 8 patients each (40%) followed by L444p/D409H (double heterozygous) in 3 patients (15%) whereas N370S/N370S (homozygous) was found in only 1 patient (5%). Allele frequency showed that L444p was found in 67.5, IVS2+1 in 20%, D409H in 7.5%, and N370s in only 5%.

Previous molecular studies of the disease mutations revealed that although more than 50 mutations were identified in the Glucocerebrosidase gene, only four of them frequently occur. These four common mutations are (N370S, L444p, 84gg, and IVS2+1) representing 90% to 95% of the mutations associated with GD in the Ashkenazi Jewish population, and 50% to 75% of the mutations in the general population **[3]** (Beutler et al. 1991) and **[4]** (Sibille et al. 1993).

The N370S mutation is the most common in patients with GD accounting for about 75% of the mutant alleles in Ashkenazi Jewish persons and about 18% of non Ashkenazi Jewish **[5]** (Zimran et al. 1989).

The L444p mutation occurs more frequently in the non-Jewish population accounting for about 4% of the mutant alleles in Ashkenazi Jewish persons and about 32% of non Ashkenazi Jewish **[6]** (Tsujii et al. 1987).

The 84GG mutation accounts for 12% in Ashkenazi Jewish persons and 1% of non Ashkenazi Jewish. **[3]** (Beutler et al. 1991), while IVS2+1 mutation account for 3% in Ashkenazi Jewish persons and 1% of non Ashkenazi Jewish **[7]** (Sidransky et al. 1994).

(Tayebi, Stubblefield et al. 2003) **[8]** found that N370S/ N370S mutation accounts for about 29%, N370S/? mutation accounts for about 20%, N370S/L444p mutation accounts for about 16%, N370S/84GG mutation accounts for about 12%, L444p/L444p mutation accounts for about 6%, L444p/? mutation accounts for about 3%, and N370S IVS2+1 mutation accounts for 3%.

(El-Beshlawy et al. 2006) **[9]** found that 14 of 22 patients were homozygous or compound heterozygous for L444p and D409H mutations.

In our study equal sex distribution was found among patients with no significant relation with either homozygosity Vs double heterozygosity or the different genotype groups. This is in agreement with Theophilus et al. (1989), who reported that Gaucher's disease occurs with equal frequency in males and females.

The mean age of the patients was 10.15 years with a median of 6 years, the eldest was 45 years; his genotype was L444p/IVS2+1. The mean age of onset of the disease was 9 months. Both age of the patients and age of onset of the disease have no significant relation with either homozygosity Vs double heterozygosity or the different genotype groups.

As regards family history we found that 80% of cases had positive consanguinity with 45% of patientsí parents being first cousins. Relation between consanguinity and gene homozygous Vs double heterozygous was found to be highly significant p (<0.01) all 9 homozygous cases showed positive consanguinity, whereas 7 cases of 11 of the double heterozygous showed positive consanguinity. Relation between consanguinity and different genotype groups was found to be statistically significant (p<0.05). All patients with the genotypes L444p/L444p and N370S/N370S were from consanguineous parents ie: 8 cases of 8 and 1 case of 1 respectively. On the other hand all 3 patients with the genotype L444p/D409H had negative consanguinity, while the genotype L444p/IVS2+1 showed 7/8 with positive consanguinity and only 1 case with negative consanguinity.

Eighty percent of cases had affected sibs; in 15% sibs were clinically free, while 5% were a first baby. Whereas relation between number of affected sibs and gene homozygousity Vs double heterozygousity was found to be near significant (p=0.061), all sibs of homozygous patients were clinically affected. All patients of the genotype L444p/L444p had affected sibs, variable percentage of affected sibs were found in other genotypes, however, these findings were not statistically significant.

(El-Beshlawy et al. 2006) **[9]** found that Two-thirds of the patients were from consanguineous pedigrees. Another previous study revealed that 80% of cases were from consanguineous parents and 30% with affected siblings (El-Gawhary et al. 1999) **[10]**.

The high frequency of positive consanguinity and the mode of the disease inheritance drive the attention to the importance of genetic counseling and pre-natal diagnosis of the disease.

Genetic counseling prior to and following testing for Gluco-cerebrosidase mutations is essential. Obtaining an accurate family history prior to testing is necessary to identify the family members who are at risk of being affected, members who are "obligate carriers," and members who are at risk of being carriers. Information about ethnic background is relevant to the interpretation of test results. The a priori risk for individuals undergoing Gaucher's disease testing is dependent upon ethnic background and family history. For example, a negative mutation analysis (four common mutations) for an Ashkenazi Jewish individual with no family history of Gaucher's disease modifies his or her risk of being a carrier from

about 1 in 10 to about 1 in 200. Bayesian analysis should be used to calculate the modified risk of being a carrier for individuals with a family history of the disease **[4]** (Sibille et al. 1993).

Mutation analysis for the four common mutations (N370S, L444p, 84gg, and IVS2+1) detects 90% to 95% of the mutations associated with Gaucher's disease in the Ashkenazi Jewish population, and 50% to 75% of the associated mutations in the general population **[3]** (Beutler et al. 1991) and **[4]** (Sibille et al. 1993).

Mutation analysis is used in combination with Gluco-cerebrosidase enzyme assay results to diagnose Gaucher's disease and is also important for identification of carriers. Gluco-cerebrosidase enzyme assay results are frequently helpful when interpreting mutation analysis results. For example, an enzyme assay that reveals a deficiency of Gluco-cerebrosidase (<30% of normal Gluco-cerebrosidase activity) enables correct interpretation of negative mutation analyses **[4]** (Sibille et al. 1993).

Mutation analysis is the method of choice for identification of Gaucher's disease carriers. Carrier detection via enzymatic assay is unreliable because approximately 20% of obligate carriers demonstrate normal Gluco-cerebrosidase activity (false negatives) ie: An individual with normal enzyme activity but has 1 copy of N370S mutation (by mutation analysis) is an unaffected carrier. Mutation analysis for carrier detection is most informative for members of a high-risk population or for individuals with affected family members whose mutations are identified. When no mutations are detected by current methods, it is important to consider that negative results modify, but do not eliminate, the risk of being a carrier **[11]** (Beutler et al. 2004).

Three of eight of our patients with the genotype L444p/L444p had neurological affection, two of eight in the form of squint and in one of them squint was accompanied with occulomotor aprexia. In the genotype L444p/IVS2+1 three of eight patients had neurological affection one in the form of hyperreflexia, hypotonia and positive stepping reflex, while the two other patients in the form of occulomotor aprexia & squint, however this was also not statistically significant.

(Koprivica et al. 2000) **[12]** stated that patients with at least one N370S allele did not develop neurological symptoms. Patients who were homozygous for the L444p mutation tend to have severe disease, with neurological complications. This mutation results in an unstable enzyme with little or no residual activity. In a study of 31 individuals with neurological complications, L444p accounted for 25 alleles (40%) (Stone et al. 2000) **[1]**. The L444p mutation occurred alone (nine alleles), with E326K (one allele), and as part of a recombinant allele (15 alleles). In another study, homozygosity for the L444p mutation was the most common genotype among individuals with GD type 3 (10/24 individuals, or 42%) (Koprivica et al. 2000) **[12]**.

Only 5% of our cases had bone affection, 35% had chest affection, and in 5% had mild cardiac affection. No significant relation was found between these symptoms and gene homozygosity Vs double heterozygosity or different genotype groups.

Two of our patients showed bone affection, both of them had double heterozygous gene mutation, one suffered from bony pains and knock knees whose genotype was L444p/D409H; while, the other one had recurrent bony crises and his genotype was L444p/IVSII+1. X-ray evaluation of both patients revealed no recent or old pathological fracture.

(El-Beshlawy et al. 2006) **[9]** recently stated that there was no correlation between severity of bone involvement and GBA genotype.

Splenic aspiration was performed in 45% of our cases, all of them had Gaucher's cells infiltration. These findings had no statistically significant relation with either gene homozygousity Vs double heterozygousity or the different genotype groups.

(Nnaito et al. 1988) **[13]** reported that under electron microscope Gaucher's cells are filled with numerous elongated, rod shaped bodies (Gaucher bodies) that contain smooth walled tubular elements, either in bone marrow or splenic aspirate.

(Seif EI-Nasr 1993) **[14]** and (El-Gawhary et al. 1986) **[15]** observed that Gaucher cells were detected in the bone marrow of all cases with neuoropathic symptoms.

El-Gawhary et al. 1986) **[15]** reported that liver function tests were usually normal or slightly elevated, which is in agreement with our results as regards prothrombin time and serum GOT.

Conclusions:

Gaucher's disease occurs with equal frequency in males and females. The most common mutations found in our study were L444p/L444p and L444p/IVS2+1; while L444p was the most common allele. Both age of the patients and onset of the disease had no significant relation with either homozygosity Vs. double heterozygosity or the different genotype groups. Patients with homozygous gene mutations tended to have consanguineous parents.

As regards the clinical manifestations, we found that neurological involvement, growth retardation and chest symptoms were the most commonly reported in the studied cases; and less frequently bone & cardiac affection. None of the previous conditions were significantly associated with certain genotype.

The nature of Gaucher disease as a genetically inherited, debilitating disease highlights the need for preventive approaches such as genetic counseling, extended gene mutation studies for prenatal diagnosis especially in consanguineous couples with previous family history or with previously affected sibs.

Mutation analysis is the method of choice for identification of Gaucher's disease carriers; common mutation screening is the method of choice for carrier detection.

Further studies with larger number of patients will be beneficial to establish the relation between the different genotypes and the clinical manifestations of Gaucher disease, as well as the effectiveness of enzyme replacement therapy in changing its natural course.

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Journal of the Egyptian Society of Haematology & Research, Vol. 2, No. 2, September: 1 55-160, 2006

Assessment of Pancreatic Function in ß Thalassemia Major Patients

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ABSTRACT

Background: In patients with ß thalassemia major, iron overload results in functional and structural changes in various organs, especially the heart, liver and endocrine glands. The pancreas is a major site of iron deposition with severe degrees of fibrosis developing in more advanced cases.

Objective: The study attempted to assess the prevalence and characteristics of pancreatic damage in a group of thalassemic patients subjected to chronic transfusional therapy.

Patients and Methods: Ninety eight patients (52 males and 46 females), previously diagnosed to have homozygous ß-thalassemia and followed-up at the Hematology Clinic of the New Cairo University Children Hospital, were randomly selected to participate in this study. Their age ranged between 5 and 32 years. None of the patients had clinical evidence of chronic pancreatitis, chronic diarrhea, malabsorption, renal failure or diabetis mellitus. The patients were further divided into 3 subgroups according to their age. Study parameters included: Serum amylase and lipase, fasting blood glucose, serum ferritin, aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT), creatinine and blood urea nitrogen (BUN), cholesterol and triglyceride.

Results: 28 patients (28.6%) showed abnormally elevated serum lipase with a range between 34 U/L to 91 U/L while 6 patients (6.1%) showed elevation in both serum lipase and amylase (range between 35 U/L to 189 U/L and between 106 U/L to 185 U/L respectively). Only 1 patient showed elevated amylase (1%) with a value of 121 U/L. Serum amylase and lipase showed a highly statistical significant increase among the group of abnormal enzymes compared to those with normal value ($p<0.001$, $p<0.001$ respectively). Blood glucose did not show any significant difference among the two groups. ALT and AST showed a statistically significant difference between the two groups ($p<0.05$). No clinical significance was observed for the patients with abnormal enzymes. In group 1 (age<12 years), 25% (5 patients) showed elevated amylase and lipase. In group 2 (age between 13-18 years), 32.1% (9 patients) showed elevated lipase only while in group 3 (age>18 years), 40% (20 patients) showed elevated amylase and lipase. Serum amylase showed a statistically significant difference between the 3 subgroups ($p<0.05$) while serum lipase showed non significant difference. On correlating serum lipase to all other parameters, lipase showed a significantly high correlation to amylase and ALT and AST ($p<0.001$, $p<0.001$, $p=0.002$ respectively). Neither serum lipase nor amylase was significantly correlated to serum ferritin or age of the patients. Serum amylase was significantly correlated to AST and ALT ($p=0.006$, $p=0.007$ respectively).

Conclusion: Our results suggest that the exocrine pancreas in ß thalassemia major patients is functionally affected in a high percentage of patients with iron overload though no clinical manifestations were observed. The possible clinical implications of this damage remain to be clarified. Further studies including more tests evaluating both the endocrine and exocrine pancreas functionally and morphologically are recommended to detect changes as early as possible.

Key Words: *ß Thalassemia major – Pancreatic function.*

INTRODUCTION

Thalassemia is considered the most common genetic disorder world wide, about 3% of the world's population carry ß-thalassemia gene **[1]** and about 60.000 children with thalassemia major are born annually **[2]**.

These ß-thalassemia patients suffer from iron overload due to increased iron absorption and regular blood transfusions. This iron results in structural and functional changes in various organs, especially the heart, liver and endocrine system resulting in progressive organ failure **[3]**.

Postmortem studies in thalassemic patients have shown that the pancreas is among the organs most severely affected by iron accumulation and fibrosis however, the frequency and the clinical relevance of these findings are little known **[4]**.

While several studies have documented an involvement of the endocrine pancreas in this disease, little is known about alterations of the exocrine pancreas **[5]**.

The aim of this study was to assess the prevalence and characteristics of exocrine pancreatic changes in *ß*-thalassemia major patients subjected to chronic transfusional therapy.

