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CONTENTS

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The Detection of Transfusion Transmitted Virus (TTV) by Polymerase Chain Reaction Among Cancer Patients and Apparently Healthy Individuals

LOBNA M. ABD EL-MEGUID, M.D.*; MOHAMMED T. MANSOUR, M.D.**; MOHAMMED M. HAFEZ, M.D.** and AZZA A.F. AHMED, M.D.***

The Departments of Clinical Pathology, Cancer Biology**, N.C.I., Cairo University and Faculty of Science***, Helwan University.*

ABSTRACT

Background: TTV was first isolated in 1997 from patients with hepatic failure, despite many attempts, no clear evidence has been found to define TTV as a causative agent in disease. The high prevalence of TTV in healthy individuals suggests that most infections have no pathogenic importance; thus, it was proposed that certain genotypes might be more pathogenic than others or that TTV might play a role in accelerating the effects of other infectious agents. According to the published reports on this newly characterized virus, it is evident that TTV is prevalent in several countries of the world. As such, it is not involved in causation of a serious problem in the body and simply acts as a bystander without much impact of its single or co-infection with other viruses. Attempts are still going on to find out exact clinical implications of TTV infection. Much is already known about the molecular biology of the virus, yet there still remains a need to develop simple techniques based on molecular and immunodiagnostics to diagnose TTV infection.

The Aim: Of this study was to investigate the incidence and genotyping of the TT Virus infection among cancer patients and apparently healthy individuals.

Materials and Methods: This study included 200 cases divided into two groups, control group which included 100 apparently healthy individuals, and the patients group which included 100 patients.

Results: Of this study showed the prevalence of TTV DNA in the sera of normal healthy persons and patients. The incidence of TTV DNA in patients group was 43%. While incidence of TTV genotype 1a was 16%. The incidence of TTV genotype 1b was 20%. The incidence of TTV non-genotype 1a & 1b was 16%. In controls group the incidence of TTV DNA was 44%. The incidence of TTV genotype 1a was 25%. While incidence of TTV genotype 1b was 61%. The incidence of TTV non-genotype 1a & 1b was 13% with no difference between the two groups.

Conclusion: High incidence of TTV infection was found among cancer patients and healthy individuals in Egypt with no difference between the two groups. TTV infection is widely spread among all type of cancer patients due to damage of their immune system and exposure to risk factors for infection but there was no relation between the type of cancer and infection with TTV. High incidence of genotype G1 (1a and 1b) among patients and controls in addition to low incidence of non 1a non 1b genotypes. Co-infection of TTV with HCV and HBV in patient and control groups were found, but TTV infection may have no effect on HCV and HBV infection. TTV infection is symptom-free, and found in normal persons and patients with different types of cancer, without any symptoms or signs. From that we conclude that TTV may be a part of the normal flora in human body. More studies are needed and also follow-up of TTV infection in patients and controls.

Key Words: *Transfusion transmitted virus (TTV) – HCV – HBV – Cancer patients – PCR.*

INTRODUCTION

Transfusion transmitted virus (TTV) is a recently discovered virus, which was suspected to be a causative agent of non-A to non-E hepatitis. TTV was first identified in the serum of a patient who was hospitalized with post transfusion hepatitis of unknown etiology in 1997. Initially, TTV was described as a non-enveloped, 3739 bases long and single stranded DNA virus, based on its genomic characteristics **[1]**.

Cloning of TTV provide the nucleotide sequences that allowed the development of methods for its detection by PCR. The original PCR method used primers from the N22 region (a part of the gene encoding the TTV structural protein) to investigate cases of post-transfusion

hepatitis and other liver diseases of unexplained etiology. The pathological role of this virus in liver disease is generally small or does not exist. High detection rates of this virus have been found in serum of healthy children **[2]**.

Hino and Miyata in 2007 reported a second virus distantly related to TTV and provisionally described as TTV-like Minivirus (TLMV) which was accidentally discovered by PCR with TTV primers that partially matched homologus sequences in TLMV. Even less is known about disease associations and epidemiology of TLMV, but it has been established that infection in human is common and that it shows genetic heterogeneity comparable to or even greater than that of TTV. TLMV was found to be with approximately 209 Kb genome **[1]**. Some TTV subtypes have less than 50% sequence identity. However, there are certain conserved regions. Primers were designed in such a way that most of the subtypes could be detected. Recently, real time PCR based methods with either SYBR Green or TaqMan Probe, designed to quantitate selectively TTV and TLMV, have been used.

The development of sensitive and reliable polymerase chain reaction (PCR) protocols allowed the detection of TTV DNA at a very high prevalence in sera of healthy populations around the world. Currently, the heteroduplex mobility assay to detect multiple infections with isolates of TTV belonging to different genotypes or subtypes has also been developed. In the simplest application of heteroduplex mobility assay, heteroduplexes are formed by denaturing and reannealing mixtures of PCR amplified DNA fragments from divergent isolates of the same virus. When these products are separated on polyacrylamide gels; a homoduplex band plus two slow moving heteroduplex bands are observed **[3]**.

MATERIAL AND METHODS

This study was performed on 200 cases which are divided into two groups, control group which included 100 apparently healthy individuals, and the patients group which included 100 patients, attending the out patient clinics of the medical and surgical oncology departments at the Egyptian national Cancer Institute, Cairo University, during the period form May 2004 to December 2005, all cases were subjected to the following:

Sample collection:

Samples were collected in 10 ml sterile tubes, centrifuged within half an hour of collection using a low speed centrifuge and separated to avoid hemolysis. Serum was divided into three 0.5 ml aliquots and immediately stored at -70°C under complete sterile conditions.

I- *Clinical assessment:*

Full history of cancer patients (including history of jaundice, blood and blood products transfusion and surgical intervention) was collected from patients' files and from apparently healthy individuals.

II- *Laboratory investigations:*

All patients and apparently healthy individuals were subjected to the following:

A- *Serological tests for viruses:*

1- Detection of hepatitis B surface antigen by ELISA technique; kits supplied by dialab system.

2- Detection of hepatitis C virus antibodies by ELISA technique; kits supplied by diasorin system.

B- *Molecular biology technique:*

Nucleic acid extraction from serum: As described by Boom et al., 1990 **[4]**.

1- Detection of hepatitis C virus RNA by polymerase Chain Reaction (PCR) for all samples (whether positive or negative for HCV-Ab). HCV-RNA can be reverse transcribed into cDNA then amplified using PCR technique (RT-PCR). PCR is a cDNA amplification technique in which template cDNA is amplified using a thermal cycler with three different temperature steps: Denaturation, primer annealing and primer extension. The one-step RT-PCR was performed in a 50ul volume containing 1x buffer containing (50mM. Tris-HCl (pH 8.3), 20mM. KCl, 0.2mM. $MgCl_2$, 0.2ml. (each) dNTPs (Sigma), 100ng RB-6A (Sense primer 5' GTG AGG AAC TAC TGT CTT CAC G 3' [nt 47 to 68]), 100ng RB-6B (antisense primer 5' ACT CGC AAG CAC CCT ATC AGG 3' [nt 292 to 312]) and 10ul of RNA **[4]**.

2- Detection of hepatitis B virus DNA for HBsAg positive samples using PCR technique. PCR amplification was performed using a published oligonucleotide primer Set selected from the highly conserved HBV core gene. 10µl of DNA was added to 90µl of reaction mixture containing 2.5 unit Taq polymerase, 100 ng of each primer, 0.2 mM. of dNTps, 1x PCR- buffer of reaction buffer (50 mM. KCl, 10 mM. Tris-HCl, PH = 8.3) and 1.5 mM. $MgCl_2$. Samples were denatured for 5 minutes at 95ºC and then subjected to 40 cycles of 1 min. at 95ºC, 1 min. at 55ºC and 2 min. 72ºC in an Eppendorf thermal cycler (Master cycler 5330 Germany) **[4]**.

3- Detection of TTV virus DNA by PCR. TTV DNA was amplified using nested PCR which used two set of primers; one set external and the other internal. All experiments included TTV positive control and negative control. Four primers sequance were used. for the 1st round PCR, 1st primers pair used were (T45as, 5' GAA GAT AAA GGC CTT ATG GCG 3') antisence (T1 5' AGT GCA CTT CCG AAT GGC TG 3'). For the nested PCR internal primers pairs used were (T2 5' -GAG TTTT CCA CG CCC GTCCG 3') and (T46as 5' GTCTGG CCCC ACT CAC TTT CG 3'). The 1st PCR round were performed in a 50µl reaction volume containing 10µl of nucleic acid, 2.5U recombinant Taq DNA polymerase (promiga, Madison, WI), 0.2 mM. of deoxyribonuclease triphosphates, 100ng of each primer pairs, 1.5 mM. $MgCL_2$, 1x buffer, The PCR was performed in 37 cycles as follow: 1 cycle at 95°C for 5 minutes, 1 cycle at 72°C for 5 minutes, 35 cycles each consisting of 30 sec. at 95°C (denaturation) and 45 sec. at 55°C (annealing) and 1 minute at 72°C (extension); 2µl of PCR products were subjected to an additional 37 cycles of amplification with the nested set of primers by using the same ingredients and condition as described above except that the volume of water was increased to 36.5µL **[5]**.

4- Identification of TTV genotype 1a and 1b using restriction enzymes. Positive samples for TTV infection obtained were used to study genotyping of TTV by using restriction enzymes. The genotyping study was performed in the following steps: The PCR products of the primer T1/T45 was subjected to another PCR round with a set of primers designed to be specific for genotype G1, the primer sequence was (T6 5'- AGCTCC CAC GCT GCT ATGT -3') anti sense (T11 5'- CGT CTA GCA GGT CTG CGT CT -3'). The PCR products then digested using restriction enzymes (Mbo1 and Ban1) to discriminate between the two subtypes of G1 (1a and 1b). the digestion performed by adding 10µL of PCR products, 30µL buffer and 1µL of restriction enzymes then incubate at 37°C for 1hr, then at 70°C for 10 minute, the products were visualized as follows: 5µl loading buffer (blue/orange loading dye, promega) was added to the digested products and mixed, then analysed by electrophoresis in 2% agarose gel, DNA species were visualized under UV light after staining with ethidium bromide **[6]**.

RESULTS

This study included 200 cases which are divided into two groups, control group included 100 apparently healthy individuals, patients' group included 100 patients, divided into two subgroups:

Subgroup I: 50 cases with solid tumors.

According to the data obtained from patients' files 16 patients with breast cancer, 15 patients with bladder cancer, 4 patients with rectum cancer, 3 patients with colon cancer, 13 patients with HCC cancer and only one case with abdominal mass.

Subgroup II: 50 cases with hematological tumors.

9 patients with AML, 9 patients with CML, 8 patients with ALL, 10 patients with CLL and 12 patients with NHL.

In patients' group, male to female ratio was 1.07:1 and their ages ranged from 35 to 75 with a mean of 55 years. Distribution of blood transfusion was 21% and surgical intervention was 15%. In control group male to female ratio 1.6:1 and their ages ranged from 21 to 49 years with mean of 35 years. A highly significant difference between patients' and control group in blood transfusion and surgical intervention was detected.

In the patients group 43/100 (43%) cases were positive for TTV DNA. While in the control group 44/100 (44%) individuals were positive for TTV DNA, Fig. (1).

Table (1): Clinical history of patients and control group.

	Patient (NO.100)	Control (NO.100)	Total	*p*-value
Blood transfusion	21 (21%)	0	21	0.000*
Surgical intervention	15 (15%)	0	15	0.000*
Mean age	55±20	35±14	—	—
Male	52 (52%)	62 (62%)	114	0.153
Female	48 (48%)	38 (38%)	86	0.153

* Significant *p*-value

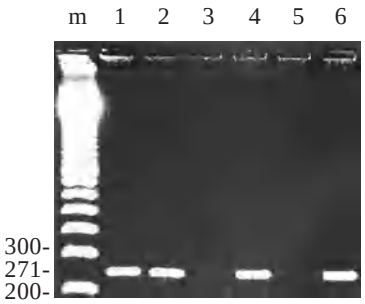

Fig. (1): Detection of TTV-CNA by PCR.
Lanes 1,2,4,6 represent positive samples.
Lanes 3,5 represent negativ samples.

TTV DNA was detected in 40.38% of solid tumor cases (21/50). The incidence of TTV DNA was 31.25% (7/14) in breast cancer cases, 46.6% (7/14) in bladder cancer cases, 50% (2/4) of rectum cancer cases, 33% (1/3) of colon cancer, 41.6% (5/12) in HCC cases and the patient with abdominal mass was found to be positive for TTV infection. TTV DNA was detected in 44% of hematological malignancies (22/50). The incidence of TTV DNA was 33.3% of AML cases (3/9), 66.6% of CML cases (6/9), 62.5% of ALL cases (5/8), 41.6% of CLL cases (5/12), 25% of NHL cases (3/12).

Regarding Hepatitis viral markers; in the solid tumor subgroup: 12/50 (24%) cases were positive for HCV-Ab with a significant *p*-value when compared with the control group. While 7/50 (14%) cases were positive for HCV-RNA; 1/50 (2%) cases were positive for HBs-Ag while 2/50 (4%) cases were positive for HBV-DNA. In the Haematological malignancies subgroup: 12/50 (24%) cases were positive for HCV-Ab while 9/50 (18%) cases were positive for HCV-RNA; 5/50 (10%) cases were positive for HBs-Ag while 5/50 (10%) cases were positive for HBV-DNA.

The solid tumor subgroup included 3/50 (6%) cases with history of blood transfusion and 14/50 (28%) with history of surgical intervention. In the Haematological malignancies subgroup: 18/50 (36%) cases with history of blood transfusion and 1/50 (2%) with history of surgical intervention. A highly statistically significant difference was detected between the solid tumor subgroup and the Hematological malignancies subgroup in blood transfusion and surgical intervention.

By studying the genotyping in our study groups; we found that: In the patients group 43/100 (43%) cases were positive for TTV DNA; genotype 1a was detected in 16/43 cases (37%), genotype 1b was detected in 20/43 cases (47%) and non genotype 1a or 1b was detected in 7/43 cases (16%). While in the control group 44/100 (44%) individuals were positive for TTV DNA; genotype 1a was detected in 11/44 cases (25%), genotype 1b was detected in 27/44 cases (61%) and non genotype 1a or 1b was detected in 6/44 cases (13%).

Table (2): The incidence of TTV DNA among patients' subgroups.

	No. of cases	No. of TTV positive cases	Percent of TTV positive cases
Breast cancer	16	5	31.25%
Bladder cancer	14	7	50%
Rectum cancer	4	2	50%
Colon cancer	3	1	33%
HCC	12	5	41.6%
Abdominal mass	1	1	100%
Total	50	21	40.38%
AML	9	3	33.3%
CML	9	6	66.6%
ALL	8	5	62.5%
CLL	12	5	41.6%
NHL	12	3	25%
Total	50	22	44%

Table (3): TTV and viral hepatitis co-infection among patients (solid and hematological tumour subgroups) and control groups.

	Positive blood transfusion	Surgical intervention	Positive HCV-RNA	Positive HCV-Ab.	Positive HBV-DNA	Positive HBs-Ag.	TTV DNA
Solid tumor (no. 50)	3 (6.0%)	14 (28%)	7 (14%)	12 (24%)	2 (4.0%)	1 (2.0%)	21 (40.38%)
Hematological tumor (no. 50)	18 (36.0%)	1 (2.0%)	9 (18%)	12 (24%)	5 (10.0%)	5 (10.0%)	22 (44%)
Total	21	15	16	24	7	6	43 (43%)
p-value	0.000*	0.000*	0.585	1.0	0.436	0.204	
Control (no. 100)	NA	NA	9 (9%)	9 (9%)	4 (4%)	4 (4%)	44 (44%)
p-value			0.134	0.004*	0.352	0.516	0.887

* Significant *p*-value NA: Not applicable

Table (4): Prevalence of TTV infection and its genotypes in patients and control groups.

	TTV DNA	Genotype 1a	Genotype 1b	Non 1a and 1b genotype
Patient (no. 100)	43 (43%)	16 (37%)	20 (47%)	7 (16%)
Control (no. 100)	44 (44%)	11 (25%)	27 (61%)	6 (13%)
p-value	0.887	0.191	0.201	0.812

DISCUSSION

Transfusion transmitted virus (TTV) is a recently discovered virus, which was suspected to be a causative agent of non-A to non-E hepatitis. TTV was first identified in the serum of a patient who was hospitalized with post transfusion hepatitis of unknown etiology in 1997. Initially, TTV was described as a non-enveloped, 3739 bases long and single stranded DNA virus, based on its genomic characteristics **[1,7]**.