MATERIALS AND METHODS

Patients:

Ninety eight patients, previously diagnosed to have homozygous *ß*-thalassemia and followed-up at the Hematology Clinic of the New Cairo University Children Hospital, were randomly selected to participate in this study.

All the patients were on standard therapy consisting of chronic blood transfusions with desferroxamine (20-40 mg/kg/day) as iron chelator. Among the ninety eight patients recruited for this study, 52 were males and 46 females whose age ranged between 5 and 32 years with a mean age of 18.3±6.4 years and a mean hemoglobin of 7.725±1.3 g/dl.

None of the patients had clinical evidence of chronic pancreatitis, chronic diarrhea, malabsorption, renal failure or diabetis mellitus.

The medical charts of the recruited patients were reviewed. Frequency of blood transfusion and chelation therapy was recorded. A complete physical exam was performed for all patients.

The patients were further divided into 3 subgroups according to their age:

Group (1): Included patients aged 5 to 12 years, mean age of 9±2.06 years accounting for 20.4% of the total number of patients (20 patients).

Group (2): Included patients' age ranging from 13-18 years old and mean age of 15.3±1.7 years accounting for 28.6% (28 patients).

Group (3): Included patients older than 18 years with a mean age of 23.6±3.2 years and accounting for the remaining 51% (50 patients).

Study parameters included:

- Serum amylase and lipase.
- Fasting blood glucose.
- Serum ferritin.
- Aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT).
- Creatinine and BUN.
- Cholesterol and triglyceride.

Collection of blood samples and assays:

Venous blood was obtained without stasis. Blood allowed to clot & serum separated and stored at -20°C till the assay time.

All the analytes were measured on Beckman Synchron CX9 PRO autoanalyzer with Beckman Kits. Serum pancreatic lipase activity was measured based on a timed enzymatic rate method in which a diglyceride is the substrate. The diglyceride substrate is hydrolyzed by pancreatic lipase in the sample to 2-monoglyceride and fatty acid. A sequence of four coupled enzymatic steps causes the oxidative coupling of N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine (TOOS) with 4-aminoantipyrine (4-AAP) to form a red quinine diimine dye. The reference value is 3-32 U/L **[6]**.

Serum pancreatic amylase activity was measured based on an immuno-inhibition method using two monoclonal antibodies with two incubation steps. In the first incubation step, the activity of human salivary α-amylase is inhibited by two monoclonal antibodies which do not affect pancreatic α-amylase. After a second incubation with the substrate, the α-amylase cleaves the substrate (4,6-Ethylidene-G7p-Nitrophenol) into fragments and these fragments are further hydrolyzed by α-glucosidase to yield p-nitrophenol and glucose with reference interval 28-100 U/L **[7]**.

Serum AST, ALT, Alkaline phosphatase and BUN activities were measured based on a kinetic rate method, Creatinine was measured based on a modified-rate Jaffé method, Cholesterol and Triglyceride were measured based on a timed-endpoint method.

Data management and statistical methods:

The data was coded and entered using the statistical package SPSS version 13. The data were summarized using mean and standard deviation for quantitative data and percent for qualitative data. The difference between studied groups were assessed using the Chi-square and Fisher's Exact tests for qualitative data and

independent samples *t*-test and ANOVA (analysis of variances) for quantitative data. The *p*-value was considered significant at a level <0.05.

RESULTS

Twenty eight patients (28.6%) showed abnormally elevated serum lipase with a range between 34 U/L to 91 U/L while 6 patients (6.1%) showed elevation in both serum lipase and amylase (range between 35 U/L to 189 U/L and between 106 U/L to 185 U/L respectively). Only 1 patient showed elevated amylase (1%) with a value of 121 U/L (Fig. 1).

Serum amylase and lipase showed a highly statistical significant increase among the group of abnormal enzymes compared to those with normal value ($p<0.001$, $p<0.001$ respectively). Blood glucose did not show any significant difference among the two groups. ALT and AST showed a statistically significant difference between the two groups ($p<0.05$) (Table 1).

No clinical significance was observed for the patients with abnormal enzymes.

In group 1, 25% (5 patients) showed elevated amylase and lipase.

In group 2, 32.1% (9 patients) showed elevated lipase only while in group 3, 40% (20 patients) showed elevated amylase and lipase.

Serum amylase showed a statistically significant difference between the 3 subgroups ($p<0.05$) while serum lipase showed non significant difference (Table 2).

On correlating serum lipase to all other parameters, lipase showed a significantly high correlation to amylase and ALT and AST ($p<0.001$, $p<0.001$, $p=0.002$ respectively) (Table 3).

Neither serum lipase nor amylase was significantly correlated to serum ferritin or age of the patients. Serum amylase was significantly correlated to AST and ALT ($p=0.006$, $p=0.007$ respectively).

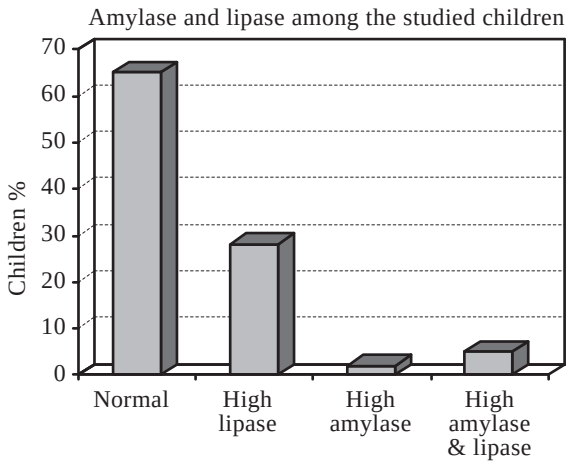

Fig. (1): Shows serum amylase and lipase among the thalassemic patients.

Table (1): Shows comparison between the mean and standard deviation of the measured parameters in patients with normal and high lipase and amylase activities.

	Patients with high lipase and amylase activity No.=34 (34.7%) 21 males & 13 females		Patients with normal lipase activity No.=64 (65.3%) 31 males & 33 females		
	Mean	SD	Mean	SD	*p* value
Age (years)	18.9	5.7	17.9	6.8	0.418
Lipase (U/L)	48.9	27.7	21.5	5.5	<0.001*
Amylase (U/L)	79.4	28.3	56.6	22.3	<0.001*
ALT (U/L)	89.7	58.1	68.1	41.4	0.036*
AST (U/L)	99.7	59.2	73.3	42.5	0.013*
BUN (mmol/L)	4.2	0.99	3.9	1.03	0.120
CRE (umol/L)	35.5	8.4	33.9	9.7	0.432
Glucose (mmol/L)	5.4	1.5	5.8	4.2	0.636
Cholesterol (mmol/L)	2.8	0.62	2.9	0.73	0.703
Triglycerides (mmol/L)	2.1	0.9	1.8	1.1	0.219
Ferritin (ng/mL)	4383	2013	3960	3290	0.495

ALT : Alanine aminotransaminase. BUN : Blood urea nitrogen. * Significant: <0.05.
AST : Aspartate aminotransaminase. CRE : Creatine.

Table (2): Shows comparison between the mean and standard deviation of the lipase and amylase in the three groups of the patients divided according to their age.

	Group 1 5-12 years No.=20 (20.4%)		Group 2 13-18 years No.=28 (28.6%)		Group 3 19-32 years No.=50 (51%)		*p* value
	Mean	SD	Mean	SD	Mean	SD	
Age	9.05	2.06	15.4	1.7	23.6	3.2	<0.001*
Lipase (U/L)	36.6	40.6	29.1	12.2	29.9	12.7	0.453
Amylase (U/L)	77.4	32.7	58.4	19.1	62.08	26.8	0.039*

Significant: <0.05.

Table (3): Shows the correlation between lipase and other parameters.

	Lipase		
	"*r*" value	*p* value	Siginificance
Age (years)	–0.073	0.476	Non
Amylase (U/L)	0.670	<0.001	High
ALT (U/L)	0.349	<0.001	High
AST (U/L)	0.304	0.002	Sig.
BUN (mmol/L)	0.026	0.798	Non
CRE (umol/L)	–0.063	0.536	Non
Glucose (mmol/L)	0.044	0.664	Non
Cholesterol (mmol/L)	0.048	0.641	Non
Triglycerides (mmol/L)	0.173	0.088	Non
Ferritin (ng/mL)	0.037	0.720	Non

DISCUSSION

The use of regular frequent blood transfusions in thalassemia major has improved the span and quality of life in these patients, but it leads to iron overload which frequently causes endocrine problems. The pancreas is a major site of iron deposition with severe degrees of fibrosis developing in more advanced cases **[8]**.

Impaired structure and function of the exocrine pancreas has been reported in patients with ß-thalassemia major. The mechanism underlying exocrine pancreatic damage in these patients is probably the infiltration of the acinar tissue with iron which causes damage to the endocrine components of the pancreas, the islets of langerhans and ß-cells in particular **[9]**.

Though measurements of both lipase and amylase are commonly used as aids in the diagnosis of pancreatic diseases, 19% of pancreatitic patients present with normal serum amylase. Thus, leaving lipase as the main hematochemical marker of pancreatic insult. **[10]**.

It was previously reported that lipase activity may be increased in the absence of clinically overt pancreatitis in thalassemic patients. This abnormality was thought to reflect a break down of the acinar-blood barrier, and their proenzymes into the blood **[11]**.

Twenty eight patients showed elevated serum lipase, six patients showed elevation in both enzymes while only one patient showed elevated serum amylase. On comparing the group of abnormal enzymes to the second group, highly significant results were observed ($p<0.001$, $p<0.001$ respectively). No subnormal results were detected among our patients.

Our results disagree with previous studies reporting a decrease in lipase enzyme in 60% and 33% respectively **[9,11]**. It was reported that on studying pancreatic enzymes (trypsin, lipase, amylase and elastase) among a group of thalassemic patients, 40% of the patients had abnormally low concentrations of one on more enzymes most commonly of trypsin and lipase being the most sensitive indicators of pancreatic insufficiency **[12]**.

During the earlier phase of pancreatic damage, pancreatic enzymes probably leak directly into the circulation causing the enzyme to increase in serum. This phase is then followed by a progressive destruction of the acinar tissue and a decline in pancreatic enzyme concentrations. These data thus confirm that the exocrine

pancreatic reserve in these patients is probably subnormal. It may be argued that the concentration of the pancreatic enzymes in serum does not reflect the extent of enzyme secretion into duodenum **[13]**.

A previous study on a group of 30 thalassemics, treated with continuous subcutaneous desferroxamine infusion for a mean period of 30 months, showed normal levels of amylase and lipase suggesting the role of iron overload on the pancreatic damage **[14]**.

The group of high lipase showed significantly higher liver enzymes (ALT and AST) than the other group (p=0.036, 0.013 respectively). This agrees with a previous study reporting that the severity of pancreatic damage is related to the progress of hepatic fibrosis and iron overload **[12]**. In a study estimating lipase activity in primary cystic fibrosis, it did not differ in any stage of the disease suggestive that pancreatic damage is not dependent on and does not parallel histological changes in the liver **[15]**. The change in cholesterol and triglycerides levels was not statistically different among our 2 groups. It was previously reported that in thalassemics with low lipase, total cholesterol decreased, thus this enzymatic activity had a major role in determining the level of lipids **[16]**.

No correlation was found between serum lipase and serum ferritin in our patients which agrees with a previous study reporting no correlation between pancreatic enzymes values and mean serum ferritin values or mean blood consumption over 3 years **[17]**. Individual tissue susceptability to iron may determine the eventual damage to pancreas as well as the degree of iron overload **[9]**.

On dividing the patients into 3 subgroups serum amylase showed statistically significant difference according to age, while no significant difference was detected between serum lipase levels. Our results disagree with a previous study denoting that the severity of pancreatic changes increased in old patient with a longer history of transfusion **[12]**.

Serum immunoreactive trypsin was supranormal in thalassemics beyond 12 years of age and subnormal in older ones **[9]**. During earlier phase of pancreatic damage, pancreatic enzymes increase to be followed by a progressive destruction of the acinar tissue and decline in the pancreatic secretion **[11]**.

Transfusion dependent thalassemic patients having major impairment of the exocrine pancreatic functions show increased echogenicity by ultrasonography **[12,18]** and higher signal intensity of the pancreas because of fatty replacement of the parenchyma by magnetic resonance imaging **[19]**.

From a clinical point of view, the possible development of a severe exocrine pancreatic insufficiency in these patients raises the question of whether this complication impairs the digestion of food and causes some degree of malnutrition especially in patients with more advanced disease **[12]**.

Though our study was not a case control study, our results suggest that the exocrine pancreas in ß thalassemia major patients is functionally affected in a high percentage of patients with iron overload inspite of absence of clinical manifestations of pancreatic insult. The possible clinical implications of this damage remain to be clarified. Further studies including more tests evaluating both the endocrine and exocrine pancreas functionally and morphologically are recommended to detect changes as early as possible.

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Journal of the Egyptian Society of Haematology & Research, Vol. 2, No. 2, September: 161-168, 2006

Study of Sepsis Related Changes in Platelet Adhesion, Aggregation and Angiogenic Growth Factor Release

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ABSTRACT

Background: Reassessment of the platelets' role in the various pathogenic mechanisms that have been recently implicated in sepsis-related coagulopathy has not yet been made. The aim of this study was to elucidate the effect of sepsis on platelet adhesiveness, aggregation, and growth factor release.

Setting: Radioisotopes department, Nuclear Research Center, in Collaboration with Clinical Pathology Department, Faculty of Medicine, Cairo University.

Patients and Methods: Platelets' count and function were investigated in 30 patients with sepsis. Agonist induced platelet aggregation was measured using three agonists (ADP, collagen, arachidonic acid [A.A]). Release of vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF), were measured using immunoradiometric (IRMA) assay. Expression of platelet adhesion molecules was measured using flowcytometry (fibrinogen binding, glycoprotein V and p-selectin expression).

Results: All patients with sepsis exhibited thrombocytopenia that was related to the severity of illness and associated with bad outcome. Agonist-induced platelet aggregation was consistently reduced in patients, specially with AA.

Platelet aggregation decreased regardless of the platelet count or thrombin generation, rather, correlated to the severity of sepsis. Flowcytometric analysis, revealed significantly reduced fibrinogen binding, maintained CD42a, and significantly elevated CD62p. VEGF and PDGF release were significantly increased as compared to normal controls.

Conclusion: These results suggest that sepsis induces a complex regulation of platelets' function, with redistribution of platelets' role from heamostasis towards other functions including inflammatory response and vascular healing. These changes occur even when the platelet count is normal, independent of thrombin generation.

Key Words: *Platelet adhesion – Aggregation – PDGF – VEGF – DIC.*

INTRODUCTION

Platelet function can be seen as a succession of overlapping events involving adhesion, aggregation, secretion, and promotion of procoagulant activity. All patients with sepsis have an activated coagulation system, which may range from minor changes in highly sensitive molecular markers for hypercoagulability to full blown disseminated intravascular coagulation (DIC) with intravascular fibrin deposition and consumption of platelets and coagulation factors **[1,2]**. Ongoing systemic activation of coagulation does not only lead to microvascular failure with obstruction of the blood supply to various organs; thereby, contributing to organ failure, but also plays a central role in the inflammatory response to severe infection and sepsis **[3]**. The various mechanisms that are important for the derangement of coagulation in sepsis have been elucidated in the last 10-15 years. The focus has mainly been on plasma coagulation proteins, activated inflammatory cells expressing cytokines and tissue factor, and perturbed endothelial cells, which play central role in the impaired anticoagulant function, fibrinolysis, and potentially as a source of tissue factor expression **[4]**. Sepsis is a clear risk factor for thrombocytopenia in critically ill patients and the severity of sepsis, correlates with the decrease in platelet count **[5,6]**. Studies on platelet function in sepsis have yielded conflicting results. Many investigators reported endotoxin-induced platelet accumulation and enhanced platelet aggregation in animal models **[7-9]**. In

vitro, bacterial compounds such as lipopolysaccharide and staphylococcus aureus lipoteichoic acid can bind to platelets and endothelial cell membranes in patients with sepsis and inhibit platelet aggregation **[10]**. On the other hand, sepsis-generated cytokines did not seem to activate human platelets either directly or via thrombin **[11]**. Several clinical studies have reported decreased platelet aggregability during sepsis **[12,13]**, whereas, Gawaz et al. **[14]** reported increased platelet aggregability.

Platelet α-granules contain vascular growth factors, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and platelet factor 4 (PF-4), which are released during aggregation. VEGF is a potent angiogenic factor regulating proliferation, differentiation, and survival of microvascular endothelium **[15]**. PDGF contributes to endothelial repair and angiogenesis **[16,17]** but little is known about their regulation during the septic response.

In the present study, we studied platelet aggregation, adhesion, and growth factor release in patients with sepsis in order better to define sepsis-related alterations in platelet functions and delineate platelets' position into the new insights in the pathogenesis of sepsis-related coagulopathy.

SUBJECTS AND METHODS

Subjects and study design:

Platelet aggregation, adhesion and growth-factor release were investigated in 30 patients with sepsis of recent onset (14 patients uncomplicated sepsis admitted to the medical, surgical, gynecological units of Cairo University hospitals, and 16 critically ill patients admitted to intensive care unit (ICU) of Cairo University hospital. Fifteen healthy volunteers matched for age and sex were included as normal controls. Healthy volunteers had no evidence of infection and had not received antibiotics or non steroidal anti-inflammatory drugs (NSAID) within the 10 days prior to the study.

Diagnosis of sepsis was established by having at least one positive blood culture and/or an identified focus of infection. Severity of the disease was assessed according to the Acute Physiology and Chronic Health Evaluation (APACHE) II score **[18]**, and the Sequential Organ Failure Assessment (SOFA) score **[19]**. Presence of at least one organ failure related to sepsis was considered as severe sepsis, presence of sustained sepsis-related hypotension necessitating the administration of vasopressor agents was defined as septic chock.

The criteria for DIC were those defined by the subcommittee on disseminated intravascular coagulation of the international society of thrombosis and haemostasis (based on platelet count, soluble fibrin monomers, prothrombin concentration, and fibrinogen concentration) score above 5 was considered as DIC **[20]**.

Exclusion criteria were; blood creatinine >2.5mg/dl at entry, administration of heparin or drugs affecting prostanoid synthesis. However, antibiotics could not be avoided, although, some of them may affect platelet functions.

Methods:

5ml of venous blood were collected in 3.8% sodium citrate tubes with the ratio of 9:1. Blood samples were withdrawn on the day of admission to the ICU from patients with severe sepsis, and at the onset of sepsis in patients who developed sepsis during hospitalization.

Whole blood (500ml) was used for flow-cytometry; platelet rich plasma (PRP) was immediately prepared from the remaining blood sample by centrifugation at 90xg for 10 min. Platelet poor plasma (PPP) was prepared from PRP by a second centrifugation at 2000xg for 15 min. PRP was used for assessment of platelet aggregation and growth factor release. PPP was frozen at -70ºC for use in the measurement of thrombin generation and growth factor release. PRP from healthy volunteers was sequentially diluted in their PPP to match the low platelet counts in PRP of the thrombocytopenic patients with sepsis.

* Thrombin generation assessment was done by enzyme-linked immunoassay (ELISA) method using 4 commercial kits to measure, thrombin-antithrombin (TAT) complex (Enzygnost TAT), prothrombin fragment 1+2 (Enzygnost F1+2) and soluble fibrin monomers (Enzygnost SF), all produced by Boehring®, Schwalbach, Germany. D-dimer was measured using Mini Vidas DDI, Biomereux®, Letoile, France Kit.

* Platelet aggregation was assessed according to the manufacturer's instructions using lumiaggregometer (Chrono-log Corp, Havertown, PA, USA). The agonists used were: 500 µg/ml arachidonic acid (Biol Data Corporation), 5µm ADP (Roche, Mannheim, Germany) and 2.5µg/ml collagen (Chrono-log corp. Havertown, PA, USA). Agonist induced aggregation was recorded graphically for 10 min. Results were given as a ratio of percentage aggregation of the patients' in relation to the healthy controls' with the closest platelets count after dilution.

**VEGF and PDGF measurement:*

To evaluate in vitro growth factor release, the supernatant of the aggregation induced by collagen was collected after centrifugation at 2000xg to remove aggregates and free remaining platelets and was kept at -70ºC for determination of VEGF and PDGF content by Immunradiometric assay (IRMA) technique using the kits (Human VEGF, IRMA, and Human PDGF-AB IRMA) produced by LINCO Research St-Charles Missouri 63304, USA. The sensitivity of the test for VEGF was 9pg/ml and for PDGF was 8.4pg/ml. The amount of growth factor released in vitro per million platelets was calculated as described by Yagouchi et al. **[13]** from the formula:

$$\text{G.F. released per } 10^6 \text{ platelets in pg} = \frac{\text{G.F in PRP} - \text{G.F in PPP}}{\text{No. of platelets in PRP sample}} \times 10^6$$

**Measurement of platelet adhesion by flow cytometry:*

- Avidity of GP IIb/IIIa for fibrinogen was studied with the use of directly fluorescein isothiocyanate (FITC) stained fibrinogen (Dako, Glostrup, Denmark).
- The adhesion molecule glycoprotein V involved in von-Willebrand factor rolling (CD42a), was studied using FITC-stained anti CD42a produced by Immunotech, Marseille, France.
- α-granule secretion was studied by detection of P-selectin expression with the use of FITC-stained monoclonal anti CD62p, produced by Becton Dickinson.

Platelets were stained with FITC-conjugated mouse monoclonal antibodies against CD42a, CD62p, fibrinogen and with the appropriate isotype matched control monoclonal antibody. After 15 min incubation at room temp, the platelets were fixed in 1ml 1% paraformaldhyde in edta phosphate-buffered saline. Data were acquired using FACScan flowcytometer (Coulter Epics, Elite). Platelets were defined as events fitting the platelet size and complexity in log scale forward and side scatter and expressing fluorescence in the FL3 channel. The median fluorescence intensity (MFI) was used to depict molecule expression on the platelet surface.

Statistical analysis:

All values were expressed as mean±SD (normal distribution) or median±SD (Scewed data). Data were analyzed by unpaired student t-test (normal distribution) or Mann-Whitney U-test. Pearson's correlation coefficient was used for correlations.

$p < 0.05$ was considered to be statistically significant.

RESULTS

Clinical diagnosis of the patients included in the study, isolated micro-organisms from blood culture, and antibiotic therapy used are shown in Table (1). Demographic data and clinical classification of the studied patients according to severity of illness and coagulation status are shown in Table (2). Of the 30 patients 14 had uncomplicated sepsis (47%) and 16 had severe sepsis or septic shock (53%). The healthy control group included 10 males and 5 females with mean age of 39±9 years, and median platelet count of $240x10^3 \pm 90x10^3/mm^3$ (range $180\text{-}380x10^3/mm^3$). Thrombocytopenia was evident in patients and correlated positively with severity of illness, 53% of patients had platelet count $<150x10^3/mm^3$, and 56.6% showed count $<100x10^3/mm^3$, as shown in Table (2).

Platelet aggregation assessment revealed significantly reduced aggregation ratio in all the patients compared to diluted matched-count PRP of the controls, regardless of the agonist used. Patients were divided into 3 groups according to their platelet counts, those with platelet count $>180x10^3/mm^3$ (8 sepsis and 2 complicated sepsis), those with platelet count between $180x10^3$ and $100x10^3/mm^3$ (3 sepsis and 9 complicated sepsis), and those with low platelet count $<100x10^3/mm^3$ (3 sepsis and 5

complicated sepsis). Results of platelet aggregation with different agonists in relation to severity of illness are presented in Table (3). Platelet aggregation was more severely reduced in patients with severe sepsis than in those with uncomplicated sepsis, the difference being particularly significant in patients with thrombocytopenia. The most sensitive pathway to sepsis-induced alteration in platelet aggregation was the cyclo-oxygenase pathway with arachidonic acid (AA) inducing 48% maximal aggregation in patients with normal platelet count and 12% in thrombocytopenic patients (Table 3). Platelet aggregation was similarly altered in patients with and without DIC, unless there was thrombocytopenia. (Table 4).

Thrombin generation assessment revealed that, there was neither correlation between soluble fibrin monomer, D-dimers, TAT and F1+2 and DIC status, nor, the aggregation ratio related to ADP, AA, and collagen in all patients. (Table 5).

Flowcytometric analysis revealed that the activated conformation of GP IIb/IIIa assessed by FITC-stained fibrinogen binding was significantly decreased in patients after platelet activation by collagen, whereas, the ex-vivo expression of the adhesion molecule CD42a involved in von-willebrand rolling revealed no significant change in patients as compared to controls. Results are shown in Table (6). Alpha-granule secretion as detected by the expression of P-selectin (CD62p) with collagen activation was significantly increased in patients compared to controls ($p<0.05$). The difference being markedly significant in patients with uncomplicated sepsis. (Table 6). VEGF release by platelets after collagen activation was significantly increased in all patients. (Table 7). PDGF release was also significantly increased in all patients. The increase in both VEGF and PDGF release was more significant in uncomplicated sepsis than in severe sepsis, and in patients without DIC than in those with DIC. Results are represented in Table (7). There was no correlation between thrombin generation and VEGF or PDGF release regardless of the patient's platelet count.

Table (1): Clinical diagnosis, isolated organisms, and therapy in patients.

	No of patients
Clinical diagnosis:	
• Purperal sepsis and septic abortions	5
• Appendicular abscess and abdominal sepsis	4
• Post-splenectomy pneumonia or overwhelming sepsis	5
• Diabetic foot	6
• Pyelitis and pyelonophritis	3
• Foreign body	3
• Major trauma and severe lacerations	4
Isolated organisms:	
• Escherichia coli	12
• Staphylococcus aureus	9
• Klebsiella	6
• Pseudomonas aeruginosa	3
Antibiotics, used:	
• Aminopenicillin	12
• Cephalosporins	27
• Aminoglycosides	16
• Vancomycin	10
Vasoactive agents in septic shock:	
• Dopamine	10
• Dopamine and norepinephrine	4
• Dobutamine	2

Table (2): Demographic data and clinical classification of the patients (n=30) according to severity of illness and the occurrence of DIC (mean ±SD).

	Severity of illness		Coagulation status	
	Sepsis	Sever sepsis/ septic shock	No DIC	DIC
Number of patients	14 (47%)	16 (53%)	13 (43.4%)	17 (56.6%)
Age (years)	40±10	50±9	40±13	56±4
Male: female	9:5	10:6	9:4	14:3
APACHE II score	9±4	15±4*	13±6	15±7
SOFA score	2±1	7±4*	4±3	8±4**
Platelet count $x10^3/mm^3$	210±90	140±100* (53.4%)	200±90	80±30** (56.6%)
Number of non survivals	0	14 (87.5%)	5 (39.3%)	17 (100%)

* $p < 0.05$ sepsis Vs. severe sepsis / septic shock, ** $p < 0.05$ no DIC Vs. DIC.