The aim of this study is to investigate the incidence and genotyping of the TTV infection among cancer patients and apparently healthy individuals. The incidence of TTV DNA in the sera of normal healthy individuals and blood donors was found to vary widely in different countries **[8,9,10]**. In the current study the incidence of TTV DNA in healthy individuals (control group) was 44% of all cases. Our results were in agreement with other study carried in Egypt. The prevalence of TTV DNA in blood donors was 35.5% **[11]**.

Our results were in disagreement with other studies which found that the prevalence of TTV DNA in healthy individuals in Eygpt was 9%, **[12]**, in Western population was 13% **[13,14]**, in Asian countries as Japan 94% **[1,6,15]**, in Thailand 62%, Koria 53% **[16]**, in national and non national United Arab Emirates 40% and 89% respectively, in Saudi arabia, using primers derived from ORFI and 5UTR, TTV DNA was detected in 5.5% and 50.5% respectively in blood donors **[17]**.

The most probable explanation for the variation in the incidence of TTV DNA among healthy individuals and blood donors, that it may be due to the difference in the geographic area, the level of health care and the primers sets.

The high prevalence of TTV in general population, may reflect its high incidence in patients and may complicate linking TTV to any pathologic states. This unusual feature among viruses aroused the proposal that TTV might be a commensal virus or part of human microflora **[2]**.

A significant difference was found between control group and cancer patient in blood transfusion and surgical intervention; this result is in agreement with other authors' findings **[18, 19]**.

Although TTV was known to be transmitted mainly by blood transfusion; from our results we found high incidence of TTV in healthy individuals, all of them have no history of blood transfusion. So the relatively high prevalence of TTV in healthy individuals and patients with no history of transfusion of blood and blood

products led to the suggestion that alternative routes of transmission of TTV infection may exist. TTV DNA has also been detected in saliva, throat swabs, breast milk, semen and vaginal fluid thus, supporting routes of transmission other than blood and blood products **[5,20,21]**. In addition the children of TTV-infected mothers apparently tend to get infected more often and earlier after birth than children of TTV negative mothers, the role of postnatal transmission of TTV is being considered. Postnatal route of transmission from mother to child and infection via frequent social contacts seem to be very important modes of transmission in children. **[22,23,24]**. We could conclude that TTV is not only parenterally transmitted. This result was in agreement with another author who reported that following implementation of viral inactivation methods in the process of clotting factors concentrates production, the risk for HCV transmission was significantly reduced and to less extent for TTV DNA **[25]**. This conclusion was supported also by other researches that prove that TTV is also transmitted by faeco-oral route, saliva, breast milk and transplacentally **[26]**.

In the current study, TTV infection was detected in different types of cancer patients with no significant difference from its prevalence among the control group. Recently, Camci et al. has reported the high prevalence of TTV in patients with various malignancies. The viral load in cancer patients was extremely high. It might result from the impaired immune reaction. Further studies are needed to explain whether the impairment is caused by the neoplasm or the virus itself **[1,18,27]**.

Our results revealed that TTV is frequently detected in patients with other types of viral infection. TTV co-infection was noted with HCV and HBV in patient and control groups. We found that TTV co-infection had no effect on HCV and HBV infection; this was in agreement with other authors **[11,28]**. TTV did not change the results of chronic hepatitis B therapy with lamivudine and TTV genome was not integrated into the host hepatocyte DNA; which is probably necessary to initiate potential neoplasm development **[29]**.

Though the role of TTV on the cancer patients is not clear. The high incidence of genotype G1 (1a and 1b) among patients and controls in addition to low incidence of non 1a non 1b genotypes may suggested G1 as a candidate for pathogenicity of TTV strains in Egypt. Co-infection of TTV with HCV and HBV in patient and control groups were found, but TTV infection may had no effect on HCV and HBV infection. TTV infection is symptom-free, and found in normal persons and patients with different types of disease not only cancer, without any effects. From that we concluded that TTV may be a part of the normal flora in human body. More studies are needed and also follow-up of TTV infection in patient and controls.

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Hemostatic Derangements in Egyptian Patients with ß-Thalassemia Major

RAGIA H. BADAWY, M.D.*; NADIA A. SADEK, M.D.* and MAHMOUD S. ELIWA, Ph.D.,MRPCPH**

The Department of Hematology , Medical Research Institute, Alexandria University and National Research Center, Cairo**.*

ABSTRACT

Background: Profound hemostatic changes have been observed in patients with ß-thalassemia major, which make it a high risk condition for development of thromboembolic events.

Aim: The present study aimed at evaluating coagulation parameters in Egyptian patients with ß-thalassemia major. Forty patients with ß-thalassemia major (Group I: 20 nonsplenectomised and Group II: 20 splenectomised) were included in the study. The study also included 16 age and sex matched healthy children as control. Hematological parameters as well as iron status and liver functions were analyzed. Coagulation was assessed by standard clotting tests; prothrombin time (PT) and activated partial thromboplastin time (APTT). The activity levels of anticoagulant proteins including protein C, protein S and antithrombin III (AT III) were also determined. In addition prothrombin fragment 1+2 (F1+2), a marker of thrombin generation was measured.

Results: Severe microcytic hypochromic anemia was present in all cases (mean Hb concentration was 6.66±1.36 and 7.19±0.19gm/dL and mean MCV was 66.42±6.26 and 68.54±6.71fL in group I and II respectively). They also suffered iron overload as both serum iron and serum ferritin were significantly higher in patients than control. PT and APTT were significantly prolonged ($p<0.001$). Protein C, protein S and antithrombin III were all significantly lower than the control ($p<0.001$) and they negatively correlated with serum ferritin in group I ($r=-0.634$, -0.616, -0.612 respectively and $p<0.01$) and in group II ($r=-0.673$, -0.666, -0.676 respectively and $p<0.001$). The level of F1+2 was significantly higher in splenectomised group than nonsplenectomised and the control ($p<0.001$).

In conclusion significant alterations in anticoagulant proteins and F1+2 exist in patients with ß-thalassemia major. Close monitoring and assessment of these parameters in thalassemic patients is recommended so that effective measures to control thromboembolic episodes can be implemented.

Key Words: *Hemostatic derangement – F1+2 – ß thalassemia major.*

INTRODUCTION

The homozygous ß-globin chain mutation in the adult hemoglobin (Hb A) gene gives rise to ß thalassemia major. In this genetic disorder, red blood cells have a short life span due to excess accumulation of the unpaired α-globin chain **[1]**. Possessing unstable molecular configuration, α-globin chains aggregate and precipitate in early hemoglobin producing cells in the bone marrow. This leads to apoptosis of these cells and ineffective erythropoiesis. The red cells that reach the peripheral blood also contain excess α-globin chains. This results in membrane damage and hemolysis of these cells preferentially with development of severe anemia **[2]**.

ß-thalassemia major is treated with regular blood transfusion and concomitant administration of chelating agents to reduce blood transfusion induced hemochromatosis **[3]**. Adequate chelation of such patients reduces iron accumulation and prevents organ damage, resulting in a consistent decrease of morbidity and mortality **[4]**. Survival of patients with ß-thalassemia major has improved by the development of comprehensive thalassemia care services. With continued improvement in survival, a number of late adverse effects has become increasingly apparent. The effects on heart, liver and endocrine system have been known for over four decades but focus on hemostatic derangement is relatively recent **[5]**.

Profound hemostatic changes have been observed in patients with ß-thalassemia major. The presence of higher than normal incidence of thrombotic anomalies in the majority of

patients, has led to the recognition of the existence of chronic hypercoagulable state in ß-thalassemia major **[6]**. The commonly seen clinical manifestations are transient ischemic attacks **[7]**, stroke **[8]**, deep vein thrombosis **[9]**, recurrent pulmonary hypertension **[10]** and myocardial infarction **[11]**. Similarly, autopsy findings in patients with ß-thalassemia major have clearly demonstrated hypercoagulability as a pathologic feature. Multiple microthrombi in the pulmonary arterioles, composed mainly of platelets were found in autopsies performed on thalassemic patients **[12]**.

Thrombotic events are associated with increased activity of the coagulation system and the generation of thrombin **[13]**. Thrombin itself is impossible to quantify because it lasts only seconds in the circulation. Thus, it is necessary to use a surrogate marker for thrombin generation. Prothrombin fragment 1+2 is an activation peptide which is generated during conversion of prothrombin to thrombin in blood coagulation **[14]**. Thus, it can be used as a biomarker for thrombin generation during blood coagulation and has the diagnostic potential for assessing thrombotic risk and monitoring anticoagulant therapy **[15]**. Studies of the coagulation proteins provide strong evidence for the existence of a chronic hypercoagulable state in ß-thalassemia major **[16]**. Profound changes in prothrombin time (PT), partial thromboplastin time (APTT), natural anticoagulants like protein C, protein S and Anti-thrombin III (AT III) have been described though the mechanisms involved in the thrombotic tendency seen in some patients have not been fully elucidated **[5]**.

ß-thalassemia major; the most common genetic disorder in Egypt, is a major health problem with an estimated carrier rate of 7% **[17]**. This study was therefore, planned with the objectives of evaluating the hemostatic derangements in Egyptian patients with ß-thalassemia major. So that, effective measures to control thromboembolic episodes can be implemented.

PATIENTS AND METHODS

This study was carried out on 40 patients with ß-thalassemia major. They were followed at the Hematology Department, Medical Research Institute, Alexandria. Their ages ranged from 8 to 14 years. The diagnosis of ß-thalassemia major was carried out by clinical signs, complete blood count and hemoglobin electrophoresis. The patients received regular blood transfusions and were under chelation therapy with Desferrioxamine (DFO).

Patients were divided into two groups; Group I: Which included 20 non splenectomised patients, 10 males and 10 females, their mean age was 10.80 years±1.10 and their median age was 10 years and Group II: Which included 20 splenectomised patients 8 males and 12 females, their mean age was 9.90 years±1.77 and their median age was 9 years. Our study also included 16 healthy normal children with matched age and sex as a control group. They were 9 males and 7 females, their ages ranged from 8 to 13 years with a mean of 10.13±1.96 and a median of 10 years.

This study was approved by the institutional review board and followed the Helsinki Declaration on human experimentation. Informed consent of the parents of all children was taken before starting the work.

All thalassemic children were subjected to the following:

• Careful history taking.

• Thorough clinical examination.

Blood samples were collected from thalassemic patients immediately before blood transfusion and from the healthy children.

• Two ml. of whole blood were collected on EDTA tube for complete blood picture and reticulocyte count **[18]**.

• Two ml. of whole blood without anticoagulant were collected for determination of AST (SGOT), ALT (SGPT), serum iron and serum ferritin.

• All assays for coagulation were carried out on blood collected in sodium citrate at a final concentration of 3.8% (w/v) and the ratio of anticoagulant to blood was 1:9 (v/v). Platelet poor plasma was prepared for all coagulation tests by centrifugation at 2000g for 15 minutes, it was kept at room temperature for all coagulation tests and kept at -70ºC until prothrombin fragment 1+2 was determined.

* Prothrombin time (PT) was estimated by Quick one stage method using calcium thromboplastin (thromborel-S) from Behringwerke, Marburg, Germany. Activated partial thromboplastin time (APTT) was estimated using C.K. prest kit supplied by Diagnostica Stago (France). PT and APTT were done using standard laboratory methods **[19]**.
* Protein C activity levels in plasma were determined by the synthetic chromogenic substrate method using Stachrom Protein C kit from Diagnostica Stago (France).
* Protein S and antithrombin III (AT III) quantitative determination was done by the immune-turbidimetric method using Liatest Protein S and Liatest AT III kits respectively, the kits were supplied by Diagnostica Stago (France).
* The levels of prothrombin fragment 1+2 (F1+2) were determined by ELISA **[20]** using Enzygnost F1+2 micro® from Dade Behring (Germany).
* Serum iron was quantitatively determined by colorimetry using Spinreact kit (Spain). Serum ferritin estimation was done by ELISA kit from Bioplus, San Fransisco, USA.

Protein C <70%, Protein S <70%, and AT III <80% were taken as low values.

Statistical analysis:

Statistical analysis was done using SPSS (version 10.0 for windows). Data were expressed as mean and standard deviation and were subjected to the Kolmogorov-Smirnov test to determine the distribution and method of analysis. As most of the data were normally distributed continuous variables, student's *t* test was used for numeric values. Pearson's correlation coefficient (*r*) was used to test the correlation between the continuous variables. *p* values <0.05 and <0.001 were considered significant and highly significant respectively.

RESULTS

The clinical data of nonsplenectomised and splenectomised groups of ß-thalassemia are summarized in Table (1). In group I, there was splenomegaly in 14 patients (70%), hepatomegaly in 12 (60%), pulmonary hypertension in 2 (10%). Right heart failure and leg ulcers were not detected. On the other hand, in group II, 15 patients (75%) had hepatomegaly, 3 (15%) had pulmonary hypertension, 2 (10%) had right heart failure and 2 (10%) had leg ulcers.

Table (2) summarizes the hematological parameters in both patient groups and control group. The red blood cell count (RBC), hemoglobin concentration (Hb), and cell indices including mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCH) were all markedly decreased in both patient groups when compared to the control indicating severe microcytic hypochromic anemia ($p<0.001$). However, the differences between group I and group II were not statistically significant. On the other hand, the two patient groups had higher reticulocyte percentage than the control group and the differences were statistically significant ($p<0.001$). Moreover, group II had higher normoblast percentage than group I and the difference was statistically significant ($p<0.001$). Regarding the platelet count, group II had statistically significant higher value than both; group I and the control ($p<0.001$).

Table (3) shows the iron status in the study groups and the controls. Both patient groups had statistically significant higher serum iron and serum ferritin levels than the control group ($p<0.001$), however the difference between the two patient groups was not statistically significant. Both levels of AST and ALT in the two patient groups were significantly higher than the control group ($p<0.001$) as shown in Table (4).

Table (5) illustrates the coagulation parameters in both groups of ß-thalassemia major and the control group and Table (6) shows the proportion of patients with abnormal results. PT and APTT were prolonged in both patient groups when compared to the control group and the differences were statistically significant ($p<0.001$). However, the difference between group I and group II was not statistically significant in PT while group II had more prolonged APTT than group I and the difference was statistically significant ($p<0.01$). The levels of naturally occurring anticoagulants namely; protein C, protein S and AT III were significantly lower in thalassemic patients whether splenectomised or not, than the control group ($p<0.001$), though both patient groups did not differ significantly from each other. There was significant

negative correlation between serum ferritin and the studied coagulation inhibitors; protein C, protein S and AT III in group I (r=-0.634, -0.616, -0.612 respectively and p<0.01) and in group II (r=-0.673, -0.666, -0.676 respectively and p<0.001). The level of F1+2 was significantly higher in group II than group I and the control group (p<0.001) (Fig. 1). However, group I had higher mean level of F1+2 than the control group but the difference did not reach statistical significance.

Table (1): Clinical findings in thalassemic patients.

Parameters	Group I n=20		Group II n=20	
	No.	%	No.	%
Splenomegaly	14	70	–	–
Hepatomegaly	12	60	15	75
Pulmonary hypertension	2	10	3	15
Right heart failure	0	0	2	10
Leg ulcers	0	0	2	10

Table (2): Hematological parameters in the patient groups and controls.

Parameters	Group I	Group II	Control
Hb g/dL	6.66±1.36 *p*a<0.001*	7.19±0.19 *p*a<0.001* *p*b>0.05	12.06±0.13
RBCsx10^{12}/L	2.88±0.64 *p*a<0.001*	3.15±0.4 *p*a<0.001* *p*b>0.05	4.43±0.29
MCV fL	66.42±6.26 *p*a<0.001*	68.54±6.71 *p*a<0.001* *p*b>0.05	86.12±4.05
MCH pg	23.38±2.29 *p*a<0.001*	22.94±3.28 *p*a<0.001* *p*b>0.05	27.41±1.41
Normoblasts/100 WBCs	9.25±3.35	19.05±4.35 *p*b<0.001*	
WBC x 10^9/L	8605±4090.36 *p*a>0.05	8500±2714.68 *p*a>0.05 *p*b>0.05	7625±1246.59
Platelet x 10^9/L	323.75±128.99 *p*a>0.05	419.30±100.30 *p*a<0.001* *p*b<0.01*	285±68.09
Reticulocyte%	4.23±1.07 *p*a<0.001*	4.8±0.89 *p*a<0.001* *p*b>0.05	0.93±0.23

*p*a: Compared to control. *p*b: Compared to group I. * Statistically significant.

Table (3): Iron status in thalassemic patients and controls.

Parameters	Group I	Group II	Control
Serum iron µg/dL	154.65±31.9 *p*a<0.001*	161.60±45.48 *p*a<0.001* *p*b>0.05	50.5±8.08
Serum ferritin ng/dL	1592.85±602.07 *p*a<0.001*	1546.65±565.83 *p*a<0.001* *p*b>0.05	34.25±8.81

*p*a: Compared to control. *p*b: Compared to group I. * Statistically significant.