Table (3): Maximal aggregation (%) induced by different agonist in patients in relation to severity of illness (mean +SD).

Platelet count x10^3/mm^3		No of patients	5 µm ADP	2.5 µg/ml collagen	500 µg/ml AA
>180x10^3/mm^3	Sepsis	8	80±1	70±5	63±6
	Severe sepsis	2	71±6*	68±3*	58±3*
<180x10^3/mm^3	Sepsis	3	81±2	70±4	51±8
>100x10^3/mm^3	Severe sepsis	9	70±2*	65±1*	48±13*
<100x10^3/mm^3	Sepsis	3	69±3	65±1	43±2
	Severe sepsis	5	40±4**	43±19**	12±2**

* $p < 0.05$ sepsis Vs. severe sepsis, ** $p < 0.01$ sepsis Vs. severe sepsis.

Table (4): Maximal aggregation (%) induced by different agonist in patients with and without DIC (mean±SD).

Platelet count x 10^3/mm^3		No of patients	5 µm ADP	2.5 µg/ml collagen	500 µg/ml AA
>180 x 10^3/mm^3	No DIC	8	80±1	70±5	63±6
	DIC	0	–	–	–
<180 x 10^3/mm^3	No DIC	5	80±6	69±3	51±8
>100 x 10^3/mm^3	DIC	7	79±2	70±1	48±13
< 100 x10^3/mm^3	No DIC	0	–	–	–
	DIC	10	45±41*	43±16*	12±2*

$p < 0.05$ thrombocytopenia Vs. normal platelet count, otherwise, $p > 0.05$ for DIC Vs.

Table (5): Thrombin generation in the studied patients (median±SD).

	Coagulation status	N	Soluble fibrin monomer mg/L	D-dimers ng/ml	Thrombin-Anti -thrombin µg/L	Prothrombin fragment 1+2
Normal values			0-17	< 500	1.0–4.1	0–1.1
All patients		30	28.1±10.3	1890±1150	15.0±21.2	1.9±0.93
>180x10^3/mm^3	No DIC	8	26.9±6.5	1531±960	10.8±10.5	1.9±0.93
	DIC	0	–	–	–	–
<180x10^3/mm^3	No DIC	5	27.3±14.4	2010±1200	14.2±14.6	1.9±1.0
>100x10^3/mm^3	DIC	7	28±6.3	2080±1233	14.9±11.9	2±0.86
<100x10^3/mm^3	No DIC	0	–	–	–	–
	DIC	10	30.4±10.1	2210±1260	20±30.4	2.4±0.5

N: number of patients. Markers of thrombin generation did not correlate to DIC status ($p > 0.05$).

Table (6): Flowcytometric analysis for platelet adhesion molecules and P-selectin expression. (MFI±SD) (median fluorescence intensity on FL3-channel linear scale).

Collagen induced activation	Control (n=7)	Patients (n=30)	
		Sepsis (n=14)	Severe sepsis/septic shock (n=16)
Fibrinogen binding	740±25	415±150*	300±96**
CD42a	481±70	400±85	403±80
CD62p	420±130	608±120**	536±100*

* $p < 0.05$ Vs. control, ** $p < 0.01$ Vs. control.

Table (7): PDGF release and VEGF release induced by collagen in patients and controls in relation to severity of illness and DIC status (median±SD).

Collagen induced release	Control n=15	Patients (n=30)			
		Severity of illness		Coagulation status	
		Sepsis (n=14)	Severe sepsis/ shock (n=16)	No DIC (n=13)	DIC (n=17)
PDGF pg/10^6 platelets	12±6	28.9±10.4**	21.4±16*	28.1±13*	20.3±11*
VEGF pg/10^6 platelets	15±11	36±15.1**	28.6±13.9*	30.1±12**	28.5±10.6*

* $p < 0.05$ Vs. control, ** $p < 0.01$ Vs. control.

DISCUSSION

In the present study, all patients with sepsis had an activated coagulation system. We observed that sepsis was a clear risk factor for thrombocytopenia in critically ill patients (53.4% <150x10^3/mm^3 and 56.6% <100x10^3 / mm^3), and the severity of sepsis correlated with the decrease in platelet count. It was previously reported that, the incidence of thrombocytopenia (platelets <150x10^3/mm^3) in critically ill patients was 34-44%, whereas, platelet count of less than 100x10^3 was seen in 20-25% of patients, and 12-15% of patients had platelet count <50x10^3/mm^3 **[21]**. Mavromattis et al. **[22]** and Akca et al. **[23]** reported that, the platelet count in patients with sepsis decreased during the first 4 days in the intensive care unit, this decrease was correlated to the severity of sepsis. The mechanism by which thrombocytopenia in sepsis occurs, however, is not completely clear. Impaired production of platelets from the bone marrow may seem contradictory to the high levels of platelet production-Stimulating pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin (IL-6), and high concentration of circulating thrombopoietin in patients with sepsis. These cytokines and growth factors should theoretically stimulate megakaryopoiesis in the bone marrow **[24]**. However, in a substantial number of patients with sepsis marked hemophagocytosis by monocytes and macrophages may occur, hypothetically, due to stimulation with high levels of macrophage colony-stimulating factor (M-CSF) in sepsis **[25]**. Platelet consumption may also play an important role in patients with sepsis, due to ongoing thrombin generation (which is the most potent activator of platelets in vivo) **[26]**.

In the present study, we observed that platelet functions can be regulated separately in sepsis, with reduced aggregation, maintenance of adhesion molecule expression, increased α-granule secretion (↑ P-selectin expression) and modulation of growth factors with increased VEGF and PDGF release. The decrease in platelet aggregation was related to the severity of sepsis. Flowcytometry confirmed this aggregation defect, demonstrating concomitant reduction in fibrinogen binding on the platelets of septic patients. These changes were observed even in patients with normal platelet count or low DIC score, and were completely independent of thrombin generation. Similar results were reported by Yaguchi et al. **[13]**. At first sight, this result can be interpreted in two ways. It may be that there is decreased platelet aggregation in sepsis, but, a more likely explanation is that an increased platelet aggregation activity in patients with sepsis will lead to circulating platelets that are already activated and will not aggregate in an ex-vivo setting. The assembly of several coagulation factor complexes, such as the tenase or prothrombinase complexes, will be markedly facilitated if a suitable phospholipids surface is available, and occurs in vivo on the membrane of activated platelets **[27]**. In the setting of inflammation induced activation of coagulation, platelets can be activated directly by endotoxin **[28]** or by pro-inflammatory mediators, such as platelet activating factor **[29]**. Once thrombin is formed, this will activate additional platelets. In the present study, regardless of the severity of sepsis, platelets preserved normal expression of the adhesion molecule CD42a (involved in von-Willebrand binding), suggesting that the ability of platelets to adhere is probably preserved, even, when aggregation induced by this agonist was profoundly affected.

This finding evokes a defect at the intracellular transduction pathway rather than down regulation of surface receptors. The most affected pathway was found to be the cyclo-oxygenase pathway with arachidonic acid. In accordance with these findings, Lundahl et al. observed that platelets had decreased fibrinogen binding capability in response to AA, which was correlated with a poor outcome **[30]**. In contrast to aggregation, α-granule secretion function was enhanced with increased p-selectin (CD62P) expression on platelet surface. Similar results have been reported by Salat et al., who observed higher expression of CD62P in patients with sepsis, but with a low level of statistical significance **[31]**. However, Gawaz et al. **[14]** and Yaguchi et al. **[13]** reported unmodified expression of P-selectin on the platelet surface. Previous studies have shown that expression of P-selectin on the platelet membrane not only mediates the adherence of platelets to leukocytes and endothelial cells, but, also enhances the expression of tissue factor on monocytes **[29]**. The molecular mechanism of this effect relies on nuclear factor kappa-B (NFK-B) activation, induced by binding of activated platelets to neutrophils and mononuclear cells **[29]**. P-selectin can be relatively easily shed from the surface of the platelet membrane and soluble P-selectin levels have indeed been shown to be increased during systemic inflammation **[32]**. We also, observed, enhanced release of VEGF and PDGF in patients with sepsis compared to the control group. This increase occurred mainly in patients with uncomplicated sepsis, no DIC, low thrombin generation and normal platelet count. Since platelets have no nucleus, this observation suggests that changes in α-granule content may occur at the megakaryocyte level, probably, as a result of the inflammatory response **[33]**. Yaguchi et al., in their study reported differential release of growth factors from platelets in patients with sepsis, with increased VEGF and unchanged PDGF release **[13]**. Results of the present study, suggest that, platelet release of inflammatory mediators and growth factors may be another link between activation of coagulation and inflammation, moreover, it adds to previously accumulated knowledge on the role of platelets in sepsis, suggesting that, sepsis induces a redistribution of platelet function from haemostasis towards other functions, including vascular healing.

Conclusion:

Platelets seem to occupy one of the essential crossroads in the complex interaction between inflammation and coagulation, not only by facilitating and propagating thrombin generation (which in itself will further activate platelets) but also, by being an important mediator of growth factor and adhesion molecule activity. More research on the role of platelet activation in sepsis will undoubtedly be helpful in further unraveling the pathogenesis of sepsis and in understanding the tight crosstalk between inflammation and coagulation.

Acknowledgment:

The authors greatly appreciate Prof. Dr. Hesham Soliman, professor of vascular surgery, Cairo University; Prof. Dr. Mohammad Zaater, Professor of nephrology, Cairo University; and Prof. Mohammed Abdel Kader, professor of gynecology and obstetrics, Cairo University, for their great help to accomplish this work.

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Journal of the Egyptian Society of Haematology & Research, Vol. 2, No. 2, September: 169-178, 2006

Evolution of the Accelerated and Blastic Phases of Chronic Myeloid Leukemia: Molecular, Cytogenetic, Flowcytometric and Electron Microscopic Studies

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ABSTRACT

Background: Chronic Myeloid Leukemia is a clonal disease that results from an acquired genetic change in a pluripotential hemopoietic stem cell. Molecular abnormalities and mutations usually accompany the accelerated and blastic crisis phases of CML.

Aim of Work: This study was conducted to explore the possible ultrastructural, molecular cytogenetic, apoptotic and morphological abnormalities that may contribute to the progression of chronic phase to accelerated and blastic crisis phases in CML patients, for focusing on high risk patients to justify accurate lines of treatment.

Subjects and Methods: The study included thirty CML patients newly diagnosed and under treatment presenting to the Medical Oncology department of the National Cancer Institute, Cairo University, and ten age-matched subjects as a control group. CD95 (FAS) and p53 were studied by flowcytometry, BCR/ABL gene was studied at the cytogenetic and molecular level by RT PCR and ultrastructural apoptotic changes were studied by EM in PB samples. Also FISH was performed on few selected cases where conventional cytogenetics was not informative.

Results: Mean level of p53% was highly increased in the accelerated and/or blastic crisis phase and the chronic phase compared with controls (p=0.04). Mean level of CD95% expression was higher when measured on the whole cell population in the (accelerated and/or blastic crisis) compared with chronic phase and controls (p=0.14). By selecting CD34+ve cells, lower levels of CD95% expression were found in the (accelerated and/or blastic crisis phase) compared with the levels expressed on the whole cell population in the same phase. Cases were divided according to follow up into Group 1: 16/30 (53.3%) chronic phase cases that remained chronic during treatment. Group 2: 12/30 (40%) chronic phase cases that developed an accelerated or blastic crisis during treatment then returned to the chronic phase. Group 3: 2/30 (6.6%) chronic phase cases that developed an accelerated or blastic crisis phase then died. Mean p53% levels showed no statistically significant difference between the groups of CML (p=0.85). Higher levels of CD95% in CD34+ve cells were expressed in Group 1 compared with Group 2 and Group 3 and were statistically insignificant (p=0.85). Mean levels of CD95% expression were higher when measured on the whole cell population in Group 2 than Group 1 and Group 3 (p=0.45). Mean level of p53% in the treated cases was higher compared to newly diagnosed cases (before treatment) showing a statistically significant difference (p=0.01). Higher mean levels of CD95% on whole cell population, and on CD34+ve selected cells were detected after treatment (p=0.30, p=0.83). The mean levels of p53% and CD95% were higher in BCR/ABL fusion gene positive cases than BCR/ABL fusion gene negative cases but didn't reach significant levels respectively (p=0.21, p=0.62).

Conclusion:

A- p53% and CD95% levels expression in the accelerated and blastic crisis phases of CML patients were higher than those in the chronic phase.

B- Comparative studies for the apoptotic markers with cytogenetic analysis and RT PCR techniques revealed higher levels of p53 and CD95 in BCR/ABL positive cases than BCR/ABL negative cases.

C- p53 and CD95 levels were higher in treated cases than newly diagnosed cases.

Key Words: *Chronic myeloid leukemia (CML) – p53 – CD95 (FAS) – CD34 – Apoptosis – FCM, BCR/ABL – Ph chromosome – FISH – Electron microscopy (EM).*

INTRODUCTION

Chronic Myeloid Leukemia is a clonal disease that results from an acquired genetic change in a pluripotential hemopoietic stem cell **[1]**. CML remains silent for sometime then usually transforms to a more aggressive disease after 2 to 4 years and occurs in at least two clinical

manifestations rarely accelerated phase and blastic crisis which are considered outcome changes occuring at the end of CML evolution **[2]**. Philadelphia negative cases and aberrant cytogenetic and molecular changes are usually associated with accelerated and bad prognostic sequence **[3]**.

Molecular abnormalities and mutations usually accompany the accelerated and blastic crisis phases of CML. Beck et al. **[4]**, suggested the association of abnormalities of p53 gene with acceleration of CML. Loss or mutation of the tumor suppressor gene, p53, is one of the most frequent secondary mutations in CML blastic crisis. The transition between chronic phase and blastic crisis is associated with increased resistance to apoptosis correlating with poor prognosis **[5]**.

p53 mutation is reported to be less frequent in hematologic malignancies, but when present, they are important determinants of disease outcome **[6]**. P53 has a very short half-life, so that the wild-type protein is present in the cell at undetectable levels by immunohistochemical assay. In contrast, mutated version of p53 protein tends to accumulate into the nuclei of malignant cells due to its longer half-life, thus can be detected immuno-cytochemically. Accumulation of p53 occurs sometimes without mutation, however it is also nonfunctioning **[7]**.

The level of expression of CD95 (FAS) has a bearing effect on the outcome of CML. CD95 triggering is involved in the apoptotic death of lymphoid cells and may be also important in myelopoiesis **[8]**.

Reports on ultrastructural findings showed that the study of the morphology of the blood and bone marrow cells remains one of the important criteria that the pathologists have to depend upon in the diagnosis of different hematological disorders. Besides the use of the electron microscopes by investigators in their research work, hematopathologists find it necessary in confirming the diagnosis of some hematological disorders, where other tests give inconclusive results **[9]**.

This study was conducted to explore the possible ultrastructural, molecular, cytogenetic, apoptotic and morphological abnormalities that may contribute to the progression of chronic phase of CML patients to blastic crisis phase, for focusing on high risk patients, to justify accurate lines of treatment.

SUBJECTS AND METHODS

Subjects:

This study was carried out on 30 chronic myeloid leukemia patients (18 de-novo and 12 under treatment) presenting to the Medical oncology department of the National Cancer Institute, Cairo University, in the period between 2003 and 2005 including 12 females and 18 males whose ages ranged from 20 to 60 years old with a median of 41 yrs. In addition, 10 age matched subjects were included as controls.

Methods:

All Patients were subjected to full history taking, full clinical examination, complete blood picture **[10]**, bone marrow aspiration and morphological examination **[11]**, as well as LAP score (Leucocyte Alkaline Phosphatase) **[10]**.

Specimen collection: Ten ml venous blood were collected from each patient and control, and distributed as follows: 2ml on EDTA for Flowcytometric (FCM) analysis of CD34, CD95 (FAS) and p53 **[12]** and 8ml on preservative free heparin for: Cytogenetic analysis for Ph chromosome (Ph) **[13]** and FISH **[14]** and RT PCR **[15]** and Electron microscopic examination **[16]**.

P53, CD95 and CD34 by flow cytometric analysis:

Fresh peripheral blood or bone marrow aspirate were used and analysis was performed by flowcytometer (Coulter Epics XL, Hialeh). Fluorescent labeled mouse monoclonal antibodies against human anti-Fas (CD95), CD34 and p53 fluorescein isothiocyanate (FITC) and phycoerythrin (PE) conjugated in addition to isotopic control and intrastain permeabilizing solution were purchased from Becton Dickinson (Mountain view, California).

Sample preparation:

Two ml fresh venous blood on EDTA was collected, TLC was done using coulter counter. Dilution of the sample with PBS was made to reach a total leucocytic count from 8-10x10^9/L). The test tubes were labeled as follows:

1- Control tube (isotype corresponding to each monoclonal).

2- CD34 (FITC)/CD95 (PE) tube.

3- P53 (FITC)/CD34 (PE) tube.

Detection of surface markers by direct staining:

For the CD34 (FITC)/CD95 (PE) Tube: Ten ul of monoclonal antibodies CD34 and CD95 were added to 100ul of diluted blood, and left in the dark for 20 minutes. Two ml lysis solution was added, well mixed and left for another 5 minutes. Tubes were centrifuged, cells were washed twice with PBS solution and suspended in sheath fluid to be ready for reading by flowcytometer.

Detection of intracellular markers by direct staining:

For the p53 (FITC)/CD34 (PE) Tube: Ten ul of monoclonal antibody CD34 was incubated with 100ul of diluted blood in the dark for 20 mins. Two ml of lysis solution was added and left for another 5 minutes. Cells were washed with PBS, supernatant removed and 100ul of fixative solution were added and left for 15mins, 2ml of PBS was added to cell suspension, centrifuged for 5mins, and supernatant removed. One hundred ul of permeabilizing solution and 10ul of monoclonal antibody p53 were added and left in the dark for 20mins. Cells were centrifuged for 5 minutes at 2000rpm, supernatant removed and cells washed twice with PBS and suspended in sheath solution to be ready for reading by flowcytometer. Sample was considered positive if ≥Cut off value for p53=0, cut off value for CD95 was 1.2 and CD34 was 10%.

Cytogenetic studies:

Conventional cytogenetic analysis was carried out on unstimulated bone marrow or peripheral blood cultures. Metaphases were trypsin/ Giemsa-banded. Karyotypic abnormalities were described according to the specifications of the International System for Human Cytogenetic Nomenclature **[17]**. Fluorescence in situ hybridization (FISH) analysis was performed when necessary using the commercial BCR/ABL locus specific identifier (LSI) probe (Vysis Downers Grove, IL, USA) according to the manufacturer's recommendation **[14]**. For cytogenetic and FISH analysis an automated karyotyping system (Quips, Vysis, USA) was used.

Real – Time RT – PCR:

Buffy coat layer was separated by density gradient centrifugation **[18]**. Cells were preserved in RPMI 10% Demso solution to keep them viable and at -80ºC until used. RNA extract was prepared from the separated cells using pure script Total RNA Isolation kit (Gentra System, USA). BCR/ABL gene expression was carried out using Real-Time RT PCR using SYBR Green **[19]**.

The procedure began with reverse transcription of total RNA using SYBR Green RT-PCR one Step (Genecraft), with reversed primer. Primer 1: (5`-TGTGATTATAGCCTAAGA CCCGGAGCTTTT-3´). The cDNA was then used as a template for realñtime PCR with gene specific primer. Primer 2: (5`-GAGCGTGCAG AGTGGAGGGAGAACATCCGG-3´) Primer 3: (5`-TTCAGC GGCCAGTAGCATCT-GACTT-3´). The reaction mixture condition consisted of Buffer 1x, SYBR Green dye and Rox reference dye, 3.5mmol Mn-acetate solution, 0.3mmol dNTPS, 7.5pmol for each primer and 1.5uTaq polymerase DNA. The thermal cycler profile consists of: 60ºC for 60min. RT-reaction, 95ºC for 15min. DNA polymerase activation, 95ºC for 15sec. denaturation and 60ºC for 1min annealing and extension. The main PCR cycle was repeated for 40 cycles. The results were obtained in the form of PCR amplification plot curves. The Stratagene Real-Time instrument system has a built in therma lcycler and 4 laser beams directed via fiber optic cables to the wells. The fluorescence emitted travels back through the cables to a CCD camera detector.

Electron microscopic examination:

Leucocytes suspension was prepared, after separation from peripheral blood and bone marrow samples, for transmission electron microscopy according Catovsky **[20]**. The cells were fixed in 2.5% glutaraldehyde (Merk) in PBS (pH=7.4) then post fixed in 1% osmium tretraoxide (Electron microscopy Sciences) in PBS (pH=7.4) for 2 hours at 4ºC, dehydrated in ascending grades of ethanol. The cells were substituted in a mixture of Epon resin (Electron microscopy Sciences) and absolute ethanol and lastly infiltrated in three baths of Epon resin at room temperature twelve hourly. The cells were embedded in EPON 812 capsules. Polymerization of the resin then follows at 60ºC for 24 hours. Ultra thin sections were prepared and examined under the electron microscope, Joel 1200 EX II.

Statistical analysis:

Statistical Package for social sciences (SPSS) version 9 was used. Quantitative variables were

summarized using mean and SD, median, minimum and maximum values. Qualitative data were summarized using frequencies and percentage.

The relation between quantitative variables was tested by ANOVA test and Spearman Correlation. 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 **[21]**.

RESULTS

Thirty randomly chosen CML cases presenting to the National Cancer Institute, Cairo University, in the period between 2003-2005 and 10 age matched normal control subjects were included in this study. The age ranged from 20-60 years with a mean of 38.03±12.81 and a median of 41. Male to female ratio was 3:2 (18 males and 12 females).

Mean LAP score of all patients and controls was 32.6±57.1 (range 0 to 237). 6/30 (20%) patients had a high LAP score, they were classified as either acute blastic crisis or accelerated phase and 24 (80%) patients had a low LAP score (chronic phase of CML).

P53% by FCM:

Mean P53% level was 1.6%±2.3 (range 0 to 9.6%). Twenty four out of 30 cases (80%) showed a positive expression and 6/30 (20%) were negative for p53.

CD95% by FCM:

Mean level of CD95+ve/CD34+ve cells was 5.9%±11.7 (range 0 to 54%). Mean CD95% level studied of the whole cell population was 12.7%±23.9 (range 0 to 98%). Twenty two out of 30 cases (73.3%) expressed a high CD95% level ≥1.2 (Positive) and 8 (26.6%) expressed a low level <1.2 (Negative).

Cases were classified according to the different phases of CML into: 3 cases in acute blastic crisis, 3 cases in accelerated phase and 24 in chronic phase. Patients were divided into groups according to follow-up:

- *Group 1:* 16/30 (53.3%) chronic phase cases that remained chronic during treatment.
- *Group 2:* 12/30 (40%) chronic phase cases that developed an accelerated or blastic crisis during treatment and returned to chronic phase.
- *Group 3:* 2/30 (6.6%) chronic phase cases that developed an accelerated or blastic crisis phase then died.

Comparative studies of p53% and CD95% between the different phases of CML are illustrated in Table (1):

- There was statistically significant higher level of p53% expressed in the accelerated and/or blastic crisis phase 2.25%±2.32 and the chronic phase 2.18%±2.75 compared with that of controls 0.049%±0.04 (*p*=0.04).
- There was higher level of CD95% expression when measured on the whole cell population in the accelerated and/or blastic crisis 20.00%± 32.82 compared with chronic phase 15.98%± 23.84 and controls 1.27%±0.28 but did not reach significant level (*p*=0.14).
- On the other hand studying the level of CD95+ve/CD34+ve cells, low levels of CD95% expression were found in the accelerated and/or blastic crisis phase 10.4%±17.7 compared with the levels expressed on the whole cell population in the same phase.

Comparative studies of p53% and CD95% with BCR/ABL fusion gene expression by conventional cytogenetics, FISH and real-time RT PCR: (Table 2):

The level of p53% was higher among cases positive for BCR/ABL fusion gene 2.49%±2.64 compared with cases negative for BCR/ABL fusion gene 0.48%±0.45 but didn ot reach significant level (*p*=0.212).

The level of CD95% was higher among cases positive for BCR/ABL fusion gene 18.36%±27.92 compared with those negative for BCR/ABL fusion gene 10.21%±11.31, but didn't reach significant level (*p*=0.629).

Comparative studies of p53% and CD95% in CML groups as regards follow-up: (Table 3):

- There was no statistically significant difference in the p53% level when studied among the 3 groups of CML, though higher levels were seen among group 3 but did not reach significant level, (*p*=0.85).
- There were higher levels of CD95 +ve/CD34 +ve cells expressed in group 1 (9.2%±14.8) (chronic phase only) compared with group 2

(7.0%±12.2) & group 3 (4.2% ±4.2), but did not reach significant level (p=0.85).

- There were higher levels of CD95% expression when measured on the whole cell population in group 2 (26.50%±35.40) when compared with group 1 (12.32%±21.65) and group 3 (16.06%±12.53), but did not reach significant level (p=0.45).

Comparative studies of p53% and CD95% among newly diagnosed cases and their response to treatment are illustrated in Table (4):

- There was a statistically highly significant difference in the level of p53% in the treated cases (3.9%±3.0), compared to newly diagnosed cases before treatment (1.4%±1.9), (p=0.01).
- There was no statistically significant difference in the levels of CD95+ve/ CD34+ve cells in the newly diagnosed cases (before treatment) (8.4%±14.8) compared to the treated cases (7.2%±9.5), (p=0.83).
- On the other hand studying the levels of CD95% on the whole cell population, higher levels were found in the treated cases (25.6% ±28.5) compared to those newly diagnosed (before treatment) (13.8%±25.7), but did not reach statistical significance (p=0.30).

Ultrastructural findings:

EM examination of selected cases with high p53% and CD95% showed some apoptotic changes. These morphological changes were divided into early and late changes. Early morphologic changes showed chromatin condensation along the nuclear envelope with subsequent thickening and irregularity, asynchrony of nucleus with the cytoplasm, with frequent nuclear fragmentation and widening of the nuclear membranes and increase in the nuclear pores.

Late morphologic changes showed cytoplasmic changes in the form of compacting of cytoplasmic organelles, increased cytoplasmic vaculations, widened smooth endoplasmic reticulum, margination of cytoplasmic mitochondria towards the cell membrane, swelling of mitochondria and loss of its christae. as well as rufflings, blebbings and thinning of the plasma membrane (Fig. 1: D,E).

Table (1): Comparative study of p53% and CD95% mean±standard deviation between the studied groups as regards phases of CML.