Table (4): AST and ALT in thalassemic patients and controls.

Parameters	Group I	Group II	Control
AST U/L	52.10±29.36 *p*a<0.001*	59.20±23.62 *p*a<0.001* *p*b>0.05	16.20±9.08
ALT U/L	59.15±34.21 *p*a<0.001*	61.05±35.34 *p*a<0.001* *p*b>0.05	18.80±7.71

*p*a: Compared to control. *p*b: Compared to group I. * Statistically significant.

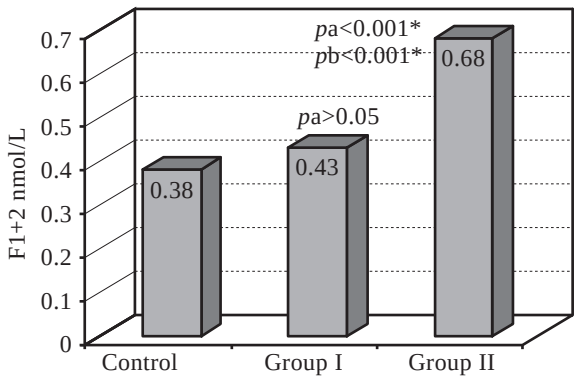
Table (5): Coagulation parameters in thalassemic patients and controls.

Parameters	Group I	Group II	Control
PT (seconds)	14.54±1.12 *p*a<0.001*	14.88±0.92 *p*a<0.001* *p*b>0.05	13.06±7.37
INR	1.23±0.18 *p*a<0.001*	1.29±0.15 *p*a<0.001* *p*b>0.05	1.0
APTT (seconds)	45.04±9.52 *p*a<0.001*	55.90±16.13 *p*a<0.001* *p*b<0.01*	32.37±2.58
F1+2 (nmol/L)	0.43±1.02 *p*a>0.05	0.68±0.22 *p*a<0.001* *p*b<0.001*	0.38±1.1
Protein C%	76.35±14.88 *p*a<0.001*	75.4±13.94 *p*a<0.001* *p*b>0.05	97.33±13.42
Protein S%	77.75±15.31 *p*a<0.001*	77.15±14.06 *p*a<0.001* *p*b>0.05	96.12±13.84
AT III%	79.35±13.83 *p*a<0.001*	80.75±14.22 *p*a<0.001* *p*b>0.05	101.44±7.06

*p*a: Compared to control. *p*b: Compared to group I. * Statistically significant.

Table (6): Proportion of patients with abnormal coagulation parameters in the two patient groups.

Parameters	Group I n=20		Group II n=20	
	No.	%	No.	%
APTT	9	45	15	75
Protein C	6	30	6	30
Protein S	5	25	7	35
AT III	6	30	5	25
F1+2	2	10	13	65



*p*a: Compared to control *p*b: Compared to group I
* Statistically significant

Fig. (1): Levels of F1+2 (nmol/L) in thalassemic patients and controls.

DISCUSSION

Patients with ß-thalassemia major are prone to develop thromboembolic complications. Zurlo et al. **[21]**, Michaeli et al. **[22]**, Borgna Pigantti et al. **[8]** and Eldor et al. **[9]** described thromboembolic complication in patients with ß-thalassemia major in previous reports. In the present study, 2 nonsplenectomised patients (10%) and 3 splenectomised (15%) complained of pulmonary hypertension, 2 splenectomised (10%) suffered from right heart failure and another 2 splenectomised (10%) had leg ulcers. Sonakul et al. **[10]** in an autopsy series found pulmonary artery obstruction in 44% of patients with ß-thalassemia major. On the contrary, Naithani et al. reported no thrombotic episodes in their patients **[5]**. Ibrahim has also studied 32 children with ß-thalassemia major and found no evidence of thrombotic manifestations. The plausible explanation for the lack of thromboembolic phenomenon in such studies could be the lower mean age of the studied groups when compared to patients evaluated by other investigators **[7]**. This might explain the lower incidence of thromboembolic events in our patients since their mean age was 10.80 years in nonsplenectomised and 9.90 years in splenectomised patients. Moreover, asymptomatic pulmonary vascular disease that could result from silent, recurrent thromboembolic events had been found in many patients with ß-thalassemia major. This was suggested by echocardiographic studies in 35 patients with ß-thalassemia major who had no clinical signs and symptoms of thromboembolic disease. Many of the patients showed pulmonary hypertension and right heart failure, which was more prevalent than left heart failure **[23]**. These findings suggest that the early right ventricular dysfunction, which precedes left heart failure in patients with ß-thalassemia, may be due to pulmonary hypertension secondary to microembolisation in the lungs and not from cardiomyopathy resulting from excessive iron deposition **[24]**.

In the present work, the mean PT and APTT were significantly prolonged than age-matched healthy children. This is in accordance with Naithani et al., who reported prolongation of the PT and APTT in their patients **[5]**. Parenchymatous liver damage or the circulating hemolysates can explain these effects. Ibrahim pointed that such prolongations could be due to a chronic activation of intrinsic coagulation and intravascular hemolysis **[7]**.

Andrew et al., investigated the reason for this contact activation and found a kallikrein like protease activity which could be released from tissues due to iron overload **[25]**. In keeping with iron overload, in the present study both levels of serum iron and ferittin were significantly higher in thalassemic patients than age-matched healthy children.

Low levels of the coagulation inhibitors, protein C, protein S, and AT III have been observed in patients with ß-thalassemia major from a variety of ethnic backgrounds **[9,16,26]**. Mussumeci et al., found low protein C activity in 94% and low AT III in 55% of cases but only 2 of their 74 patients had clinical thromboembolic manifestations **[27]**. Similarly, Ibrahim found low activity of protein C, protein S and AT III in 27,23 and 32% of patients, respectively but none had thromboembolic features **[23]**. In the present work low activity of protein C, protein S and AT III was found in 30,25, and 30% of cases respectively in group I and in 30,35 and 25% of patients respectively in group II. Furthermore, significant lower levels of the three parameters were observed in the patient groups when compared to age-matched healthy children. Inherited deficiencies of these factors are unlikely owing to such a high percentage of patients being deficient. Cappellini et al., explained that the deficiencies of these coagulation inhibitors may be due to a possible role of the liver dysfunction since protein C, protein S and AT III are very sensitive to mild degrees of impairment of the synthetic function of the liver **[26]**. This was evident in our study by the significant increase in liver enzymes. Moreover, severe iron overload can result in progressive organ failure **[6]**. In accordance with our results there was significant negative correlation between serum ferritin and the studied anticoagulant proteins; protein C. protein S and AT III in all patients whether splenectomised or not.

In the present study, F1+2; a marker for thrombin generation, was significantly higher in splenectomised patients than both non splenectomised patients and controls. Our results go in line with those of Opartkiattikul and his colleagues who found high level of F1+2 in splenectomised patients when compared to non-splenectomised patients and age-matched

healthy children **[28]**. On the contrary, Cappellini et al., reported normal levels of F1+2 in both splenectomised and non-splenectomised thalassemia major patients, however a much higher level of F1+2 was detected in his splenectomised patients with thalassemia intermedia when compared to non-splenectomised thalassemia intermedia patients and those with thalassemia major **[26]**. The reason for this difference might have been that thalassemia major in Italy undergo regular blood transfusion more than do our patients who suffered severe microcytic hypochromic anemia (mean Hb concentration in our study was 6.66 and 7.19gm/dL and mean MCV was 66.42 and 68.54 fL in group I and II respectively). This could be explained by the irregularity of blood transfusion in our patients due to lack of health education which makes the availability of matched blood for thalassemic patients a hindering problem. Atichartakran and his colleagues reported that smaller sized RBCs are more thrombogenic especially after splenectomy **[29]**. In addition Cappellini et al. postulated that thalassemic red cells and erythroid precursors present in the blood of splenectomised patients with ß-thalassemia would act as activated platelets and enhance the conversion of prothrombin to thrombin in the final stage of blood coagulation **[26]**. This is in agreement with our results as splenectomised patients had significantly higher percentage of normoblasts and F1+2 than non-splenectomised patients. Moreover, thalassemic red cells may provide a source of negatively charged phospholipids such as phosphatidyl serine which can increase thrombin generation **[30]**. Phosphatidyl serine exposed on the surface of thalassemic red cells functions as a signal for their recognition by phagocytes and their subsequent removal from the circulation and for the occurrence of apoptosis **[31]**. It is plausible that this phenomenon is more marked after removal of the spleen because splenectomy favors the persistence of these damaged red cells in the circulation **[26]**. These findings may explain that the incidence of thromboembolic events in our study was greater in splenectomised than non-splenectomised patients.

In conclusion, significant alterations in the hemostatic system exist in ß-thalassemia major particularly irregularly transfused patients, which make it a high risk condition for development of thromboembolic events. We suggest regular blood transfusion programs and close monitoring of thalassemic patients specially with increasing age so that effective measures to control thromboembolic episodes, can be implemented.

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Annexin V and Apoptosis in Peripheral Blood Lymphocytes of Children with Down's Syndrome

SOLAF M. EL-SAYED, M.D. and GHADA M. EL-SAYED, M.D.*

Genetics Unit, Children Hospital, Ain Shams University and Clinical Pathology Department, National Cancer Institute, Cairo University.*

ABSTRACT

Down's syndrome (DS) is the most common and best-known chromosomal disorder and is associated with several other pathologic conditions including immunodeficiency which makes a significant contribution to morbidity and mortality. Various immunological theories and observations to explain the predisposition of individuals with DS to various infections have been published, one of which is increased apoptotic cells.

The aim of this study was to identify the phenotype of apoptotic immune cells (T and B lymphocytes) in children with DS using Annexin V staining of phosphatidyserine (PS) as a specific marker of early apoptosis.

The study included 17 children with karyotypically ascertained DS (7 males and 10 females). Their ages ranged from 4 months to 14 years with mean age of 5.7± 4.35 and a median of 7 years. Seventeen age matched healthy children were included in the study as controls. Complete blood picture, relative and absolute number of CD3+ (T-cells) and CD19+ (B-cells) and analysis of apoptosis using Annexin V were done to all children included in this study using flow cytometry.

Complete blood picture, relative and absolute number of CD3 and CD19 did not show significant differences between DS children and control group. However, the relative and the absolute number of early apoptotic CD3 positive T lymphocytes were significantly higher (p=0.001) in DS children which may explain the functional impairment of these cells and the defect in adaptive immunity in theses patients. On the other hand, the relative number of early apoptotic CD19 positive B lymphocytes was significantly higher in DS children but no significant difference was detected when the absolute number was compared (p=0.022 and 0.286 respectively).

In conclusion, increased apoptotic CD3 cells may contribute to the impaired cellular immunity in DS children in spite of the normal absolute number of these cells. Further studies on apoptotic cellular phenotype in DS children and its correlation with the infection episodes in these children are recommended.

Key Words: *Annexin V – Apoptosis – Down's syndrome.*

INTRODUCTION

Down syndrome (DS) is the most common and best-known chromosomal disorder and is associated with several other pathologic conditions including immunodeficiency which makes a significant contribution to morbidity and mortality **[1]**.

Various immunological theories and observations to explain the predisposition of individuals with DS to various infections has been published **[2,3,4]**. Neutrophil and monocyte dysfunction is well documented in DS. Impaired function of chemotaxsis, phagocytosis, and oxidative response has been reported **[5]**. Additionally aspects of humoral immunity have been examined. Despite normal levels of circulating B-cells, immunoglobulin levels of DS may differ from normal values with tendency towards high immunoglobulin G (IgG) and low IgM serum levels **[6]**. Although the circulating numbers of cells bearing the T-cell marker CD3 are generally normal, the ratio of CD4 to CD8 cells is decreased in DS. In addition, the proportion of cells expressing the α, β chains of T-cell receptor (TCR) is decreased in peripheral blood (PB) of patients with DS and the proportion of γ, δ chains is increased. Another lymphocyte subset, natural killer (NK) cells, shows abnormalities in DS; circulating numbers of NK cells are increased while functional activity is impaired **[7]**.

On the other hand, DS patients show signs of precocious aging of various organs and tissues, one of which is the immune system and

according to some investigations, DS ranks first among human "segmental progeroid syndromes" defined as those genetic disorders in which multiple major aspects of the senescent phenotype appear **[8,9,10]**.

Apoptosis is a mechanism of programmed cell death, which plays an important role in a number of biological processes. Increased apoptosis has been suggested to be responsible for many aspects of DS pathologic condition; as it has been detected in cell lines obtained from neurons of patients with DS **[11]**. Also the percentage of apoptotic granulocytes from DS was found to be significantly higher than that from healthy subjects and this may contribute to the risk of infections in these patients **[12]**.

Apoptosis in the peripheral blood of Down syndrome have been studied before by means of electron microcopy, in situ nick translation (ISNT), and DNA electrophoresis **[13]**. However the previous methods do not identify the phenotype of apoptotic cells.

The aim of this study is to identify the phenotype of apoptotic immune cells (T and B lymphocytes) in children with DS using Annexin V staining of phosphatidyserine (PS) as a specific marker of early apoptosis.

During apoptosis, externalization of PS and phosphatidylethanolamine is a hallmark of the changes in the cell surface. These phospholipids are normally sequestered within the cell surface on the cytoplasmic side of the plasma membrane. This occurs relatively early just after segmentation of the nucleus during which the cell membrane remains intact **[14]**.

The permeability of the plasma membrane is a central difference between necrosis and apoptosis. Large molecular DNA binding dyes, such as propidium iodide (PI), can not enter intact cells because of their large size and without permebilization treatment, do not label apoptotic cells until the final lysis stage. Annexin V, a Ca^{2}- dependent phospholipids binding protein, which possesses high affinity for PS can be used specifically for detecting early apoptotic cells. When used with promidium iodide (PI), Annexin V staining allows the quantitation of cells at early stages of apoptosis and the simultaneous identification of cell surface markers **[15]**.

PATIENTS AND METHODS

The study included 17 children with karyotypically ascertained DS (7 males and 10 females) from the genetics clinic, Ain Shams University. Their ages ranged from 4 months to 14 years with mean age of 5.7±4.35 and a median of 7 years. Seventeen age matched healthy children were included in the study as controls. The following was done to all children included in this study:

1- *Complete blood count and immunophenotyping:*

The relative and the absolute leukocyte counts were determined with a Sysmex SE-9500 hematology analyzer (Sysmex, Kobe, Japan). The region of lymphocyte population (lymphocyte gate), was set manually, based on the forward-scatter and side-scatter characteristics (Beckman Coulter flow cytometer, USA). The relative count of each lymphocyte subpopulation was expressed as a percentage within the total lymphocyte population. The absolute count of each lymphocyte subpopulation (CD3+ and CD19+ lymphocytes) was calculated from the relative count of the total lymphocyte subpopulation (% of CD3+ and CD19+ lymphocytes), the relative count of the total lymphocyte population (%), and the absolute leukocyte count **[16]**.

2- *Apoptosis in peripheral T and B lymphocytes:*

Apoptosis in T and B lymphocytes was measured by staining with Fluorescein isothiocyanate (FITC) conjugated annexin V and Propidium iodide (PI) using IQ products Phosphatidyl Serine Detection Kit (IQP-116F) following manufacture instructions. Indotricarbocyanine (Cy5) coupled to Phycoerythrin (PE) conjugated anti-CD3 (PE-Cy5) IQP-519 and anti-CD19 (PE-Cy5) Dako-C7066 antibodies were used to identify apoptotic cell phenotype.

Aliquots of whole blood (50µl) were lysed using IQ lyse (IQP-199 ready to use), then washed with calcium buffer (1x stored at 4ºC). Cy5-labelled monoclonal antibodies and FITC labeled annexin V were incubated at the same time for 20 minutes on ice and in the dark. The cells were washed with calcium buffer for another time, then 10µl of PI were added and incubation for 10 minutes on ice was done. The cells were kept on ice till analyzed by flow cytometer **[14]**.

Three-color flow cytometry analysis was performed on a BECKMAN Coulter equipped with a single 488 nm argon ion laser. At least 10,000 events were acquired for each sample. The voltages and compensation were set according to the standard procedure, using negative controls and tested cells stained in a single color or a combination of colors. The proportion of FITC+/PI-, corresponding to early apoptosis in T and B lymphocytes, was evaluated by gating for CD3-PE-Cy5 and CD19-PE-Cy5.Cells that are FITC+/PI+ which are late apoptotic and those who are FITC-/PI+ necrotic cells were excluded.

Statistical methods:

Data management and analysis were performed using Statistical Analysis Systems. 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 chisquare test or Fisher's exact for small sample size. All *p*-values are two-sided. *p*-values < 0.05 were considered significant **[17]**.