	Chronic phase (a)* (n=24)	Acc. & blastic phases (a)* (n=6)	Controls (b)* (n=10)	p value
p53%	2.18±2.75	2.25±2.32***	0.049±0.04	0.049***
CD95+ve/CD34+cells	8.4±13.9	10.4±17.7	1.27±0.28	0.291
CD95%	15.98±23.84	20.00±32.82**	1.27±0.28	0.143**

*Groups with different letters are with statistical significance. ** High levels but did not reach significant level.
*** Significance <0.05.

Table (2): Comparative study of p53% and CD95% with the cytogenetics, FISH and PCR results.

	BCR/ABL Gene (-ve) (n=3)	BCR/ABL Gene (+ve) (n=20)	p value
p53%	0.48±0.45	2.49±2.64*	0.212
CD95%	10.21±11.31	18.36±27.92*	0.629

* High Levels but did not reach significant level. ** Significance <0.05.

Table (3): Comparative study of p53% and CD95% in different groups of CML as regards follow-up.

	Group 1 (n=16)	Group 2 (n=12)	Group 3 (n=2)	p value
p53%	2.00±2.71	2.35±2.48	3.05±3.46*	0.85
CD95+ve/CD34+cells	9.2±14.8*	7.0±12.2	4.2±4.2	0.85
CD95%	12.32±21.65	26.50±35.40*	16.06±12.53	0.45

* High levels but did not reach significant level. ** Significance <0.05.
Group 1: CML patients who started and remained chronic during treatment.
Group 2: CML patients who started as chronic and developed an acceleration and/or blastic crisis phase then returned to chronic during treatment.
Group 3: CML patients who started as chronic & developed an acceleration and/or blastic crisis phase then died during treatment.

Table (4): Comparative study of p53% and CD95% mean ± standard deviation as regards newly diagnosed cases and their response to treatment.

	New cases (n=18)	Treated cases (n=12)	*p* value
p53%	1.4±1.9	3.9±3.0**	0.01**
CD95+ve/CD34+cells	8.4±14.8	7.2±9.5	0.83
CD95%	13.8±25.7	25.6±28.5*	0.30

* High Levels but did not reach significant level. ** Significance <0.05.

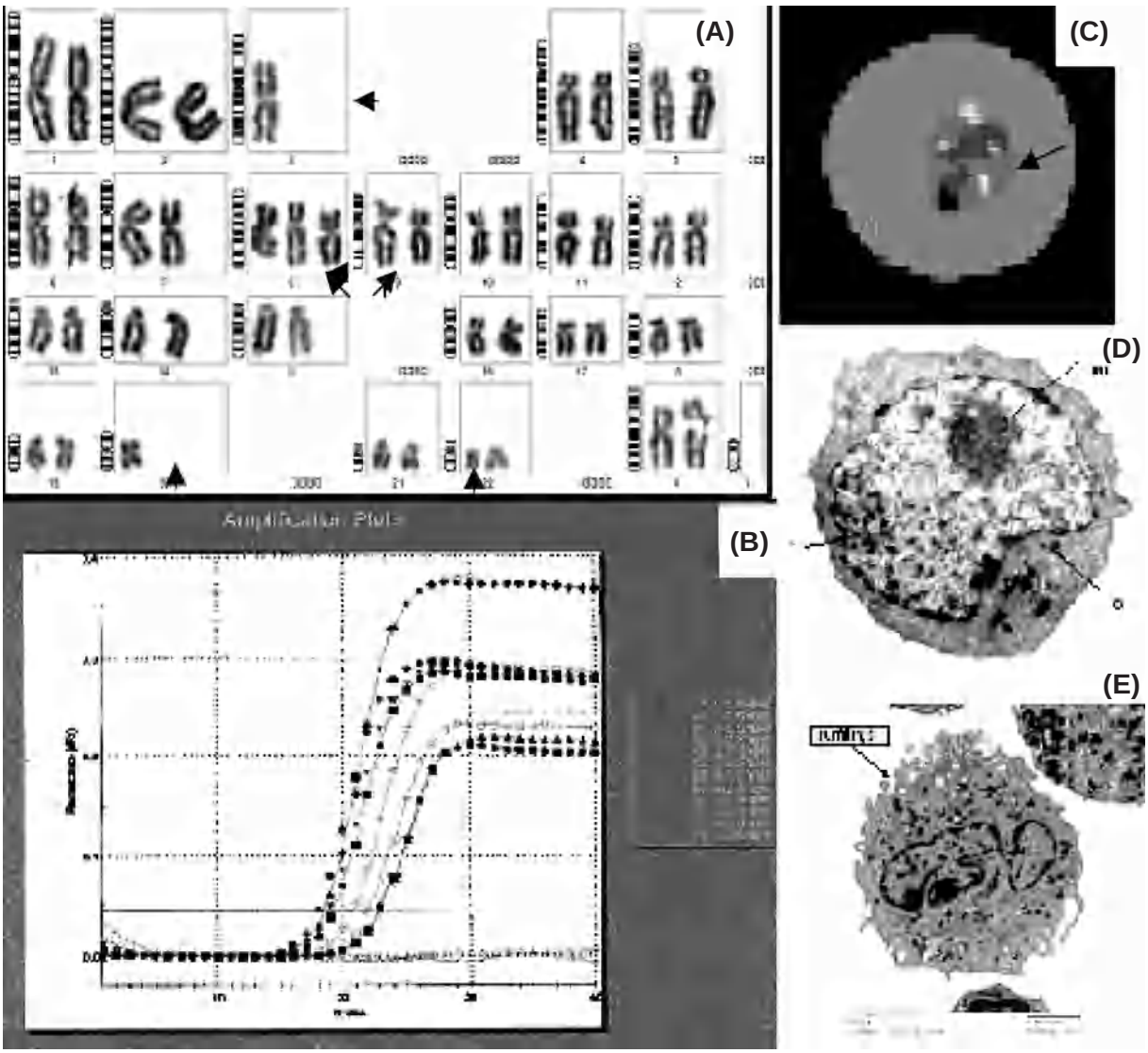

Fig. (1)

A: Karyotype of a 27 years old female presentedwith ABC on top of CML showing 45, XX, t (9;22) (q34;q11),-3 ,+8, -20.

B: PCR amplification plot.

C: FISH using BCR/ABL probe (Vysis) for a 20 years old case showing positive fusion signal.

D: An electron micrograph showing a myeloblast from a case of ABC of CML having a large immature nucleus (N) consisting mainly of euchromatin with small aggregations of heterochromatin dispersed all through the nucleus. A large prominent nucleolus (Nu) is present. The cytoplasm contains rare peroxidase positive (G) granules. The figure shows condensation of the mitochondria to one side of cell membrane (x10,000).

E: An electron micrograph of a monocyte showing increased rufflings, (x2,000).

DISCUSSION

This study was carried out on 30 randomly chosen chronic myeloid leukemia patients. Ten age and sex matched normal subjects were included as a control group.

The present study was designed to investigate the regulation of proliferation and survival of CML progenitors and the apoptotic pathway triggering in the elimination of the Ph+clone. The levels of apoptotic markers p53 and CD95 were measured by flowcytometry and compared among the different phases of CML and controls.

In this study, the mean age of the patients was 38 years and a median of 41 years, with a range of 20-60 years, most of the patients in chronic phase aged between 40-60 years and the male to female ratio was (3:2). It was found that 3 cases were younger patients of age 20–35 years presenting with blastic crisis. This finding was in agreement with previous studies **[22]**. In the present study, all the patients presented by leucocytosis with higher levels or doubling of the TLC among accelerated and/or blastic crisis phase. When compared with the control group, these findings were in agreement with previous reports **[23]**, where TLC count was $25x10^9$/L and reached $100x10^9$/L in the accelerated or blastic crisis phase.

A low LAP score was diagnostic of the chronic phase, in the present study. Only 3 cases (10%) with blastic crisis and 3 cases (10%) in accelerated phase presented with a high LAP score. This is in accordance to previous studies **[24]** which stated that the LAP score is an important diagnostic hematological data in distinguishing typical CML from other myeloproliferative disorders and reactive leucocytosis or leukemoid reactions.

p53% was expressed in high levels in the accelerated and blastic crisis phase (20% of cases) and chronic phase (80% of cases) when compared with controls with a statistically significant value (p=0.049). This is in accordance to previous studies **[25]** which reported that high percentage of p53 protein expression may indicate neoplastic transformation of chronic phase to accelerated or blastic crisis phases. Previous research **[26]** found that p53 protein expression in B-CLL is strongly associated with p53 mutation. Detection of p53 gene mutation is complex and unlike wild type p53, mutated p53 has a prolonged intracellular half-life and thus becomes detectable by immunohistochemistry and flowcytometry **[27]**. Tumour suppressor gene p53% elevations, in the accelerated and/or blastic phases, in the present study, are in agreement with a previous report **[28]** in which 14% of CML cases in accelerated and/or blastic phase expressed an elevated level of p53% whereas 65% of CML cases in chronic phase expressed low levels. These findings are in favour that alterations and elevation of p53 expression plays an important and central role in CML evolution.

In the present study, the mean level of CD95% expression on CD34+cells showed no statistically significant difference between accelerated/blastic crisis phases compared to the chronic phase and controls (p=0.29). On the other hand, high mean level of CD95% was expressed on the whole cell population in the different phases of CML but didn't reach a statistically significant difference. Higher levels of CD95% were detected among cases of accelerated, blastic crisis phases and chronic phase CML patients when compared with controls but of statistically nonsignificant value (p=0.14). These results were similar to previous reports **[29]** where the high levels of CD95% detected by flowcytometry in the chronic phase CML patients were compared to controls with statistically significant value (p<0.05), and higher levels of CD95% among accelerated phases compared to chronic phase with highly significant value (p=0.001).

In this work a comparative study was done between the p53% and CD95% expression among the studied group as regards newly diagnosed cases and their response to therapy (before and after treatment). A statistically significant higher p53% level was found among treated CML patients compared with the newly diagnosed patients (p=0.01). Higher levels of CD95% were also detected among the treated CML patients compared with newly diagnosed ones (p=0.30) not statistically significant.

p53 contributes to the cytotoxic action of many chemotherapeutic agents and protects the genome from mutagenic insult **[30]**. In quiescent cells, levels of p53 protein are low owing to its short half-life. After DNA damage, the half-life of p53 becomes prolonged **[31]** and the protein accumulates in the nucleus **[32]**, where it regulates the transcription of a number of genes.

p53 mutations typically prolong the half-life of the protein in the absence of DNA damage and are associated with increased basal levels **[33]**. However, when activated, mutant p53 protein cannot regulate gene expression because of its inability to bind to specific DNA sequences **[34]**. It was reported that IFN-alpha can greatly up-modulate Fas-R expression, an effect that seems to be more pronounced in CML cells compared to normal cells **[35]**.

The present study confirms a previous report where a high CD95% level in treated CML cases was compared with newly diagnosed cases with a high statistically significant value (p= 0.01). It was concluded that the activity of Fas triggering on hematopoietic progenitor cells derived from patients with CML is related to the levels of Fas-R induced by therapy **[36]**. The high level is possibly due to the effect of chemotherapy on apoptosis translation or RNA transcription and induction of apoptosis at all phases of the cell cycle **[37]**.

In our study the use of flow cytometry for the detection of the levels of p53% and CD95% expression, during different phases of CML patients, was an accurate method giving high statistically significant and accurate values. It was reported that flowcytometric analysis is the most precise assay because it enables the exclusion of contaminating population of non-neoplastic cells such as lymphocyte subsets and allows accurate identification of the specific immunephenotype in cases with heterogonous activity **[38]**.

Although, philadelphia negative cases and aberrant cytogenetic and molecular changes are usually associated with accelerated and bad prognostic sequence **[3]**. In this study, increased p53 expression level in BCR/ABL fusion gene positive cases compared with BCR/ABL fusion gene negative but did not reach significant levels (p=0.212). This might be due to the small number of BCR/ABL fusion gene negative cases.

In the present study, the studied group was subjected to cytogenetic analysis and real-time RT PCR techniques to define typical CML cases from atypical CML. Philadelphia chromosome (Ph) was negative in 20% of CML cases by cytogenetic analysis. Using real-time RT PCR and FISH, 10% only were negative for BCR/ABL gene confirming the results of cytogenetic analysis and the other 10% were positive for BCR/ABL.

In agreement with this comparative study, a recent study **[39]** reported that the introduction of real-time RT PCR has largely replaced the competitive quantitative procedures and more sensitive than the conventional cytogenetic method. It was stated that the fluorescence based technology enhances the reproducibility since quantitation is determined during the exponential phase of the PCR. These data were also in agreement with Xing et al. **[40]**, who described that Real-time RT PCR is a reliable more sensitive and accurate method to detect minimal residual disease, monitor the treatment outcome and predict the blastic crisis of CML patients when compared with other conventional cytogenetic and quantitative PCR techniques.

FISH technique provides an important means to complement data obtained by conventional cytogenetics. FISH has the advantage of studying larger number of cells, hence increasing the sensitivity of cytogenetic analysis **[41]**. This technique with chromosome-specific probes enables several new areas of cytogenetic investigation by allowing visual determination of the presence and normality of specific genetic sequences in single metaphase or interphase cells. In this approach, termed molecular cytogenetics, the genetic loci to be analyzed are made microscopically visible in single cells using in situ hybridization with nucleic acid probes specific to these loci **[42]**. One of the uses of FISH is in the diagnosis of CML, monitoring treatment outcome and chimerism analysis following bone marrow transplantation using XY probes if sex mismatched transplantation or BCR/ABL probes **[43]**.