RESULTS

1- *Complete blood picture:*

Red blood cells (RBCs) count, haemoglobin level (Hb), heamatocrite (Hct) level, mean corpuscular volume (MCV), mean corpuscular heamoglobin (MCH), platelets and total leucocytic counts did not show significant difference between DS children and control group (Table 1).

No significant difference was detected between the percentage and the absolute values of neutrophils, lymphocytes, monocytes or eosinophils between DS and normal children. The percentage of basophils was significantly higher in DS children but no significant difference was detected when absolute values were compared (Table 2).

2- *Immunologic markers:*

Although the absolute value of CD19+ B lymphocytes was lower in DS children, no significant difference was detected. On the other hand, both the relative and the absolute values of CD3+ T lymphocytes showed no significant difference between DS and normal children (Table 3).

3- *Apoptosis in peripheral blood T and B lymphocytes:*

The relative and the absolute number of early apoptotic CD3 positive T lymphocytes were significantly higher (p=0.001) in DS children than age matched controls. On the other hand, the relative number of early apoptotic CD19 positive B lymphocytes was significantly higher in DS children but no significant difference was detected when the absolute number was compared (p=0.022 and 0.286 respectively) Table (4), Figs. (1,2).

Fig. (1): Flow cytometric analysis of apoptotic CD3+ T-lymphocytes in a healthy control.

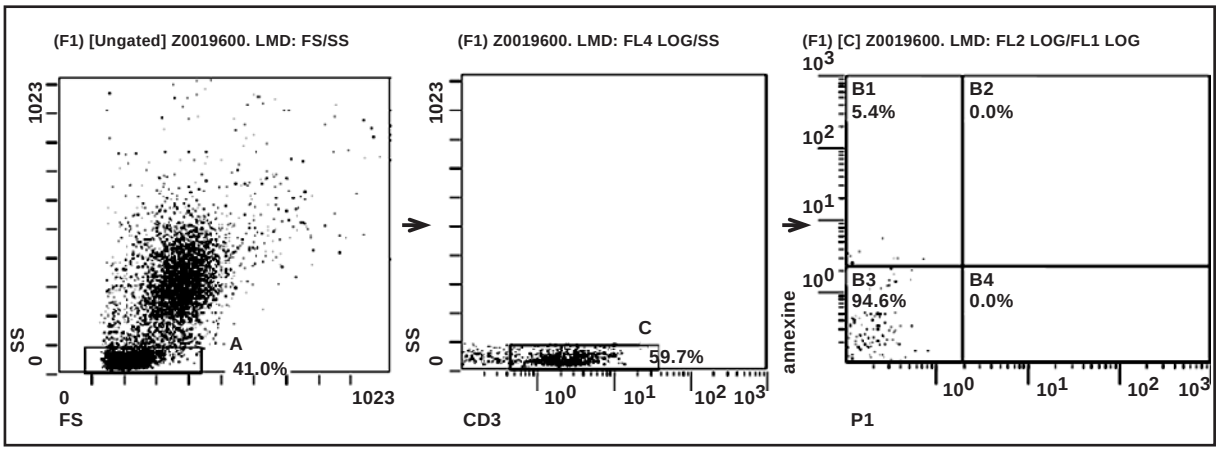

Fig. (1): From left to right: Dot plot showing gating on total lymphocytes, dot plot showing gating on CD3+ T-lymphocytes, and in the right is a dot plot showing the proportion of early apoptotic cells in the upper left quadrant of a control.

Fig. (2): Flow cytometric analysis of apoptotic CD3 positive T-lymphocytes in a patient with down's syndrome.

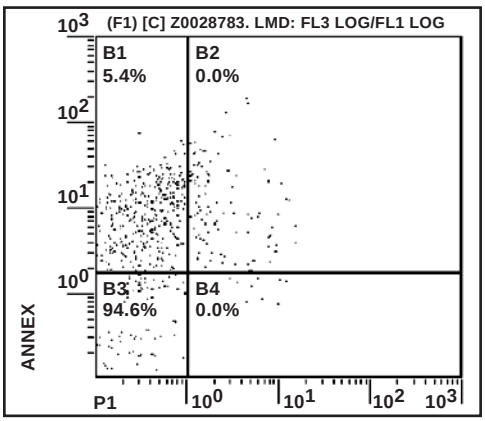

Fig. (2): Showing the percentage of FITC+/PI-in the upper left quadrant (early apoptotic cells), and the FITC+/PI+ in the upper right quadrant (late apoptotic cells) as well as the FITC-/PI+ (necrotic) cells in the lower right quadrant after gating on CD3 cells in a DS child.

Table (1): Hematological values for 17 DS patients and control group.

Variable	DS (Mean ± SD)	Controls (Mean ± SD)	*p*-value
RBC (x10^{12}/L)	4.4±0.48	4.3±0.58	0.534
HGB (g/dl)	11.71±1.70	11.47±1.21	0.856
HCT (%)	34.75±3.88	32.92±3.60	0.115
MCV (fl)	72.75±14.92	75.07±12.08	0.463
MCH (pg)	26.24±4.09	26.64±2.30	0.478
PLT (x10^{9}/L)	398.82±171.73	348.02±132.15	0.942
TLC (x10^{9}/L)	7.85±6.17	10.34±13.94	0.868

Table (2): The Differential and absolute counts of white blood cells for the 17 DS patients and age matched control group.

Variable	DS (Mean ± *SD)	Controls (Mean ± SD)	*p* value
Neutrophils:			
Percentage	44.94±19.04	42.78±14.20	0.758
Absolute (x10^{9}/L)	4.26±5.59	4.43±5.06	0.850
Lymphocytes:			
Percentage	40.30±16.65	38.06±15.28	0758
Absolute (x10^{9}/L)	2.55±1.05	3.16±1.92	0.320
Monocytes:			
Percentage	8.25±4.27	7.74±4.44	0.950
Absolute (x10^{9}/L)	0.59±0.39	0.53±0.32	0.776
Eosinophils:			
Percentage	4.01±6.11	4.81±3.38	0.113
Absolute (x10^{9}/L)	0.25±0.41	0.62±1.17	0.066
Basopils:			
Percentage	1.89±1.07	0.96±0.76	0.007
Absolute (x10^{9}/L)	0.20±0.42	0.06±0.07	0.12

* Standard deviation (SD)

Table (3): The relative and absolute counts of CD3 and CD19 of 17 DS patients and age matched control group.

Variable	DS (Mean ± *SD)	Controls (Mean ± SD)	*p* value
CD3:			
Percentage	67.19±11.81	62.11±10.31	0.163
Absolute (x10^{9}/L)	2.44±3.05	2.16±1.17	0.687
CD19:			
Percentage	13.20±5.74	17.39±7.27	0.136
Absolute (x10^{9}/L)	0.40±0.31	0.67±0.61	0.076

* Standard deviation (SD)

Table (4): The relative and absolute number of annexin V positive CD3 and CD19 in 17 DS patients and age matched control group.

Annexin V positive cells	DS (Mean ± *SD)	Controls (Mean ± SD)	*p* value
CD3:			
Percentage	32.11±13.57	9.65±8.04	0.001
Absolute (x10^{9}/L)	0.79±1.19	0.18±0.13	0.001
CD19:			
Percentage	27.85±16.68	13.54±12.36	0.022
Absolute (x10^{9}/L)	0.10±0.07	0.08±0.11	0.286

* Standard deviation (SD)

DISCUSSION

An impairment of both specific and non-specific defence mechanisms has been documented in patients with DS. Neutrophil chemotaxis, leucocytes opsonization, and phagocytosis as well as leucocytes bactericidal activity were found to be decreased in children with DS **[18]**.

Several studies have focused their attention on the role of the thymus, and have described a variety of structural and anatomic alterations present in DS **[19]**. Although studies of T-cell phenotype and function have frequently resulted in conflicting results, the overall evidence strongly points to a primary and profound impairment of T-cell mediated immunity in DS individuals **[20]** Quantitative studies of peripheral blood T lymphocytes reveal a reduction, often quite small, in the percentage and/or absolute number of T lymphocytes, although normal proportions or numbers of T and B lymphocytes in DS children have also been reported **[2]**.

Other authors found that, there was no early expansion of T and B lymphocytes in the peripheral blood of children with DS in the first

year of life. The T-lymphocyte subpopulation gradually approaches those of normal children over time contradicting the theory of precocious aging and do not explain the observed disturbance in the adaptive immune system in DS **[15]**.

In this study, both the relative and the absolute number of apoptotic (CD3 positive annexin V cells) were significantly higher in DS than that of normal children. This is in spite of the absence of significant difference in their number which may explain the functional impairment of these cells and the defect in adaptive immunity in these patients after the first year of life.

Also, T cells were more severely affected than B cells which may further support the impairment of cellular immunity in these children. The higher percentage of apoptotic CD19 positive B lymphocytes in DS children should not be ignored as it may indicate impairment of humoral immunity as well, although not as severe as that of the cellular immunity.

Although, a direct evidence is lacking for a major intrinsic defect of the B-cell compartment. Some, if not all, of the deficit of the humoral response might be explained by a lack of proper T-cell helper activity **[21]**.

Previous investigators evaluated apoptosis in peripheral blood of patients with DS by different methods such as electron microscopy, (ISNT) and agarose gel electrophoresis of DNA **[13]**. But unlike our study they did not identify the cellular phenotype of apoptotic cells in these patients.

Another unique advantage of the method used in this work is that it was both sensitive and specific for detecting very early cellular apoptotic changes as it was able to discriminate between three different types of cells, early apoptotic (Annexin V positive PI negative), late apoptotic (Annexin V positive PI positive), and necrotic cells (Annexin negative PI positive). Both late apoptotic and necrotic cells were excluded during analysis. None of the methods used before could differentiate between early and late apoptotic changes.

Although ISNT is considered a good method for the detection of early stages of apoptosis, Annexin V staining was found to detect apoptosis at an earlier stage **[15]**.

The most specific assay for the detection of apoptosis is perhaps the oldest, which is the detection of nuclear shape changes in the early stages of apoptosis using microscopy **[22]**. But unlike, the annexin V staining it can not identify the cellular phenotype of apoptotic cells and unlike gel electrophoresis, the method used in this study is rather simple and does not requires the extraction of large number of cells **[22]**.

In conclusion, increased apoptotic CD3 cells may contribute to the impaired cellular immunity in DS children with normal absolute number of these cells. Further studies on apoptotic cellular phenotype in DS children and its correlation with infection episodes in these children are recommended.

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Prame Gene Expression in Acute Lymphoblastic Leukemia

SOMAYA EL-GAWHARY, M.D.*; MANAL MAHMOUD, M.D.*; SALMA MEKHEMER, M.D.**; AMINA EL-SHAKANKIRY, M.D.*** and HAMDY ABD EL-AZIM, M.D.***

*The Departments of Clinical Pathology and NEMROCK***, Faculty of Medicine, Fayoum* and Cairo** Universities.*

ABSTRACT

PRAME (Preferentially expressed antigen of melanoma) was first isolated as a human melanoma antigen recognized by cytotoxic T cells (CTL). PRAME was demonstrated to be a useful marker for detection of minimal residual disease (MRD) in patients with leukemia, particularly those leukemias in which tumor specific markers are currently unavailable.

In our study, we identified the expression of PRAME gene in 26 patients with acute lymphoblastic leukemia (ALL); 14 cases were pre-B type, 6 cases were C-ALL, and 6 cases were T-ALL.

This was done using RT-PCR for RNA amplification of PRAME gene and Gel-pro analysis for the gene expression using Gel pro-analyzer version (3.1); and also using Real-time PCR for PRAME expression, and then the results of expression of PRAME gene with both techniques were compared.

Gel documentation system and Real-time PCR showed positive relative PRAME gene expression of all cases.

Comparison between different IPT groups regarding Gel document system and Real-time PCR relative expression of PRAME gene was highly significant having (p<0.01).

There was a highly significant statistical positive correlation between Real-time PCR and Gel documentation system relative expression of PRAME gene as (p<0.01) and (r=0.998).

Key Words: *PRAME gene – Acute lymphoblastic leukemia (ALL) – Minimal residual disease (MRD) – RT-PCR – Gel documentation system – Real-time PCR – Human melanoma antigens.*

INTRODUCTION

Acute lymphocytic leukemia is the most common malignant disease affecting children, accounting for approximately 30% of childhood cancer **[1]**. In Egypt, acute leukemia account for 40% of pediatric malignancy and ALL for 70% of cases, and the peak incidence is between 3-7 years **[2]**.

New methods of cancer classification involving gene expression profiling may eventually supercede cytogenetic analysis in the diagnosis and prediction of outcome in leukemia. It is more likely that they will be used in a complementary approach alongside cytogenetic, FISH, and molecular analysis to guide patient management in childhood ALL **[3]**.

PRAME (Preferentially expressed antigen of melanoma) was first isolated as a human melanoma antigen recognized by cytotoxic T cells (CTL) **[4]**. Recently,the function of PRAME has been elucidated by Epping et al. **[5]** PRAME binds to retinoic acid receptor-alpha, thereby inhibiting retinoic acid-induced differention, growth arrest and apoptosis. Suppression of high levels of endogenous PRAME in retinoic acid-resistant melanoma cells by RNA interference restores sensitivity to the anti proliferative effect of retinoic acid, suggesting that PRAME over expression contributes to oncogenesis by inhibiting retinoic acid signaling **[5]**. It was highly expressed in various solid tumor cells and normal testis. This gene was also expressed in some of the hematological malignancies, AML, chronic myelogenous leukemia in blastic crisis, acute lymphocytic leukemia, lymphoma and multiple myeloma. In addition, PRAME was demonstrated to be a useful marker for detection of minimal residual disease (MRD) in patients with leukemia, particularly those leukemias in which tumor specific markers are currently unavailable **[6,7]**.

The PRAME peptide can also be a target leukemia antigen for T-cells, so being useful

for development of new diagnostic & treatment methods for patients with ALL.

Quantitative real-time PCR (qr-PCR) allows a highly sensitive quantification of transcriptional levels of the gene of interest in a few hours with minimal handling of the samples **[4]**.

Quantitative real-time PCR is a method to rapidly and precisely quantify gene activity by detecting mRNA levels of the gene of interest,it quantitates the initial amount of the template most specifically, sensitively and reproducibly, and is a preferable alternative to other forms of quantitative RT-PCR, which detects the amount of final amplified product.

Aim of work:

This work aims to identify the expression of PRAME gene in patients with acute lymphoblastic leukemia (ALL) as a target to throw some lights on pathogenesis of ALL. It can also be useful for detection of minimal residual disease (MRD) & prediction of relapses, especially in patients without known genetic markers.

PATIENTS MATERIALS AND METHODS

Subjects: Study consisted of 36 cases, 26 patients of newly diagnosed acute lymphoblastic leukemia (ALL) cases, 14males and 12 females, age ranging from 2 years to 24 years; as well as10 age and sex matched controles, 7 males and 3 females, age ranging from 10 years to 22 years, obtained from Naser institute and New children hospital, Cairo university.

Methods: All studied groups were subjected to:

1- Full clinical assessment & follow-up of patients for prognosis.

2- Complete Blood Picture (CBC).

3- Bone marrow aspirate for FAB classification.

4- Immunophonotyping by Flowcytometry.

5- R-T PCR for PRAME expression.

6- Gel Pro-analyzer version 3.1 for gel documentation system quantitation.

7- Real-time PCR for PRAME quantification, using Light-Cycler system by Roche Molecular Biochemicals.

Procedures:

1- Samples were taken as whole blood of 5 mL peripheral blood on EDTA vaccutainer under sterile conditions.

2- MNCs separation was performed by Ficoll-density centrifugation as previously described by Matsushital et al., 2001**[5]**.

3- *RNA extraction:*

Total RNA extraction from MNCs using Gentra kit supplied by Life-trade, USA was done as previously described by **[5,6]**.

Procedure:

1- Three ml cell lysis solution was added to the tube containing the resuspended cells to lyse them.

2- Protein DNA precipitation 1mL protein DNA precipitation solution was added to the cell lysate. The tube was gently inverted 10 times and placed into an ice bath for 10min. Centrifugation was done at 15.000xg for 5min.

3- *RNA precipitation:*

The supernatant containing the RNA was poured off into a clean centrifuge tube rated for high speed containing 3mL 100% Isopropanol. The sample was mixed by inverting gently 10 times. Centrifugation was done at 15.000xg for 5min; the RNA was visible as a small translucent pellet. The supernatant was poured off and the tube was drained on clean absorbent paper. 3mL of 70% ethanol was added to wash the RNA pellet. Centrifugation was done at 15.000xg for 2min. The ethanol was poured off carefully. The tube was inverted and drained on clean absorbent paper and allowed to dry for 15min.

4- *RNA hydration:*

100µl of RNA hydration solution was added (100µl gave a concentration of 100µg/mL as the total yieldwas 10µg RNA), the RNA was allowed to rehydrate on ice for at least 30min., vortexed vigorously for 5sec., pulse spinned and the sample was carefully transferred to 1.5 mL microfuge tube and stored at -70º to -80ºc until it was used (Gentra kit; **[7,8]**).

4- *RT-PCR (2-step):*

Reverse transcription:

Reverse transcription and was done as previously described by **[5]** with modification.

The RNA was transcripted to cDNA using 2µg of total RNA in 5µl of Reverse transcriptase buffer (Finnzymes, Finland); 1µl of 10 mM of dNTPs mix (Promega Biotec., USA); 1µl of 20 MM of Random hexamer (IDT, USA); 2µl of Rnasin (Finnzymes, Finland); and 20 u of AMV Reverse transcriptase (Finnzymes, Finland).

The reaction was performed by incubation at 42ºc for 60 min. Thermal cycler (Biometra unoII) is used.

Amplification:

Complete cDNA product was then supplemented with 2.5µl of 10xPCR buffer with $MgCl_2$ (Promega biotec, USA); 1µl of a 10 mM of dNTPs mix (Promega biotec, USA) 1µl of 0.25MM of solution of each primer (IDT, USA); 2.5u of Taq-polymerase (Promega biotec, USA), and water to a final volume of 25µl.

The PCR conditions for PRAME were 5 min at 94ºc, followed by 34 cycles of 1 min at 94ºc, 2 min at 63ºc, 3 min at 72ºc. The PCR conditions for ß-actin were 5 min at 94ºc, followed by 25 cycles of 30 s at 94ºc, 1 min at 68˚c and1 min at 72ºc. **[5]**. Thermal cycler (Biometra unoII)is used. Primers (by IDT, integrated DNA technologies, USA): OPC189 and OPC 190 for PRAME amplification: (OPC189, 5´-CTGTACTCATTTCCAGAGCCAGA-3´; OPC 190, 5´TATTGAGAGGGTTTCCAAGGGGTT-3´) and A-F, A-R for ß-actin amplification: (A-F, 5´-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3´; A-R, 5´-CGTCATACTCCTGCTGATCCACATCTGC-3´).

Calibration curve:

Five ß-actin standard controls with known copy number were used to do the calibration curve. Total RNA of healthy donors (Roboscrene, Germany) was serially diluted in log step from 10^7 copies to 10^3 copies in µl volume. A calibration curve was created by logarithmic plotting of the optical density (OD) versus a known copy number, for each template in the dilution **[7,9]**.

Detection of the amplification product: by Agarose gel electrophoresis as previously described by **[10]** using ethidium bromide.

Quantification:

The input copy no and the densities of the bands stained with ethidium bromide (photographed gel) were measured using Gel pro-Analyser, version 3.1 (MEDIA CYBERNETICS, USA) and compared with those of the standards to get the maximum optical density of the PCR products of each cycle which represented the concentration of cDNA **[9,11]**.

Expression:

Relative PRAME expression was calculated by: PRAME max O.D./ß-actin max O.D. **[12,13]**.

5- Real- time PCR for PRAME quantification:

PCR amplification & data analysis in real-time were performed using the Light-Cycler™ system (Roche Molecular Biochemicals) and SYBR Green I dye (DyNAmo™ SYBR Green qPCR Kit, Finzymes, Finland).

For each assay, a reaction mixture was performed on ice containing: 10µl of 2x SYBR Green I master mixture, 5 µl of 4x of the primers, 2µl of cDNA (of concentration 500ng/2 µl volume) and water to a final volume 20 µl.

Primers: Supplied by IDT,USA. OPC189 and OPC190 for PRAME amplification: (OPC189, 5´- CTGTACTCATTTCCAGAGC CAGA-3´; OPC190, 5´-TATTGAGAGGGTTTC-CAAGGGGTT-3´) and A-F, A-R for ß- actin: (A-F, 5´ATCTGGCACCACACCTTCTACAATGAGCTGCG-3´; A-R, 5´-CGTCATACTCCTGCTGATCCACATCTGC-3´).

Initial denaturation at 95ºc for 10 min; followed by 40 cycles of denaturation at 95ºc for 0s; annealing for 5s at 63ºc for PRAME gene and at 68˚c for ß-actin gene **[5]**; and extension at 72ºc for 20s for PRAME and 40s for ß-actin depending on the length of the product (bp/25). The temperature transition rate was set at 20ºc/s. The amount of fluorescent product was measured in single-acquisition mode after each cycle at 84ºc for PRAME and 89ºc for ß-actin. (Roche Molecular Biochemicals, Light-Cycler system).

After PCR a Melting Curve was constructed to distinguish specific from non-specific products and primer-dimer.

Calibration curve:

To determine the copy number of target transcripts (PRAME mRNA), the ß-actin standard was used to generate a calibration curve. Total RNA of healthy donors (Roboscrene,

Germany) was serially diluted in log step from 10^7 copies to 10^3 copies in µl volume. A calibration curve was created by plotting the threshold cycle (ct) versus a known copy number, for each template in the dilution. The copy numbers for all unknown samples were determined by real-time soft ware, according to calibration curve.

Expression:

Relative PRAME expression =copy no of PRAME/copy no of ß-actin=conc. of PRAME/ conc. of ß-actin.

(12,13, DyNAmo™ SYBR Green qPCR Kit, Finzymes, Finland).

RESULTS

- Twenty six patients suffering from acute lymphoblastic leukemia (ALL) were included in the present study, as well as ten normal age and sex matched controls.
- The age of the controls group ranged from 10 years to 22 years with a mean of 16.8±4.13 years. They were 7 (70%) males and 3 (30%) females.
- The age of the patients group ranged from 2 year to 24 years with a mean of 10.04±7.04 years. They were 14 (53.8%) males and 12 (46.2%) females.

Clinical data:

On examination of patients; splenomegaly was found in 21 cases (80.8%), hepatomegaly was found in 10 cases (38.5%), Lymphadenopathy was found in 9 cases (34.6%), CNS affection was found in 6 cases (23.1%) and fever as a symptom in 8 cases (30.8%).

As regard prognosis: good prognosis (C.R.) was for 10 cases (38.5%) and bad prognosis (death & CNS relapse) was for 16 cases (61.5%); death was for 14 cases (53.8%) and relapse was for 2 cases (7.7%).

No statistical significance was found in comparison between different prognostic groups regarding clinical data and sex.

Haematological data:

- Hemoglobin level ranged from 5 to 10 g/dl with a mean value of (7.67±1.27).
- White blood cell count ranged from 5 to 150 x 103/µl with a mean of (44.15±35.63).
- Platelet count ranged from 10 to 75 x 103/µl with a mean value of (44.04±19.66).
- The blast cell count in peripheral blood revealed a range of 33 to 95% with a mean of (72.81±14.52).
- Bone marrow was hypercellular in all 26 cases. The blast cell count ranged from 65 to 98% with a mean of (86.27±9.52).
- Normal TLC was found in 2 cases (7.7%), mild leukocytosis in 3 cases (11.5%) and marked leukocytosis in 21 cases (80.8%).
- Mild anemia was found in 2 cases (7.6%), moderate anemia in 12 cases (46.2%) and marked anemia in 12 cases (46.2%).
- Moderate thrombocytopenia was seen in 11 cases (42.3%) and marked thrombocytopenia in 15 cases (57.7%).
- Comparison between prognostic groups and age was highly significant with (p<0.01), as good prognosis had a lower age mean (5.2± 2.4), while bad prognosis had a mean age (13.0±7.3) (Table 1).
- Comparison between prognostic groups and TLC was highly significant with (p<0.01), as good prognosis had a lower TLC mean (17.5± 6.8), while bad prognosis had a mean TLC (60.8±36.2) (Table 1).
- Comparison between hematological data and age of cases and controls is seen in Table (2).
- Comparison between hematological data and age of cases and controls was highly significant as (p<0.01).
- As regarding age, cases had a mean age (10.03±7.04), while controls had a mean age (16.8±14.3). TLC (x10^3/ul) of cases had a higher mean (44.15±35.62), while that of controls had a mean (9.88±1.71). Hemoglobin (g/dl) of cases had a lower mean (7.66±1.26), while that of controls had a mean (13.6±1.53). Platelets (x10^3/ul) of cases had a lower mean (44.03±19.66), while that of controls had a mean (291.7±53.33). Blasts % in peripheral blood of cases had a mean (72.8±14.52) and was absent in controls. Blasts % in bone

marrow of cases had a mean (86.26±9.52) and was absent in controls.

Classification of ALL cases:

- According to FAB classification 5 cases (19.2%) were L1; while 21 cases (80.8%) were L_2.
- Immunophenotypic characterization of cases showed 14 cases pre-B type (53.8%), 6 cases were T-ALL (23.1%), and 6 cases were C-ALL (23.1%).

Results of gel documentation system:

- All of the 26 cases (100%) were positive for relative PRAME expression.
- M → (Marker) [2000,1600,800,700,600, 500,400,300,200,100] bp.
- C → controls.
- L → lanes. (In all gel photos).
- All cases were positive for *ß*-actin at [1018 bp] → 1st band.
- All cases were positive for PRAME at [517 bp] → 2nd band.
- Primer dimmer was shown in → 3nd band.
- Max O.D. → maximum optical density.
- All amplification curves of gel documentation system were done by Gel pro-analyzer version (3.1).

Results of Real-time PCR (generated by light-cycler software):

All 26 cases were positive for *ß*-actin and PRAME gene expression.

Descriptive statistical data of relative PRAME expression among cases is shown in Table (3).

Gel documentation system showed positive relative PRAME gene expression ranging (0.22-0.97) with a mean (0.706±0.27).

Real-time PCR showed positive relative PRAME gene expression ranging (0.22-0.98) with a mean (0.713±0.27).

There was a highly significant statistical positive correlation between Real-time PCR and Gel documentation system relative expression of PRAME gene as (p<0.01) and (r=0.998), as shown in Table (4), Fig. (2).

Table (1): Comparison between prognostic groups regarding age and hematological data.

Item	Bad (n=16)	Good (n=10)	*p* value	Sig.
Age (y)	13.0±7.3	5.2±2.4	0.001	HS
TLC (x10^3/µl)	60.8±36.2	17.5±6.8	0.00	HS
Hb (g/dl)	7.5±1.3	7.94±1.2	0.40	NS
Plat (x10^3/µl)	43.7±19.0	44.5±21.63	0.927	NS
PB blast %	71.9±16.4	74.2±11.46	0.707	NS
BM blast %	86.7±10.4	85.5±8.31	0.752	NS

**p*>0.05= Non significant. ***p*<0.05= Significant.
****p*<0.01= Highly significant.

Table (2): Comparison between hematological data and age of cases and controls.

Item	Cases (n=26)		Control (n=10)		*p* value	Sig.
	Mean	±SD	Mean	±SD		
Age (y)	10.03	7.04	16.8	4.13	0.008	HS
TLC (x10^3/µl)	44.15	35.62	9.88	1.71	0.005	HS
Hb (g/dl)	7.66	1.26	13.6	1.53	0.00	HS
Plat (x10^3/µl)	44.03	19.66	291.7	53.33	0.00	HS
PB blast %	72.80	14.52	0	0	0.00	HS

**p*>0.05= Non significant. ***p*<0.05= Significant.
****p*<0.01= Highly significant.

Table (3): Descriptive statistical data of relative PRAME expression among cases.

Item	Range	Mean	±SD
Gel document system relative expression	0.22-0.97	0.706	0.27
Real time PCR relative expression	0.22-0.98	0.713	0.27

Table (4): Correlation between Real-time PCR and Gel documentation system relative expression of PRAME gene.

Item	*r*	*p*	Sig.
Gel documentation system relative expression	0.998	<0.01	HS

**p*>0.05= Non significant. ***p*<0.05= Significant.
****p*<0.01= Highly significant. *r* = correlation coefficient.

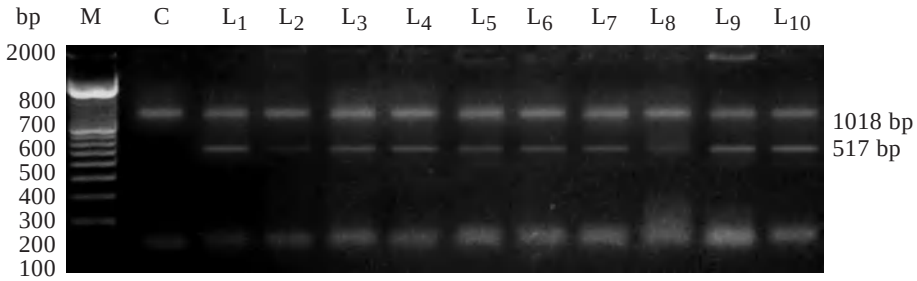

Fig. (1): Agarose gel analysis for cases 1→10.

L_1→ L_{10}: for cases 1→10, all cases showed a band at (1018bp) representing ß-actin expression and a band at (517bp) representing PRAME expression.
$L_{1,3,4,7,9,10}$ showed high expression.
$L_{5,6}$ showed moderate expression.
$L_{2,8}$ showed low expression.

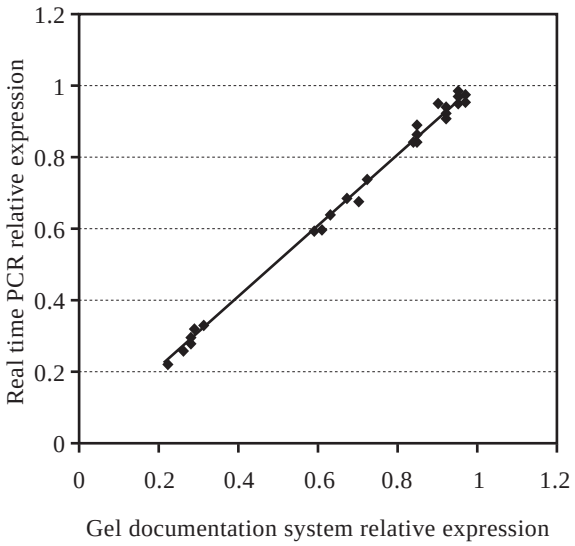

Fig. (2): Correlation between Real time PCR and Gel documentation system relative expression of PRAME gene

DISCUSSION

The molecular diagnosis of human cancer will hasten the development of treatments tailored to the abnormalities present in each patient's tumor cells. Recent gene expression profiling studies of pediatric acute lymphoblastic leukemia suggest that the molecular diagnosis of these diseases is right around the corner **[15].**

With newer drug protocols the five year survival rate is now close to 80%. Various factors have been reported to be important for prognosis and should be considered when planning **[16].**

New methods of cancer classification involving gene expression profiling may eventually supercede cytogenetic analysis in the diagnosis and prediction of outcome in leukemia.

Previous studies have extensively evaluated the PRAME expression in various hematological malignancies and demonstrated high expression of the PRAME gene in subsets of AML, chronic myelogenous leukemia in blastic crisis, acute lymphocytic leukemia, lymphoma and multiple myeloma. In addition, PRAME was demonstrated to be a useful marker for detection of minimal residual disease (MRD) in patients with leukemia, particularly those leukemias in which tumor specific markers are currently unavailable. Since PRAME was first identified as a tumor antigen recognized by CTL, the possibility that PRAME is a leukemia antigen recognized by CTL was evaluated, and it was found that PRAME-positive leukemia cell lines and fresh leukemia cells were susceptible to lysis by the PRAME-specific CTL. Five CTL epitopes associated with either HLA-A *0201 or HLA-A *2402 in the tumor Antigen PRAME have been recently identified by proteasome mediated digestion analysis. The PRAME peptide can also be a target leukemia antigen for T-cells, so being useful for development of new diagnostic & treatment methods for patients with ALL. Immunotherapy for patients with MRD is a particularly interesting strategy. It is, therefore, an attractive strategy to apply PRAME specific immunotherapy on patients with PRAME positive leukemia in MRD condition **[6,15-24,].**

Real-time reverse-transcriptase (RT) PCR quantitates the initial amount of the template

most specifically, sensitively and reproducibly, and is a preferable alternative to other forms of quantitative RT-PCR, which detects the amount of final amplified product. Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle (i.e., in real time) as opposed to the endpoint detection by conventional quantitative PCR methods **[12]**.

In the current study, we identified the expression of PRAME gene in 26 patients with acute lymphoblastic leukemia (ALL) as a target to throw some lights on pathogenesis of ALL.

This was done using RT-PCR for RNA amplification of PRAME gene and Gell-pro analyses for the gene expression, and also using real-time PCR for PRAME expression, and then the results of expression of PRAME gene with both techniques were compared. We also tried to asses the impact of PRAME gene expression on the clinical and hematological presentation, and also prognosis of the studied cases.