Apoptosis plays a role in the pathogenesis as well as in the pathologic effects of many benign and malignant diseases. Furthermore, there is increasing evidence that the process of neoplastic transformation, progression and metastasis involves alteration in the normal apoptotic pathways **[44]**.

At the ultrastructural level, using the EM technique, the morphological features of apoptosis in blastic crisis and accelerated phase cases were studied. They were divided into early changes in the form of shrinkage of cell size,

condensation of the nuclear chromatin along the nuclear envelope, reduction of the nuclear size, nuclear fragmentation and disintegration, indenting of the nuclear membrane and nuclear splitting.

The late apoptotic changes that were detected in this study were in the form of condensation of the cytoplasmic organelles, their margination towards the cell membrane, shrunken mitochondria with loss of cristae, abnormal phagosomes in the cytoplasm, cytoplasmic vaculations, thinning and perforation of the plasma membrane and lastly the formation of apoptotic bodies which finally get released from the cell.

These findings are in agreement with the morphological features of apoptosis described by Aboul-Enein **[45]**, and Khorshed et al. **[16]**. The early apoptotic changes were described in the form of condensation of the nuclear chromatin along the nuclear envelope with subsequent thickening and irregularity, reduction in the nuclear size with frequent nuclear fragmentation. They also described the late morphologic features of apoptosis to include compacting of cytoplasmic organelles, margination of cytoplasmic organelles towards the cell membrane, rufflings, blebbings and thinning of the plasma membrane and finally nuclear fragmentation into membrane bound apoptotic bodies. These apoptotic changes could be mediated either through caspase dependent or non-caspase dependent pathways **[46]**.

In conclusion, p53 and CD95 can be used as predictors of impending accelerating and/or blastic crisis phases i.e. as tumour markers for both phases, as their expression in the accelerated and blastic crisis phases of CML patients were higher than the chronic phase. In this work, the comparative studies for the apoptotic markers in cases studied by both conventional cytogenetic analysis and PCR techniques revealed higher levels of p53 and CD95 in BCR/ABL positive cases than negative cases. Finally, we conclude that the proper diagnosis, with effective classification of CML cases by cytogenetic and molecular techniques, is essential to predict and prevent the evolution of more aggressive course among chronic phases of CML patients and to justify the accurate lines of treatment.

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Journal of the Egyptian Society of Haematology & Research, Vol. 2, No. 2, September: 179-186, 2006

Association between Matrix Metalloproteinase2, Membrane Type 1-Matrix Metalloproteinase and Hepatocyte Growth Factor in Breast Cancer Patients

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ABSTRACT

Membrane type-1 matrix metalloproteinase (MT1-MMP) is essential for breast cancer invasion and metastasis. Many studies have shown that MT1-MMP has an important role in MMP-2 activation in cell membranes but only few reports about its clinical value are valid. In this study, we investigated the relationship between MT1-MMP protein expression and matrix metalloproteinase type 2 (MMP2) activity as well as hepatocyte growth factor (HGF) serum level in human breast cancer. For this purpose 34 human breast cancer tissues, also blood samples from the patients and 15 healthy controls were collected and sera were separated and analyzed for HGF determination. MT1-MMP protein expression was detected by Western Blot, MMP2 by zymographic analysis and HGF was measured by ELIZA. The results revealed that 79.4% of breast cancer tissue exhibited positive MT1-MMP expression, while it was 26.4% in the normal surrounding tissues. (p-value <0.001). There was a significant association between MT1-MMP expression and MMP-2 activity (p-value=0.007) in the tumor tissue. Also a significant association was detected between MT1-MMP expression and lymph node involvement (p-value=0.014). Neither grade nor tumor size showed significant correlation with MT1-MMP expression. MMP2 activity displayed a highly significant difference (p-value=<0.001) between the tumor tissues and their normal correspondence, while no significant association was detected between MMP2 activity and any of clinicopathological features. The serum levels of HGF were significantly increased in group of breast cancer patients as compared to the control group (p-value: 0.001). The best cut-off value for HGF was ≥430pg/ml with 91.2% and 73.3% sensitivity and specificity respectively. There was a highly significant association between serum level of HGF and MMP-2 activity in cancer breast tumor but the association between its serum level and MT1-MMP protein expression didn't reach a significant level. There was no significant correlation between serum level of HGF and any of clinicopathological features.

In conclusion: MT1-MMP can be used as a predictor for the ability of breast cancer invasion and metastasis and the association between it and MMP2 in the tumor tissue confirms that MT1-MMP is the tumor-specific activator of proMMP-2. The significant correlation between MMP2 and HGF level may reflect the regulation role of HGF on transcription of MMPs genes.

Key Words: *Breast cancer – MMPs – HGF.*

INTRODUCTION

Matrix metalloproteinase (MMP)-2 in breast cancer, is a protease produced essentially by stromal cells. In vitro studies have clearly demonstrated that it degrades molecules that are abundant in the extracellular matrix (ECM) **[1]**. MMP-2 is also one of the major targets of recently developed synthetic MMP inhibitors **[2]**. The recently literature demonstrates that the mechanism of action of MMP-2 is complex and that other molecules modulate its activity **[3]**.

MMP-2 is secreted in an inactive pro-enzymatic form and, unlike other MMPs, its activity is modulated by tissue inhibitor of metalloproteinase (TIMP)-2 and the membrane type-1 MMP (MMP-14) **[4]**.

Membrane type matrix metalloproteinases (MT-MMPs) localized to the invasive front of highly motile cancer cells **[5]** were shown to be directly involved in matrix breakdown **[6]**. So far, six members of the MT-MMP subfamily have been identified and partially characterized **[7]**. MT1-, MT2-, and MT3-MMP strongly contribute to tumor cell invasion **[8]**. MT1-MMP, the first member of a more recently established group of MMPs containing a membrane-spanning sequence, has been shown to have an important role in MMP-2 activation in cell membranes implicated in tissue-remodeling

events that range from tumor invasion and angiogenesis to growth and development **[9]**.

Current evidence indicates that MT1-MMP regulates matrix turnover by means of its ability to degrade matrix-associated molecules either directly or via the activation of downstream MMPs. As rate of activation of MMP-2 in tumor tissue is well-controlled to the expression levels of MTI-MMP and to the tumor spread, **[10]** thus MT1-MMP is believed to be the in vivo proMMP2 activator during cancer cell invasion. Animal studies showed that carcinoma cell lines transfected with MT1-MMPproduced higher levels of active MMP-2 and developed more lung metastases compared to parent tumor cells **[11]**.

Invasion and metastasis involve a large number of molecules, including angiogenic factors, growth factors and their receptors, adhesion molecules, proteases, intracellular signaling molecules, and transcription factors **[12-14]**.

Hepatocyte growth factor (HGF) was suggested to play an important role in the regulation of mitogenesis, motogenesis, angiogenesis, migration and invasion for various types of cells, and acts through a specific membrane receptor encoded by c-met proto-oncogene **[15]**.

Elevated hepatocyte growth factor content in tumor tissue was reported to predict a more aggressive biology in non-small cell lung cancer patients. However, there is still limited knowledge about the role of HGF in breast cancer. Hepatocyte growth factor (HGF) was previously reported to induce expression of Ets-oncogene family transcription factor (E1AF) gene whose product in turn positively regulates transcription of MMP genes **[16]**.

This study was carried out to investigate the expression of the activated form of gelatinase A (MMP2), MT1-MMP and serum level of pre operative HGF in breast cancer patients and to evaluate the correlation between them and clinicopathological features.

MATERIAL AND METHODS

This study was performed on 34 newly diagnosed breast cancer patients, the tumors were used for Western Blot and zymography to measure expression level of MT-1MMP and MMP2 respectively. The normal surrounding corresponding of each tissue sample was taken as control. Also venous blood samples (5ml) were collected from the patients before surgery and venous blood samples (5ml) were collected from 15 apparently healthy age matched females who serve as controls for determination of serum Hepatocyte Growth Factor (HGF). The patients under went surgical excision in National Cancer Institute, Cairo university. All the patients met the following criteria: (a) having been diagnosed as having primary invasive breast cancer (b) having no clinical manifestation of infection, (c) having received no blood transfusion during the previous 3 weeks, (d) having no known liver, renal dysfunction.

The patients were 34 females with mean age of 54.8±13.6 years (range, 28-76 years). The average tumor size was 4.6±2.4cm (range, 1.5-11cm). There were 24 tumors of differentiation grade 2 while 7 tumors of grade 3 (3 patients were unclassified). There were 24 tumors with +ve lymph nodes while 10 with –ve lymph nodes.

The tumor material was fixed in 10% neutral formalin and embedded in paraffin. The diagnosis of tumors were based on a light microscopic examination of H&E stained section.

Western blot:

To detect MT1-MMP expression, a piece of each tumor (1cubic cm) was homogenized in 1% (w/v) sodium dodecyl sulfate and diluted to one fold in water before measuring protein content against bovine serum albumin using Bradford srandard, (1979). Samples were diluted with SDS-PAGE sample buffer in the presence of mercaptoethanol, heated at 100C in boiling water bath for 5min. and allowed to cool to room temperature. Each aliquot containing 100µg was separated on 10% SDS-PAGE at 30mA and transferred to nitrocellulose membranes (Bio-RAD, USA) in transfer buffer at 10V constant for 30min (Semi-dry Bio-Rad unit, USA). The membranes were blocked for two hours at room temperature in 2.5% non fat milk powder in PBS. The membranes were then incubated overnight with monoclonal antibody specific for MT1-MMP (Santa Cruz Biotechnology, Inc.). The bands were visualized by using a horseradish-peroxidase conjugated goat antiserum against mouse IgG (Sigma Chemical CO., St. Louis, Mo), hydrogen peroxide (BDH) and diaminobenzedine (Sigma Chemical CO.,

St. Louis, Mo). The bands were evaluated (area under the peak of each sample) using Dual-Wavelength flying spot scanning Densitometer P, N206 (Shiamadzu. CO, Japan). Value of normal tissue has been taken as 100% for each sample.

Zymography analysis:

The proenzyme and activated forms of MMP2 were detected by zymography using SDS-polyacylamide gels copolymerized with 1mg/ml gelatin to detect gelatinolytic activities **[17]**.

Fifty µg from surgical specimen was homogenized in sample buffer. Samples were separated by electrophoresis on a 10% polyacrylamide gel containing 0.1% SDS and 1mg/ml gelatin as substrate. After electrophoresis, Gels were washed at room temperature in renaturation buffer containing 2.5% Triton-X 100 for 10 minutes and for 20 minutes in water. Thereafter the gels were incubated for 18 hours at 37°C in Tris-based buffer. Gels were stained with a solution containing 30% methonal, 10% glycial acetic acid containing 0.5% Coomassie brilliant blue R250. Destaining was done in 10% methanol and 10% acetic acid, and gelatinolytic activity was detected as clear bands; 68kDa and 62kDa and analysed by densitometric scanning using a computer-assisted analysis. The band of active form was classified as negative (same as control) and positive (10% more than the control). Unstained areas corresponded to zones of MMP proteolytic activities.

Determination of HGF by quantitative sandwich enzyme, immunoassay (ELISA) technique:

Using a kit from R and D systems, Minneapolis MN, USA. The samples and standard were added to micro wells precoated with the captured antibody, after washing any unbound substances, an-enzyme linked polyclonal antibody for HGF was added. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of HGF. The intensity of the color was measured **[18]**.

Statistical analysis:

Statistical package for social sciences (SPSS) version 9 was used. Quantitative variables were summarized using mean and SD. Qualitative data were summarized using frequencies and percentage. The relation between quantitative variables was tested by Spearman correlation. Chi-square test was used to test the association between the different qualitative variables. *t*-test and Mann-Whitney U were used to compare numerical data between groups. Cut-off value was calculated. Sensitivity, specificity, positive predictive value, and negative predictive value were calculated using ROC curve.

RESULTS

Detection of MTI MMP expression in tumor tissue and corresponding surrounding normal tissue by Western blot:

Table (1), showed that MT1-MMP expression was positive in 79.4% in breast tumor tissue compared to 26.4% in normal corresponding. The difference was highly significant (*p*:value=<0.001) Fig. (1).

Table (1): Comparison between MT1-MMP expression in tumor tissue and normal surrounding tissue of breast cancer patients.

MT1-MMP Expression	Normal tissue		Tumor tissue		*p* value
	No.	%	No.	%	
Negative	25	(73.6)	7	(20.6)	**<0.001
Positive	9	(26.4)	27	(79.4)	

***p*-Value is highly significant (<0.001).

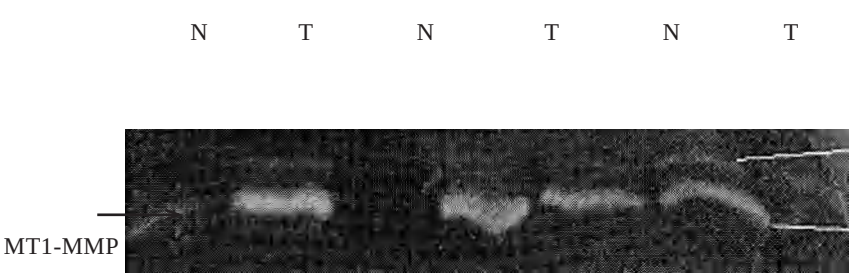

Fig. (1): Showed western blot analysis of MT1-MMP expression in breast cancer patients.
N: Normal surrounding tissue T: Tumor tissue from the same patients.

Table (2) showed the association between MT1-MMP expression and lymph nodes status in cancer breast patients. There was a significant association between MT1-MMP expression in tumor tissue and the presence of positive lymph nodes (*p*value=0.014). However there was no significant association between MTI-MMP expression and tumor grade or tumors size.