The age of ALL patients ranged from 2 years to 24 years with a mean value (10.04±7.04). Age at diagnosis is one of the most important prognostic factors. Patients below two years old and above 10 years old have bad prognosis in comparison with patients between 2-10 years. This is may be due to occurrence of multiple high risk factors in early life and adolescence. This is in agreement with **[25-28]**.

Concerning the hematological picture, decreased hemoglobin level was found in almost all of our patients with a mean value (7.67± 1.27).

Of all the studied cases 92.3% presented with hemoglobin level <10g/dl. This is in consistent with the work of Miller et al., 1983; who stated that hemoglobin level below 10g/dl is present in the majority of ALL cases. They also stated that this group of patients has a higher remission induction rate, a lower relapse rate and a longer survival rate than those with no anemia. With explosive disease, symptoms evolve before anemia, as anemia needs time to develop whereas, with indolent leukemia, disordered bone marrow function becomes clinically apparent before anemia **[29]**. On the other hand Robinson et al., 1980 stated that normal hemoglobin level is associated with bulky extramedullary involvement and higher percentage of blasts. Because of this and other risk factors with which it is associated, the hemoglobin level loses much of its predictive power in multivariate analysis **[30]**.

Regarding WBC count, it ranged from 5 to$150x10^3$/ul with a mean of (44.15±35.63). Karimi et al., 2002 upon studying prognostic factors in childhood ALL revealed that among all variables a worse prognosis was associated with WBC counts greater than $50x10^9$/L at presentation **[16]**.

The bone marrow and peripheral blood from the 26 patients with ALL at diagnosis were studied. According to FAB classification 21 of the studied cases (80.8%) were L_2 morphology and 5 cases (19.2%) were of L_1 morphology. This disagree with Bennett et al., 1981 who found that approximately 80-85% of ALL cases were L_1, 14-18% L_2, and 1-3% L_3 **[31]**. This may be due to less number of patients **[26]** used in our study,as Bennett et al. assessed the degree of concordance in the morphological classification of ALL by the FAB group after two successive reviews of 200 and 100 slides respectively.

A among the 26 ALL cases in this study, 76.9% were assigned to the B-lineage phenotype where 53.8% were (pre-B ALL), 23.1% were common ALL (C-ALL), and 23.1% were assigned to the T-lineage (T-ALL). This is in partial agreement with previous studies identifying high prevalence of B-ALL, but it also show lower frequency of T-ALL 12-15% of cases **[32,33]**. On the other hand studies done by Ritterbach et al., 1998 showed a marked prevalence of common ALL (77%) followed by pre-B (22%) **[34]**. This may be due to less number of patients **[26]** in our study; as Ritterbach et al. studied 278 patients.

Pydas et al., (2005) showed that approximately 25% of the acute leukemia samples were positive for PRAME expression. Remarkably, all acute myeloblastic leukemias that carried the chromosomal translocation t (8: 21), which fuses the genes AML1 and ETO expressed PRAME at a high level **[24,35]**.

PRAME gene was expressed in 35% of the acute myeloid leukemia (AML) samples tested. The AML-M2 subtype was the most frequently involved, followed by AML-M3 and AML-M6

whereas most of the AML-M0 and AML-M7 samples scored negative **[24]**.

Thirteen samples of CML in chronic phase were tested, 10 of these CML had a Philadelphia chromosome (Ph+) visualized by karyotypic analysis. An additional (Ph-) sample had a detectable BCR-ABL rearrangement. All the samples were negative for PRAME expression **[24]**. It is worth mentioning that expression of PRAME is associated mainly with acute leukemia subtypes that carry a relatively favorable prognosis: AML with t (8: 21), APL with t (15: 17) and childhood B-ALL with or without t (12: 21). A prospective study is needed to assess the importance of PRAME expression as an independent prognostic factor in acute leukemias. If it appears that PRAME-positive leukemias have a lower risk of relapse after chemotherapy, the possibility that this could be due to anti-PRAME immune responses will deserve further investigation **[24]**.

As PRAME gene is transcribed in leukemic cells, but not in normal bone marrow or peripheral blood mononuclear cells, RT-PCR with PRAME-specific oligonucleotides might be a valuable tool for detecting leukemic cells. It could be used to quantitate the response to induction chemotherapy or to detect minimal residual disease **[24]**.

Steinbach et al. (2002); showed that fifteen percent of the acute lymphoid leukemias (ALL) tested expressed PRAME. All the positive samples were childhood pre-B ALL (14/61 tested) (23%). None of the 10 adult pre-B ALL, two B-ALL and 17 T-ALL samples tested were found positive. All biphenotypic leukemias tested were negative except one. No PRAME expression was detected in normal bone marrow or peripheral blood mononuclear cells RT-PCR results remained negative when a subset of the normal samples was tested with five additional cycles of PCR. It is estimated that this detected as few as one positive cell in 5000 cells, which indicated that it would be unlikely that a small subpopulation of normal bone marrow cells , for example CD 34 cells which account for 1-5% of bone marrow cells could express PRAME at a significant level **[24]**.

Matsushita et al., 2001, showed that PRAME expression was detected in 42% of their samples. Also the PRAME expression was detected in high percentages in AML-M2 (45%), AML-M3 (75%), CML-BC (42%), and ALL (64%) mainly pre-B ALL but in a relatively low percentage of lymphoma cases (23%) this expression pattern was almost the same as previously reported data in Caucasian patients, except the relatively high percentage in ALL. The percentages of leukemic cells in samples that were determined morphologically ranged between 12.6% and 99.5% (mean: 70.0%) for highly PRAME-positive samples and between 3.4% and 97.0% (mean: 58.5%) for other patients. No correlation was observed between the levels of the PRAME expression and the percentage of affected leukemic cells, suggesting that the PRAME expression in leukemic cells differs among patients **[5]**.

Greiner et al., 2000; used RT-PCR to prove that the previously described antigen PRAME showed different expression levels in AML. In their peripheral blood, (47%) AML patients showed high expression, (12%) lower, and (41%) no expression of PRAME. High expression also was detected in the cell lines K562 (which was used as positive control) and HL-60, and moderate expression in the human cell lines Oci-5 and KG-1. In contrast, no expression of PRAME was found in PBMN from healthy volunteers and in $CD34^+$ separated cell samples from healthy donors or patients without hematological malignancies **[21]**.

Contrary to previous reports; Steinbach et al., 2002; showed that the PRAME gene is expressed by $CD34^+$ stem cells. This might constitute a problem in using PRAME for tumor immunotherapy. Using quantitative reverse transcriptase polymerase chain reaction, over expression of PRAME was found in 62% of patients. The rates of overall and disease-free survival in this group were higher than in patients with no or low expression.

Twenty six peripheral blood samples from newly diagnosed patients with ALL were examined for PRAME gene expression using gel-documentation system and real-time PCR. Five standard controls were used to create caliberation curve (ß-actin standard curve) for both techniques. All 10 controls were positive for ß-actin and negative for PRAME expression regarding both techniques, while all the 26 patients (100%) showed positive relative

PRAME gene and ß-actin expression regarding both techniques. Gel documentation system showed positive relative PRAME gene expression ranging (0.22-0.97) with a mean (0.706± 0.27). Also real-time PCR showed positive relative PRAME gene expression ranging (0.22-0.98) with a mean (0.713±0.27).

On the other hand, Van Baren et al., 1998; showed that 15% of the acute lymphoblastic leukemias (ALL) tested expressed PRAME gene, while no PRAME expression was detected in normal bone marrow or peripheral blood mononuclear cells RT-PCR results remained negative when a subset of the normal samples was tested with five additional cycles of PCR. It is estimated that this detected as few as one positive cell in 5000 cells, which indicated that it would be unlikely that a small subpopulation of normal bone marrow cells, for example CD 34 cells which account for 1-5% of bone marrow cells could express PRAME at a significant level **[24]**. This difference may be due to different age groups, race, and the probability of less sensetive method of detection.

This also partialy goes with Matsushita et al., 2001, who screened bone marrow (BM), Peripheral blood (PB) and lymph node (LN) samples from 98 japanese patients using semi-quantitative RT-PCR, and PRAME expression was detected in 42% of these samples, the PRAME expression was detected in high percentages in AML-M2 (45%), AML-M3 (75%), CML-BC (42%), and ALL (64%) mainly pre-B ALL, this expression pattern was almost the same as previously reported data in Caucasian patients, except the relatively high percentage in ALL **[5]**. This partial difference may be due to racial differences from Egyptian population, more number of cases studied, and comparing the band dencity with the standard K562 cDNA dilutions.

In our study, no statistical significance was found in comparison between different prognostic groups (fate) regarding relative PRAME expression among cases by Gel documentation system or by Real-time PCR. No significant statistical correlation was found between Gel documentation system or Real-time PCR relative PRAME expression and all the studied parameters (age, sex, clinical data, hematological data and fate) with (p>0.05).

This is in agreement with Watari et al., 2000, who showed that relative PRAME expression was not correlated to age, sex, prednisone response, percentage of leukemic cells in peripheral blood, or the enlargement of liver or spleen. **[20]**.

This is also in partial agreement with Steinbach et al., 2002, who stated that the rate of disease-free survival was higher and white blood cell counts at diagnosis were lower in patients with an over expression of PRAME. However; in the group of ALL patients these findings were not statistically significant. The levels of expression at diagnosis corresponded well with those at relapse **[36]**.

In the current study, comparison between different IPT groups regarding Gel documentation system relative expression of PRAME gene was highly significant having (p<0.01), as C-ALL (n=6) had a low mean (0.27±0.03), Pre B (n=14) had a high mean (0.91±0.04), and T-ALL (n=6) had a moderate mean (0.65±0.05). Also comparison between different IPT groups regarding Real-time PCR relative expression of PRAME gene was highly significant having (p<0.01), as C-ALL (n=6) had a low mean (0.28±0.04), Pre B (n=14) had a high mean (0.92±0.04), and T-ALL (n=6) had a moderate mean (0.65±0.05). This is in partial agreement with Van Baren et al., 1998, who stated that all the positive samples were childhood pre-B ALL (14/61 tested) (23%). None of the 10 adult pre-B ALL, two B-ALL and 17 T-ALL samples tested were found positive **[24]**. Also Matsushita et al., 2001, stated that pre-B ALL may express PRAME, as 12 out of 14 ALL samples in their study and all 14 PRAME-positive ALL samples in other studies were pre-B ALL **[5]**.

In our study we also found that, there was a highly significant statistical positive correlation between Real-time PCR and Gel documentation system relative expression of PRAME gene as (p<0.01) and (r=0.998).

However real-time PCR showed some advantages over the conventional PCR.

- Traditional PCR is measured at End-Point (plateau), while Real-Time PCR collects data in the exponential growth phase.
- An increase in Reporter fluorescent signal is directly proportional to the number of amplicons generated.

- The cleaved probe provides a permanent record amplification of an Amplicon.
- Time saving.
- Detection is capable down to a 2-fold change.

Real-time PCR assays are characterized by a wide dynamic range of quantification of 7-8 logarithmic decades, a high technical sensitivity (<5 copies) and a high precision (<2% standard deviation). Another advantage of this method is that no post-PCR (processing) are required, thus avoiding the possibility of cross-contamination dues to PCR products. This advantage is of special interest for diagnostic applications. Together with lower turn-around times and decreases costs it has revolutionized the filed of molecular diagnostics. New systems for field use, which can detect microorganisms in less than 10 minutes, have been developed. Taken together, these advantages have enabled the shift of molecular diagnostics toward a high-throughput and automated technology **[37]**.

Conclusion:

In Egypt, acute leukemia account for 40% of pediatric malignancy and ALL for 70% of cases. The five year survival rate is now close to 80%.

PRAME was first isolated as a human melanoma antigen recognized by CTL.

There was a highly significant statistical positive correlation between Real-time PCR and Gel documentation system relative expression of PRAME.

As PRAME gene is transcribed in leukemic cells, but not in normal bone marrow or peripheral blood mononuclear cells, RT-PCR with PRAME-specific oligonucleotides might be a valuable tool for detecting leukemic cells. It could be used to quantitate the response to induction chemotherapy, early diagnosis of leukemia relapse, monitoring and following up patients with leukemia, or to detect minimal residual disease especially in leukemia without known genetic markers.

Because of the lack of expression in control groups and high frequency of PRAME expression in AML, PRAME seems to be a favorable candidate for further vaccination studies and immunotherapy, and also an indicator of favorable prognosis and could be a useful tool for monitoring minimal residual disease in childhood AML.

HLA-A *0201 - presented CTL epitopes in the tumor antigen PRAME are expected to be applicable for immunotherapeuic purposes (adoptive CTL therapy, vaccine design, and/or immuno-monitoring) in a high percentage of cancer patients.

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Gelatinases A and B (MMP-2 & MMP-9) and interleukin 18 (IL-18) gene in Adult Acute Myeloid Leukemia: Expression and Clinical Relevance

MANAR MOHMAD M. ISMAIL, M.D.[1]; ABEER MOSTAFA E.ASHMAWY, Ph.D.[2]; MANAR M. MONEER, M.D.[3] and AMANY M. HILAL, M.D.[4]

The Departments of Clinical Pathology[1], Molecular Biology and Biochemistry[2], Epidemiology and Biostatistics[3] and Medical Oncology[4], National Cance Institute, Cairo University.

ABSTRACT

Introduction: Acute myeloid leukemia is an aggressive heterogeneous disease which progresses quickly with unpredictable behavior and response to therapy. Previous studies suggested angiogenesis as one of the controlling mechanism for blast cell mobilization, the induction of which has been proposed to be through angiogenic factors, among these are gelatinases A and B. Interleukin-18, originally called IFN-γ inducing factor (IGIF), is a multi-functional cytokine. Recent data have suggested that inappropriate production of IL-18 contributes to the pathogenesis of cancer and may influence the clinical outcome of patients. The role of IL-18 in tumor progression and angiogenesis is an area of conflicting data.

Aim of the Work: This study was conducted to analyze the cellular expression of gelatinases A and B as well as IL-18 gene in adult AML, evaluate their clinical relevance regarding response to induction therapy and survival.

Material and Methods: A cohort of 42 newly diagnosed adult AML patients was analyzed for expression of gelatinases A and B (MMP-2, MMP-9) by flowcytometry as well as IL 18 gene expression by RT-PCR using gel documentation for semiquantitation.

Results: Gelatinase A was expressed in 26/42 (61.9%) of adult AML cases showing no difference among FAB subtypes (p=0.545), while gelatinase B was expressed in 14/42 (33.3%) showing significantly higher expression among M3 FAB subtype (p=0.001). The median expression level of IL18 in AML group was 1.5 (0.8-2) which was significantly higher than the control group (p<0.001). Within the AML group, it showed a higher expression level in M4 and M5 FAB subtype (p=0.003). Blast cell distribution ratio (BCDR) and splenomegaly were significantly higher in gelatinase A positive group (p=0.004 & <0.001 respectively). As regards to response to induction therapy, a higher incidence of CR was achieved within the gelatinase A positive group (p=0.018), while there was a significantly lower incidence of CR within both the gelatinase B positive group and in the group that expressed IL 18 >1.5 (p=0.002 & 0.021 respectively). There was no significant difference regarding gelatinases A or B expression and overall survival (OS) (p=0.078 & 0.082 respectively). However, the overall survival for patients who expressed IL 18 >1.5 was significantly lower (p=0.019). Regarding combined marker expression and response to induction therapy, 7/21 (33.3%) patients who achieved CR showed double or triple marker expression compared to 14/21 (66.7%) who failed to express any of the studied markers or expressed only one marker (p=0.038), but equivalent result could not be reached regarding OS.

Conclusion: The expression of gelatinase A (MMP-2) by AML blasts supports the hypothesis that, it may be a marker of the invasive phenotype. Gelatinase B (MMP-9) expression might carry poor prognosis in AML regarding response to induction therapy. IL18 over expression may be considered as a poor prognostic factor. Combined marker expression seems to have cumulative effect on response to induction. However, to validate these assumptions, a study on a larger number of patients is required.

Key Words: *Gelatinase A – Gelatinase B – MMP-2 – MMP-9 – IL-18 – AML.*

INTRODUCTION

Acute myeloid leukemia is an aggressive heterogeneous disease which progresses quickly with unpredictable behavior and response to therapy. Only about one-third of those between ages 18-60 years who are diagnosed as AML can be cured **[1]**.

One of the prominent features of the heterogeneity of this disease is the difference in mobility of leukemic cells (blasts) from bone marrow to peripheral blood and various organs. This mobility is normally restricted to functionally mature leukocytes. Cells have to cross matrix-barrier and penetrate blood vessels wall, and this depends on the catalytic modifications of extra-cellular matrix (ECM) and basement membrane. Previous studies have suggested a role of angiogenesis as the controlling mechanism for blast cell mobilization **[2]**.

Angiogenesis is the formation of new blood vessels from pre-existing endothelium-lined vessels. Angiogenesis is mediated by a balance of various positive [e.g. vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), hepatocyte growth factor (HGF), interleukin-8 (IL-8), matrix metalloproteinases (MMPs)] and negative [e.g. platelet factor-4 (PF-4), tissue inhibitor of metalloproteinases (TIMPs)] angiogenic molecules released by tumor cells **[3]**. It has been recognized that migration of proliferating endothelial cells requires a regulated degradation of the basement membrane and surrounding ECM, which involves the production of ECM-degrading proteinases such as MMPs **[4]**.

MMPs, a family of zinc-dependent endopeptidases that degrade all proteins in the ECM, are categorized according to their substrate specificity and domain structure into collagenases, gelatinases, stromelysins and membrane-type (MT) MMPs **[5]**. Gelatinases A and B (MMP-2/72 KDa and MMP-9/92 KDa); play important roles in angiogenesis, inflammation and cancer metastasis owing to their ability to degrade components of basement membrane such as gelatin (denaturated collagen), native collagen, fibronectin, vitronectin, elastin and aggrecan **[6]**. Gelatinases are synthesized as latent proteins that are activated by caspases and other secreted MMPs. They exist in three forms: cytoplasmic, membrane-bounded and free soluble form **[4]**, and produced by many cell types including endothelial, epithelial, normal and malignant haematopoietic cells **[7]**.

Interleukin-18, originally called IFN-γ inducing factor (IGIF) is a multifunctional cytokine consisting of a single 18.3 KD peptide synthesized by Kuppfer cells and activated macrophages **[8]**. It is an immunoregulatory cytokine, which is known to be involved in many pathological processes such as autoimmune diseases as well as infections. Recent data have suggested that inappropriate production of IL-18 contributes to the pathogenesis of cancer and may influence the clinical outcome of patients **[9]**. The role of IL-18 in tumor progression and angiogenesis is an area of conflicting data. It has an angiogenic role through increase in vascular cell adhesion molecules, vascular endothelial growth factor **[10,11]** and gelatinase A **[6]**. On the other hand, IL-18 has an antiangiogenic role via increasing antiangiogenic factor (IL-10) and decreasing both angiogenine and fibroblast growth factor-2 that stimulate proliferation of capillary endothelial intima. This suggests that IL-18 may be a malignancy associated protein **[12]**.

Aim of the work:

The aim of the present work was to evaluate the cellular expression of gelatinases A and B as well as IL-18 gene expression in leukemic cells from newly diagnosed adult acute myeloid leukemia and correlate data with their significance in extramedullary invasion, disease progression, response to treatment and survival as well as their potential use as target molecules in therapy.

SUBJECTS AND METHODS

Forty-two newly diagnosed adult patients with acute myeloid leukemia (AML) who presented to the National Cancer Institute, Cairo University in the period from April to September 2006 were included in this study following an informed consent. AML was diagnosed according to the standard criteria including morphological and cytochemical examination. French-American-British (FAB) subtype was determined **[13]**. Immunophenotyping was done to verify the leukocyte differentiation antigens which reflect commitment to the myeloid lineage as well as the level of differentiation **[14]**.

Significant organ infiltration at initial diagnosis was defined by ultrasonographic extension of the liver edge ≥2 cm below the right costal margin in the right mid-clavicular line or the spleen edge ≥2 cm below the left costal margin in the left mid-clavicular line or multiple lymph node enlargements **[13]**.

Blast cell distribution ratio (BCDR) which is the ratio between peripheral blood blasts and bone marrow blasts was used to measure the potential capacity of the blasts to disseminate from BM to PB **[4]**.

Twelve apparently normal adults of comparable age and sex not suffering from malignant diseases were used as a control.

Methods:

A- *Flowcytometric detection of gelatinases:*

Purified monoclonal antibodies (MoAbs) against gelatinases A and B (MMP-2 & 9) were purchased from Zymed Invitrogen (Cat No.35-

1300) and Santa Cruze (Cat No.SC-21733) respectively. Suitable isotype control (IgG1 Kappa) was used.

Procedures of the indirect intracellular staining:

1- Bone marrow or peripheral blood EDTA samples were used provided they have malignant myeloid cells more than 20%, the cell count was adjusted to 10^4 and 50 µl were added to 100 µl of DAKO intra stain reagent A (fixative) (Cat No 60659) into 3 separate tubes, mixed gently and incubated at room temperature for 15 minutes.

2- The tubes were washed with 3mL PBS (0.01mol/L), centrifuged at 1500g and the supernatant was discarded.

3- One hundred µl of reagent B (permeablising solution) were added to all tubes and 5 µl of each monoclonal and isotype control were added to corresponding tubes, labeled and incubated for 30 minutes.

4- Step 2 was repeated, 50 µl of diluted (1:10) FITC labeled rabbit antimouse immunoglobulin (RAM) (DAKO. Cat. No. F0313) were added and incubated in the dark for 20 minutes. Step 2 was repeated, and cells were suspended in 500µl PBS for acquisition.

5- Samples of the control group were subjected to the same procedures.

Flowcytometric analysis:

Gelatinases expression was measured by (FACS Vantage: Becton Dickinson, San Diego, USA) using the Cell Quest software program (Becton Dickinson).

1- Full alignment procedures were performed each time using the standard Nile Red Beads (BD. Cat. No.347240) for adjusting forward scatter, side scatter and photomultiplier tubes.

2- Ten thousand events were passed in front of the laser for each sample tube and twenty thousand events for the control tube.

3- Analytical gates were set on the desirable viable cells based on forward and side light scatters combined with exclusion of normal cells using a CD45 tube. Threshold for positivity was based on isotype negative control. Antigen expression was determined as percent positivity of stained cells within the blast population.

4- The control sample: Lymphocytes were selected by their bright expression for CD 45 and analyzed for the expression of gelatinases A & B.

B- *RT-PCR for detection of IL-18 gene:*

1- Total RNA was extracted from 10^6 cells from B.M. EDTA sample using QIA Gen kit (Cat. No.52304).

2- One step RT-PCR QIA Gen kit (Cat. No. 210212) that combines cDNA synthesis from RNA with PCR amplification to provide a rapid, sensitive method for analyzing gene expression was used.

3- A pair of IL-18 specific primers: sense: 5'-GATGGCTGCTGAACCAGTAG-3' anti-sense 5'-GCTAGTCTTCGTTTTGAACAGTG-3' was designed to amplify 584 bp fragment of human IL-18.

4- An internal control, human glyceraldehydes 3-phosphate dehydrogenase (GAPDH) was also amplified using its specific primer: sense: 5'-GAAGGTGAAGGTCGGAGTC-3' anti-sense: 5'- GAAGATGGTGATGGGATTTC-3', that amplifies 1020 bp fragment of human GAPDH.

5- The total reaction volume was 50µl containing 2µg RNA, 400µM of each dNTP, 20pMol from each primer (forward and reverse primer for both IL-18 & GAPDH) and the enzyme Mix included in the kit (reverse transcriptases and hot start Taq polymerase) in 1 X RT reaction buffer.

6- The thermal cycle program included a step for reverse transcription (30min.50ºC), initial PCR activation step (15min.95ºC), 30 cycles consisted of denaturation (1 min. 94ºC), annealing (1 min. 58ºC) and extension (1 min.72ºC) and a final extension step (10 min.72ºC).

7- Ten microliters of the PCR products were subjected to electrophoresis on 2% agarose gel containing ethidium bromide. A molecular weight marker (200bp) was used to asses the positions of the defined DNA band. The gels were visualized under UV light.

8- Semi-quantitative determination of the PCR products was performed using complete gel documentations and analysis system (Biometra, Germany). Relative expression of IL-18 gene (R) was calculated as follow: R= Densitometrical Units of IL-18 / Densitometrical Units of GAPDH **[9]**.

9- Control samples were subjected to the same amplification procedures and analysis.

C- *Response to induction chemotherapy:*

All patients received induction chemotherapy; their response was evaluated after 2 weeks. Complete remission (CR) was defined as normocellular marrow with ≤5% blasts, no circulating blasts, no evidence of extramedullary leukemia and recovery of granulocytes to 1500/µl and platelets to 100.000/µl **[13]**. Unfavorable outcome includes refractory cases (did not achieve complete remission) and early death (death within 14 days after first diagnosis).

D- *Chemotherapy protocol:*

Patients with newly diagnosed AML (except APL/M3) received induction chemotherapy with combination of 7 days of cytosine arabinoside (100mg/m^2) and 3 days of adriamycin (45 mg/m^2). Patients who achieved CR and had favorable cytogenetics[t (8; 21) and inv 16] received consolidation chemotherapy with combination of high dose Ara-C (1gm/m^2 IV infusion over 3 hours/12 hours for 3 days) and mitoxantron (12mg/m^2 IV short infusion days 3-5) for total of 3-4 cycles. Patients with high risk cytogenetics (monosomy 7 or 5, deletion of 5q and abnormalities of 3q and those with a complex karyotype) or intermediate risk cytogenetics (those with normal cytogenetics and other changes not associated with high risk or favorable groups) were transferred for allogenic bone marrow transplantation if they had matched sibling donor after achieving CR. Patients who did not have matched donor received consolidation chemotherapy as in favorable group, then they were transferred for autologous bone marrow transplantation (ABMT). Patients who relapsed after conventional chemotherapy or failed to achieve CR despite optimal induction treatment received second induction and then were transferred for Allogenic BMT if they had matched sibling donor or ABMT if they did not have a donor. Patients who relapsed after bone marrow transplantation received palliative chemotherapy (as HAM, AVVV or FLAG).

Patients with acute promyelocytic leukemia (APL) received induction treatment with ATRA (45mg/m^2 p.o daily until CR or maximum of 90 days) and adriamycin (45mg/m^2 IV days 1-3 for 3 courses every 28 days). These patients received maintenance treatment after achieving CR with ATRA (45mg/m^2 p.o daily for 2 weeks every 3 month), 6-mercaptopurin (60mg/m^2 daily) and methotrexate (20mg/m^2 IV once weekly) for 2 years.

Statistical methods:

Data were analyzed using SPSSwin. version15. Numerical data were expressed as median and range because the data were not normally distributed. Qualitative data were expressed as frequency and percentage. Chi-square test (Fisher's exact test) was used to examine the relation between qualitative variables. For quantitative data, comparison between two groups was done using Mann-Whitney test. Comparison between 4 groups of FAB classification was done using Kruskal-Wallis test followed by post-Hoc comparison test "Schefe test" on the rank of variables to compare pairs of groups. Survival analysis was done using Kaplan-Meier method. Comparison between two survival curves was done using Log-rank test. Receiver Operating Characters (ROC) curve was used to determine the prognostic cut off for IL18 gene expression. *p*-value ≤0.05 was considered significant.

RESULTS

Clinical criteria of the studied AML patients are shown in Table (1).

Cellular expression of gelatinases A & B (MMP-2 & MMP-9) and IL-18 gene:

The median and range of gelatinase A and B expression levels in the lymphocytes in the control samples were 8 (2-19%) and 2.6 (0.3-9.5%) respectively. Accordingly, 20% and10% was considered the cut off for the expression of gelatinase A & B respectively.

Gelatinase A (≥ 20%) was expressed in 26/42 (61.9%) and gelatinase B (≥10%) in 14/42 (33.3%) of the studied cases. The median expression of gelatinase A was not significantly different among different AML FAB subtypes (p=0.545). However, gelatinase B expression was significantly higher in M3, where all the studied five M3 cases were highly expressing it (p=0.001) (Table 2 and Figs. 1, 2).

The median expression level of IL18 gene in the AML group was 1.5 with a range of (0.8-2) which was significantly higher than the control group (median=1.0, range 0-1.2) (p<0.001). Within the different FAB subtypes, the expression level of IL-18 gene showed a significantly higher expression in M4 and M5 subtypes (p= 0.003) (Table 2 and Figs. 3, 4).

Relation between gelatinases A & B (MMP-2 & MMP-9) expression and hematological data at presentation:

There was no significant association between gelatinase A & B expression and any of the hematological findings except for a significantly higher blast cell distribution ratio (BCDR) in gelatinase A positive group (p=0.004) and a significantly lower hemoglobin in the gelatinase B positive group (p=0.012) (Table 3).

Relation between IL-18 gene expression and initial TLC:

The studied group was risk stratified regarding TLC at presentation into TLC $\leq 50x10^9$/L and $>50x10^9$/L. The median expression level of IL-18 in the former group was 1.4 (0.8-2) and 1.6 (1.3-2) in the latter group; the difference was not statistically significant (p=0.081).

Relation between gelatinases expression and extramedullary infiltration:

There was a statistically significant higher incidence of splenomegaly among gelatinase A positive group regardless of the FAB subtype [22/26 (84.6%)] compared to 4/16 (25%) in the gelatinase A negative group (p<0.001). However, there was no statistical difference regarding hepatomegaly or lymphadenopathy (p=0.130 & 0.397, respectively).

On the other hand, there was no significant association between gelatinase B expression and splenomegaly, hepatomegaly or lymphadenopathy (p=0.261, 0.060 & 0.266, respectively).

Relation between the studied markers expression and response to induction therapy:

To examine the influence of gelatinases expression on remission probability, we compared their expression in patients who achieved complete remission with the unfavorable responders (refractory & early death). CR was significantly higher among the gelatinase A positive group (p=0.018), while it was significantly lower among the gelatinase B positive group (p=0.002).

As regards IL-18, a ratio of 1.5 was considered a prognostic cut-off. Accordingly, the studied group was classified into 2 groups: group that expressed IL18 ≤1.5 [23/39 (58.9%)] and group that expressed IL18 >1.5 [16/39 (41.1%)]. CR was significantly lower in the latter group (p=0.021) (Table 4 & Fig. 5).

To verify the effect of combined marker expression on response to induction, the patients were classified into two groups: a group that expressed 2 or 3 of the studied markers (19/39) and a group that failed to express any of the studied markers or expressed only one marker (20/39), showing a significantly lower incidence of CR with double or triple marker expression (p=0.038) (Table 4).

NB. Three cases were excluded from this analysis as their data were missing.

Survival analysis:

The median follow-up duration was 16 weeks (range 1.3-64 weeks). The overall survival (OS) was 55.6%. There was no significant difference regarding gelatinase A or B expression and OS (p=0.078 & 0.082 respectively). However the overall survival was significantly lower in patients with IL-18 ratio >1.5 (p= 0.019). Regarding combined marker expression and OS, the median survival was 7 week with combined marker expression compared to 32 weeks in the other group without a statistically significant difference between the two group (p=0.120) (Table 5).

N.B. Leukemia free survival (LFS), defined as the period lasts from achievement of CR till relapse, could not be studied in this work as relapse was documented only in two cases at 8 & 12 weeks after CR.

Table (1): Clinical criteria of the studied AML patients at diagnosis.

Number of patients	42
Male / Female (ratio)	20/22(0.9)
Age (years) median (range)	35 (18-60)
WBC (x 10^9) median (range)	25 (3-103)
FAB morphology (no., % of total):	
M1	12 (28.6)
M2	13 (31.0)
M3	5 (11.9)
M4	10 (23.8)
M5	2 (4.8)
Hepatomegaly (no., % of total)	27 (64.3)
Splenomegaly (no., % of total)	26 (61.9)
L.N enlargement (no., % of total)	8 (19.0)
Hb (gm/dl) median (range)	7.7 (4.5-10)
Platelets(103/µl) median (range)	44 (10-142)
P.B blasts median (range)	33 (0-89)
B.M blasts median (range)	61 (20-98)
BCDR* median (range)	0.7 (0-1)
*Response to induction chemotherapy (no., % of total)**:*	
Complete remission	21 (50)
Refractory	14 (33.3)
Early death	4 (9.5)

* Blast cell distribution ratio (BCDR): PB blasts/BM blasts.
** Three cases were missing.

Table (2): The median expression of the studied markers in relation to FAB subtypes in 42 AML cases.

	M1	M2	M3	M4+M5	*p* value
Gelatinase A (%)	24 (5-57)	31 (12-47)	18 (2.9-29)	17.5 (7-61)	0. 521
Gelatinase B (%)	2 (0.1-19) [a]	9 (0.9-37) [a]	47 (34-51) [b]	6 (0.3-11) [a]	0. 001*
IL-18 gene/GAPDH ratio	1.4 (0.8-1.5) [a]	1.6 (1.3-2) [a]	1.6 (1.5-1.9) [a]	1.8 (1.3-2) [b]	0.003*

Data as median (range). Groups with different letters are statistically significant. *Significant.

Table (3): Relation between gelatinases A & B (MMP 2 & MMP 9) and hematological data at presentation in 42 AML cases.