Detection of MMP2 expression in tumor tissue and corresponding surrounding normal tissue by zymography:

61.8% of tumor cases showed high activity of MMP2 while 38.2% showed the same activity of control correspondence (*p*value:0.001) Fig. (2).

There was strong association between MMP2 activity and MT1-MMP Expression in tumor tissue of breast cancer patients (*p*-value: 0.007).

The association between MMP2 activity with other clinical and pathological parameters were evaluated. As shown in Table (4), there was no relationship between the expression of MMP2 and lymph node status, tumor grade or tumor size.

Determination of HGF serum level by (ELISA) technique:

A highly significant increase of serum HGF levels was found in breast cancer patients when compared to controls (*p*:value=<0.001).

The cut-off value of HGF was ≥430pg/ml. It detects breast cancer patients with sensitivity 91.2% and exclude normal control with specificity of 73.3%, the positive predictive value was 88.6% and the negative predictive value 78.6%.

There was a highly significant increase in the serum levels of HGF in patients with positive MMP2 activity than those with negative MMP2 activity (*p*value:0.001). Meanwhile, there was also increase in its levels in patients with positive MTI-MMP expression compared to those with negative MT1-MMPbut the difference did not reach to significant level.

The serum level of HGFdid not correlated with age of the patients (*r*=–.139, *p*value:0.44) or with tumor size (*r*=0.146, *p*:value=0.41) and tumor grade (*r*=0.084, *p*:value=0.65). Also, there was no statistically significant difference in its level between cases with positive lymph nodes and those with negative lymph nodes (mean ± SD, 553.13±105.96pg/ml and 521.1± 97.06pg/ml respectively) with *p*value=0.42.

Table (2): Association between MT1-MMP expression and lymph nodes status, tumor size and tumor grade in cancer breast patients.

Clinical parameters	MT1-MMP expression		*p* value
	–ve No. (%)	+ve No. (%)	
Total patients no.	7	27	
Lymph nodes:			
(–)	5 (71.4)	5 (18.5)	*0.014
(+)	2 (28.6)	22 (81.5)	
Tumor size:			
≤2cm	2 (28.6)	5 (18.5)	0.59
>2cm	5 (71.4)	22 (81.5)	
Tmor grade:			
II	5 (71.4)	18 (66.7)	0.18
I11	0 (0)	8 (29.6)	

*p-value <0.05 significant (2 tailed).

Table (3): Showed the association between the MT1-MMP expression and the MMP2 activity in tumor tissue.

MTI -MMP Expression	MMP2 activity		*p* value
	–ve (no=13)	+ve (no=21)	
Negative (no=7)	6 (85.7%)	1 (14.3%)	*0.007
Positive (no=27)	7 (25.9%)	20 (74.1%)	

*p-value <0.05 is significant (2-tailed).

Table (4): Showed the association between MMP2 activity and lymph nodes status, tumor size and tumor grade.

Clinical parameters	MMP-2		*p* value
	–ve no (%)	+ve no (%)	
Total patients no.	13	21	
Lymph nodes:			
(–)	5 (38.5)	5 (23.8)	0.36
(+)	8 (61.5)	16 (76.2)	
Tumor size:			
≤2cm	4 (30.8)	3 (14.3)	0.25
>2cm	9 (69.2)	18 (85.7)	
Tmor grade:			
I	10 (83.3)	14 (73.7)	0.68
II	2 (16.7)	5 (26.3)	
III			

*p-value <0.05 is significant. (2-tailed).

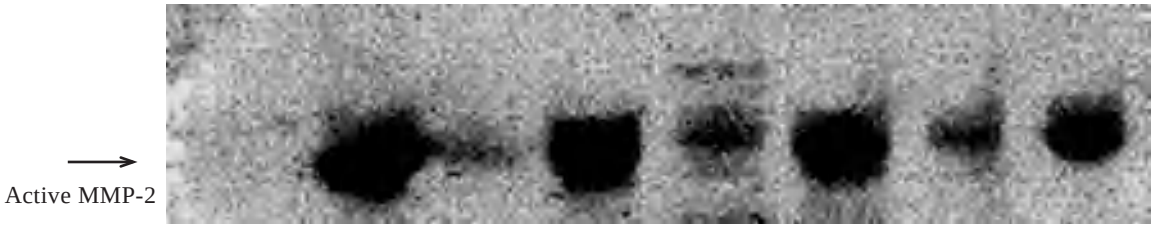

Fig. (2): Showed zymographic analysis of MMP2 in breast cancer patients.

Table (5): Serum levels of HGF in healthy controls and breast cancer patients.

Parameter	Healthy control (no=15)	Patients (no=34)	*p* value
HGF (pg/ml) Mean ± SD	409.67± 104.89	543.71± 103.03	**<0.001

***p* value <0.001 highly significant (2-tailed).

Table (6): Comparison between Serum HGF levels in patients and MT1-MMP and MMP2 expression.

Parameter	MMP2 activity		MT1-MMP expression	
	+ve (no=21)	–ve (no=13)	+ve (no=27)	–ve (no=7)
HGF (pg/ml) Mean ± SD	602.95± 79.48	448± 50.99	560.74± 103.2	478± 76.7
p: value	** 0.001		* 0.07	

**p*-value >0.05 not significant.
***p*-value <0.001 highly significant.

DISCUSSION

MT1-MMP is a major MMP because, it has been thought to be exclusively involved in the breakdown of the ECM components including collagens and laminin-5 **[19]**, and in the activation pathway of soluble MMPs, i.e. MMP-2 and MMP-13 **[20]**. The precise localization of MT1-MMP between cancer cells and surrounding stromal cells has been the subject of controversy. MT1-MMP was originally identified as an activator of pro MMP2 on the surface of invasive tumor cells **[9]**. MMP2 is thought to be responsible for the degradation of the basement-membranes as it degrades type IV collagen. In our study, by using western blot analysis, MT1-MMP was expressed in of breast cancer tissue of 79.4% of breast cancer patients and only in 26.4% of normal corresponding adjacent tissues (*p*:value=0.0001). Dalberg et al. **[21]** detected MT1-MMP mRNA expression in all invasive breast tumor biopsies investigated and found that it was mainly localized in the tumor cells. However, some authors stated that MT1-MMP is detectable in normal tissues, but the expression of this protease is strongly associated with aggressive, invasive malignant cells **[22]**.

MMP2 (gelatinase A) activity in this study was detected almost in all normal surrounding tissue of the breast tumors, however there was increase in the levels of its activity in breast cancer tissue in comparison with adjacent ones as 61.8% of the tumor cases showed high activity of MMP-2 while 38.2% showed the same activity of control correspondence (*p*-velue <0.01). similarly, Lee et al. **[23]** and Remacle et al. **[24]** were found by using zymography that breast cancers patients expressed higher levels of activated MMP-2 than benign lesions. The mechanism by which MMP-2 is regulated in normal tissue by malignant cells is likely to be important for the escape of neoplastic cells from tumor margins allowing invasion of adjacent normal tissues and possibly entry into the blood or lymphatic systems. However, it is difficult to imagine these mechanisms being involved in the infiltration of malignant cells at distance sites where a much more rapid regulation of MMPS is likely to be required **[25]**.

In this study a significant association between MT1-MMP expression and MMP-2 activity in tumor tissue was detected (*p*:value= 0.007). In consistence with our result Velasco et al. **[26]** and Murphy et al. **[27]** who showed that MT1-MMP may have the ability to activate pro-MMP2 through the association of an MTI-MMP with tissue inhibitor of matrix metalloproteinase-2 (TIMP-2) extracellulary, this complex then binding pro-MMP2. The MMP2 is then brought into close proximity with a second MT-1MMP, resulting in cleavage of MMP2 prodomain. Also Cox et al. **[28]** reported that the expression of gelatinase A can frequently be

superimposed on to that of MT1-MMP. This suggests that the transcription of these two genes may be coordinated and that MT1-MMP then activates progelatinase A allowing ECM degradation. Similarly Ratnikov et al. **[29]** stated that Activation of the latent MMP-2 zymogene involves its binding to the cell surface MT1-MMP*TIMP-2 (membrane type-1 matrix metalloproteinase/tissue inhibitor of matrix metalloproteinase-2) complex with subsequent cleavage of proMMP-2 by TIMP-2-free adjacent MT1-MMP. This is followed by autolytic maturation of the activation intermediate and the release of the mature MMP-2 species from cell surfaces into the extracellular milieu.

In our study, no association between MMP2 and MT1-MMP in normal breast tissue has been found. This resultes in agreement with Naw eroke et al. **[30]**, who stated that there was no association between gelatinase A and MT1-MMP in normal lung parenchyma, so the correlation of MT1-MMP with MMP2 activation in breast cancer tumor tissue again suggests that MT1-MMP is a tumor specific activator of proMMP2.

Clinical parameters such as tumor size, grade and lymph node status have long been used to characterize breast phenotypes in relation to prognosis. In the present study, we investigated MTI-MMP and MMP-2 for their prognostic significance. We found that MT1-MMP was expressed in 91.7% of positive lymph nodes cases, while expressed in 50% only of negative lymph nodes cases. (p:value=0.014). This significant association has previously mentioned by Yao et al. **[31]** who reported that MT-1 MMP protein expression in human breast cancers had positive correlation with lymph node metastasis. Mimori et al. **[32]** also determined that the highest expression of MT1-MMP mRNA was found in breast cancer specimens showing lymph-node metastasis and/or lymph-vessel invasion.

Meanwhile there was only a tendency of increased MMP2 activity in positive lymph nodes cases as it expressed in 66.7% of positive lymph node cases comparing to 50% in negative lymph node ones, but the difference was not statistically significant. In agreement with our result Têtu et al. **[33]** who did not find a significant association between MMP2 activity and lymph nodes metastasis in cancer breast patients.

The association of MT1-MMP but not MMP-2 expression with lymph nodes metastasis may be explained by the fact that MT1-MMP has many substrates other than MMP-2 **[21]**. However our study demonstrated no association between MT1-MMP or MMP2 and tumor size or tumor grade. In contrary to our result He-Cheng et al. **[34]** and Yao et al. **[31]** who found that, the expression of MMP2 and MT1-MMP correlated with tumor grade and tumor size, this discrepancy could be explained by the different method of detection used.

The serum levels of hepatocyte growth factor have already been shown to be of prognostic value in other malignancy **[35]**. Semi quantitative analysis by immunohistochemical staining of tumor specimen has several drawbacks; as it is sometimes not accurate enough to evaluate the intermediate patterns of staining although it is sufficient to differentiate negative versus positive reaction, also different results could due to possibility of heterogeneity within tumor specimens. So the choice of serum for a quantitative analysis in this study could possibly avoid the abovementioned disadvantages of a semiquantitative analysis by immunohistochemical staining.

The present study showed highly statistically significant elevated levels of serum HGF in cancer breast patients when compared with controls. (p-value<0.001). Our results agree with Sheen-Chen et al. **[36]** who reported that there was a highy significant increase in preoperative serum level of HGF in cancer breast patient in comparison with control. Also HGF is found to be involved in carcinogenesis, Jeffers et al. **[38]** reported that co transfection of HGF was able to induce morphologic transformation in vitro and tumorigenicity in vivo in a nontumorigenic mouse cell line C127.

The cut-off value of HGF was ≥430pg/ml, it detected breast cancer patients with sensitivity 91.2% and exclude normal control with specificity of 73.3% so, serum HGF could be used as a non invasive marker for diagnosis of breast cancer. On the other hand the present study showed that there no significant correlation between HGF serum level in cancer breast patients and lymph nodes status, tumor grade or tumor size. In disagreement with our result, Sheen-Chen et al. **[36]** who stated that the preoperative level of serum HGF may reflect the severity of invasive breast cancer.

Furthermore the results of the present study showed highly significant increase in the serum levels of HGF in cancer breast patients with positive MMP2 activity in their tumor tissue than those with negative MMP2 expression with *p*-value<0.001. So there was a strong positive association between MMP2 and HGF. Meanwhile there was a tendency of increased serum levels of HGF in cancer breast patients in cases with positive MT1-MMP expression than those with negative MT1-MMP expression but the difference did not reach a significant level (*p*:value=0.07). Previous reports have shown that PEA3/E1AF/ETV4 gene (which encodes an Ets-related transcription factor that is expressed in the epithelial cells of the mammary gland) can up-regulate promoter activities of many genes associated with tumorigenesis. A significant fraction of those encode matrix metalloproteinases (MMP genes) **[38,16]**. Also Motoaki Hanzawa et al. **[16]**, reported that the levels of MMPs mRNAs increased in cells treated with HGF and correlated with E1AF up regulation.

Conclusion:

- MT1-MMP can be used as prognostic factor predicts the possibility of breast cancer invasion and metastasis, so the enzyme may be used a therapeutic target in breast cancer.
- The significant association between MT1-MMP protein expression and MMP2 activity in tumor tissue confirms the previous results that MT1-MMP is the tumor-specific activator of proMMP-2.
- Serum HGF may be used as diagnostic marker for breast cancer and the highly significant association between it and MMP2 activity reflects and confirms the regulatory role of HGF on MMPs genes.
- Follow-up of cases and further study with larger number of patients may be of great value to achieve more substantial conclusion and confirms the usage of the three parameters which investigated as a diagnostic or prognostic markers.

Acknowledgment:

The authors acknowledg Dr. Amany A. El basmy. (Department of biostatistics, Cancer epidemiology), National Cancer Institute, Cairo University for their help.

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