	Gelatinase A expression		*p* value	Gelatinase B expression		*p* value
	Positive	Negative		Positive	Negative	
TLC	25 (3-69)	44 (23-60)	0.151	25 (3-79)	33 (5-103)	0.679
Hb	7.8 (4.5-9.8)	7.6 (4.7-10)	0.726	6.1 (4.5-9.8)	8.1 (5.6-10)	0.012*
Platelets	41 (10-142)	69 (23-119)	0.143	34 (11-109)	45 (10-142)	0.262
P.B. blasts	41 (12-89)	16 (0-85)	0.060	40 (6-86)	33 (0-89)	0.915
B.M. blasts	61 (20-98)	67 (40-96)	0.182	68 (51-97)	61 (20-98)	0.069
BCDR	0.8 (0.2-1)	0.3 (0-0.9)	0.004*	0.6 (0.1-1)	0.7 (0-1)	0.749

Data as median (range). *Significant.

Table (4): Relation between studied markers expression and complete remission in 39 AML cases.

		Complete remission (CR) (n=21)	Non responders (n=18)	*p* value
Gelatinase A	positive	17 (81.0%)	8 (44.4%)	0.018*
	negative	4 (19.0%)	10 (55.6%)	
Gelatinase B	positive	2 (9. 5%)	10 (55.6%)	0.002*
	negative	19 (90.4%)	8 (44.4%)	
IL-18 gene/GAPDH ratio	≤1.5	16 (76.2%)	7 (38.9%)	0.021*
	>1.5	5 (23.8%)	11 (61.1%)	
Combined marker expression	0/1marker	14 (66.7%)	6 (33.3%)	0.038*
	2/3markers	7 (33.3%)	12 (66.7%)	

Table (5): Impact of the studied marker expression on survival in 39 AML cases.

		Cumulative survival %	Median±SE	95% confidence interval	*p* value
Gelatinase A	positive	71.2	31.0±8.3	14.8-47.2	0.078
	negative	28.5	4.0±0.6	2.8-5.2	
Gelatinase B	positive	18.7	5.0±0.8	3.5-6.5	0.082
	negative	70.3	32.5±4.1	24.4-40.6	
IL-18 gene/GAPDH ratio	≤1.5	73.2	32.5±1.1	30.2-34.7	0.019*
	>1.5	31.2	4.0±0.5	2.9-5.0	
Combined marker expression	0/1marker	39.3	7.0±1.5	3.9-10.0	0.120
	2/3markers	70.0	32.5±1.0	30.4-34.5	

SE: Standard error. *Significant.

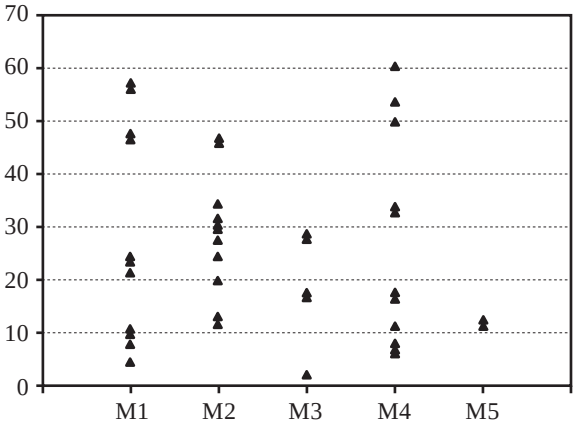

Fig. (1): Gelatinase A expression in relation to FAB subtypes in 42 AML cases

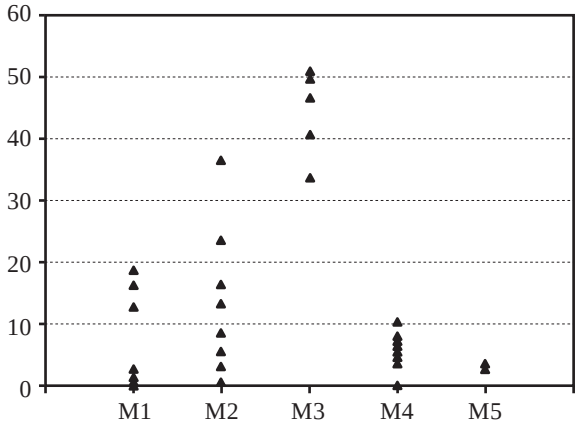

Fig. (2): Gelatinase B expression in relation to FAB subtypes in 42 AML cases

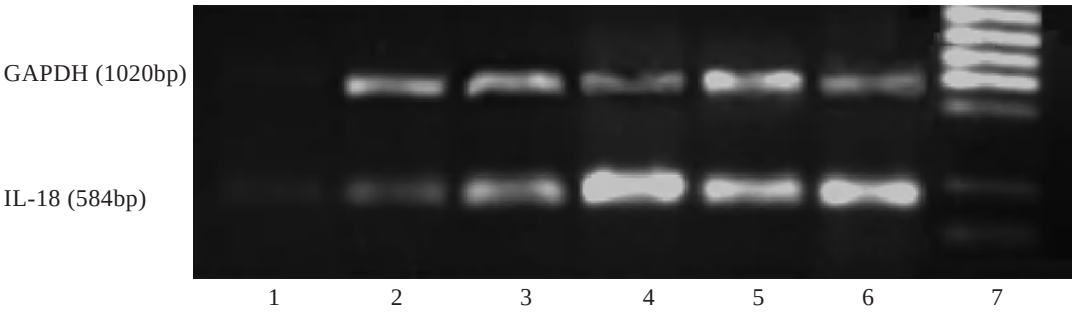

Fig. (3): IL-18 expression in bone marrow of patients with AML and controls.
Lane 1: negative control; 2: normal control; 3: M1; 4: M4; 5: M2; 6: M5 & 7: molecular weight marker.

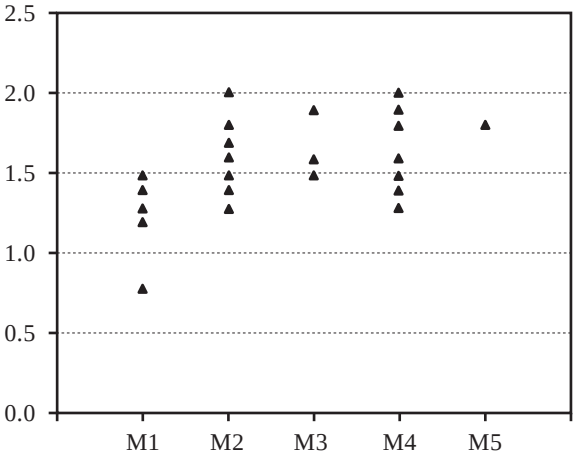

Fig. (4): IL18 gene expression in relation to FAB subtypes in 42 AML cases.

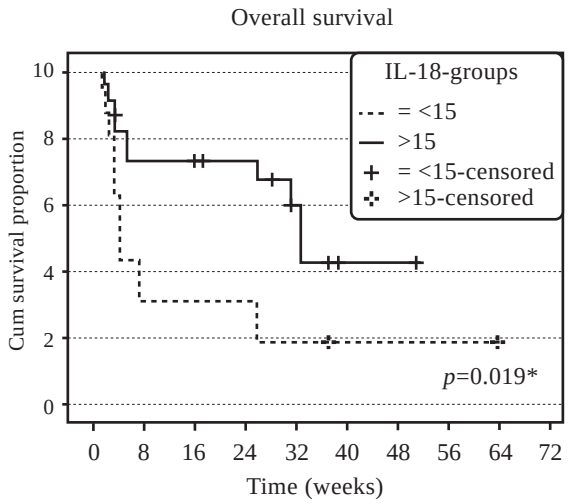

Fig. (5): IL18 gene expression in relation to overall survival in 39 AML cases

DISCUSSION

Knowledge about the biological and clinical role of gelatinases A & B (MMP-2 and MMP-9) in solid malignancies is rabidly increasing. However, in the field of hematological malignancies, the data are scarce **[4]**.

In the present work, expression of gelatinases A & B as well as IL 18 gene in blast cells from de novo adult AML cases was studied. Also, their significance in extramedullary invasion, disease progression, response to induction therapy and survival was evaluated.

In the current study, gelatinase A (MMP-2) was expressed in 26/42 (61.9%) in adult AML cases with no significant difference in its expression among different FAB subtypes (p= 0.545). Our data regarding the level of expression of gelatinase A are comparable to several published reports which ranged from 71-83% **[15,16,17]**. In agreement with our results Aref et al. found no correlation between pretreatment soluble gelatinase A (sMMP-2) levels and different FAB subtypes **[4]**.

In this study, gelatinase B (MMP-9) was expressed in 14/42 (33.3%) in adult AML cases with a significantly higher expression in M3 subtype (p=0.001). Our results regarding the level of expression of gelatinase B is higher than that reported by Kuittinen et al. **[15]** who detected MMP9 in only 1/35 (2.8%) of cases using immuocytochemistry (IHC). Our higher results could be explained by the fact that we measured expression of gelatinase B using flowcytometry which is known to be a more sensitive technique compared to IHC. However, our percentage of gelatinase B expression (33.3%) is lower than Janowska et al who detected MMP-9 mRNA by PCR in 4/6 (66.6%) of their cases **[17]**. Their higher percentage may be related to the small sample size which could not represent the studied group fairly or alternatively to using a different method.

In our work, gelatinase B (MMP-9) showed a significantly higher expression among FAB-M3 subtype and this supports what was previously reported by Ries et al. who found MMP-9 expressed predominantly in promyelocytic HL-60 cell line, which may be explained by the fact that gelatinase B normally secreted from the neutrophilic series specially the more mature stages **[18]**. However, other reports regarding expression of MMP-9 among AML FAB subtypes were conflicting with either no correlation **[19]** or a significantly higher expression among FAB-M5 **[20]**.

In this study, the incidence of extramedullary infiltration was significantly higher among gelatinase A (MMP-2) positive group as seen by the higher BCDR (a measure of blasts to mobilize peripherally) (p=0.004) as well as, the higher incidence of splenomegaly (p<0.001). However such correlation between extramedullary infiltration and gelatinase B (MMP-9) expression could not be obtained. Our results are in agreement with the previously published data by Sawicki et al. **[21]** who found that MMP-2 but not MMP-9 is involved in the invasive process of AML blasts by matrigel assay. However, our finding is not in accordance with that reported by Aref et al. **[20]** who found that serum level of MMP-9 correlated significantly with PB blasts, BCDR and extramedullary infiltration and Yang et al. **[22]** who found that both MMP-2 and MMP-9 participated in the extramedullary leukemic invasion of AML patients. Our results regarding the relation between gelatinase A expression and extramedullary infiltration support the suggestion that MMP2 might enhance the degrading function of leukemic cell, thus making the crossing of them through the bone marrow barrier easier with their subsequent release into blood.

In this study, the incidence of CR was significantly higher among gelatinase A (MMP-2) positive group (p=0.018). However, the overall survival (OS) did not differ significantly among gelatinase A positive and negative groups (p=0.078). Literature reports regarding correlation between OS and MMP-2 expression are conflicting. While some reports showed better OS among MMP-2 positive AML patients than among MMP-2 negative patients **[15,21,23,24]**; others showed that high serum levels of MMP-2 was associated with short OS in AML patients **[4]**. This discrepancy between results regarding MMP-2 protein expression and good prognosis in AML may be related to measuring the MMP protein or mRNA levels instead of measuring the enzyme activity.

In this work, CR was significantly higher among gelatinase B (MMP-9) negative group (p=0.002). However, no correlation between gelatinase B expression and OS was found (p=0.082). Lin et al. **[19]** found that lower marrow levels of MMP-9 was significantly associated with both higher CR and better OS rates (p=0.001 & 0.012 respectively). Also, Aref et al. **[20]** found that a lower serum level of MMP-9 is associated with higher incidence of CR and better OS. Absence of statistical significance regarding OS and gelatinase B expression may be due to the small sample size.

In the present work, IL18 gene expression in AML group was significantly higher than the control group (p<0.001). All FAB subtypes had

significantly higher values than the control, with highest expression detected in M4 and M5 FAB subtypes (p=0.003). Similar results were obtained by many authors **[9,12]**. The possible source of augmented IL-18 gene expression may be the leukemic cells or the stromal cells or both. The higher levels obtained in monocytic leukemia may be due to the fact that IL 18 is mostly expressed in activated macrophages and monocytes derived cells **[9]**.

While Zhang et al. **[9]** found a significantly higher median IL 18 gene expression among AML patients with high initial TLC (p=0.02), we could not confirm this finding in our work though the median IL 18 gene expression was higher among patients with elevated TLC group (p=0.081) which may be related to ethnic variation.

In this work, the median expression of IL18 was higher among the non responders (refractory & early death) and those with short overall survival (p=0.021 & 0.019 respectively). To our knowledge the relation between IL18 expression and response to treatment and survival has not been studied before. From our results, IL 18 may be considered as a poor prognostic factor in AML as evidenced by its correlation with poor response to treatment and short OS. Additionally IL 18 may be considered a positive regulatory factor for proliferation of leukemic cells as seen by its tendency to be higher among patients with high initial TLC and its correlation to FAB-M4 & 5 subtypes. However, further studies are needed to understand the full significance of IL 18 in leukemogenesis.

To our knowledge, this study is the first to address the relation between combined marker expression (Gelatinases A, B & IL18) and their clinical relevance regarding response to induction and OS, where only 7/19 (36.8%) patients that showed double or triple marker expression achieved CR compared to 14/20 (70%) in patients who failed to express any of the studied markers or expressed only one marker (p=0.038) suggesting an additive or cumulative effect of these markers on response to chemotherapy. However, we could not reach such results with OS although the median survival was 7 weeks with combined marker expression compared to 32 weeks in the other group.

Conclusion:

The expression of gelatinase A (MMP-2) by AML blasts supports the hypothesis that in AML, MMP-2 may be a marker of the invasive phenotype and may suggests a novel therapeutic approach in AML. Gelatinase B (MMP-9) expression might carry a poor prognosis in AML. IL18 may be considered as a poor prognostic factor acting as a positive regulator for proliferation. Combined expression of gelatinases and IL18 gene overexpression could have cumulative effect on response of AML patients to chemotherapy. In summary; these data suggests that angiogenic factors may have a role in the leukemic process and that anti-angiogenic therapy could be a new therapeutic strategy in AML. However, to validate these assumptions, a study on a larger number of patients is required.

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Outcome of Patients with Non-Hodgkin's Lymphoma: Correlation with Apoptotic Markers

YOUSREYIA A. AHMAD, M.D.; SOHEIR M. AHMAD*, M.D.; MAHMOUD A. ASHRY, M.D.; OSAMA A. IBRAHIEM, M.D.; HANAN G. ABDEL-AZEEM, M.D.* and MOHAMAD R. ABDEL-HAMEED, M.D.

The Departments of Internal Medicine and Clinical Pathology Faculty of Medicine, Assiut University, Assiut.*

ABSTRACT

Abnormalities in specific cell cycle control genes as B-cell lymphoma-2 (Bcl-2) family proteins and regulator of apoptosis as Fas/APO-1(CD95) commonly found in lymphoid neoplasm. The study aims to identify factors that can help in predicting the behavior of non-Hodgkin's lymphoma, selecting patients with higher response to different chemotherapeutic modalities and early detection of recurrence.

Thirty newly diagnosed Non-Hodgkin's lymphoma patients before treatment (group I), after receiving 4 cycles of chemotherapy (group II) and ten apparently healthy volunteers were chosen as controls (group III) were included in the study. All groups were studied for presence of Bcl-2 and human Fas/APO-1by ELISA.

Bcl-2 is significantly higher (p<0.001) in patients groups I and II compared to group III. Also, Bcl-2 serum levels of patients after treatment (group II) had highly significant reduction (p<0.001) comparable to group I. In addition, Fas/APO-1 represents significantly higher values in patients groups I and II compared to group III (p< 0.001) with statistically significant elevation among patients who attained complete remission than in those with partial or no remission after treatment.

It could be concluded that the elevated levels of Bcl-2 in NHL patients has been contributed to its role as a major anti-apoptotic factor in tumor genesis. However, its significant reduction after chemotherapy indicates that function of Bcl-2 is dependent upon which other members of the Bcl-2 family that is involved in controlling the upstream and downstream events. While Fas/APO-1 could be considered as an important apoptotic marker in NHL and can be considered as a good predictor for response to chemotherapy.

Key Words: *NHL – Apoposis – Bcl-2 – CD95.*

INTRODUCTION

Apoptosis is an intrinsic cell death program that plays critical roles in tissue homeostasis, especially in organs where high rates of daily cell production are offset by rapid cell turnover **[1]**. The hematopoietic system provides numerous examples confirming the importance of cell death mechanisms for achieving homeostatic control. It is characterized by a series of morphological and biochemical changes **[2,3]**.

Non-Hodgkin's lymphomas are heterogeneous group of disorders with different clinical characteristics that usually relapsed and became difficult to control. Therefore, identification of factors that can help in predicting the behavior of the disease and selecting patients with higher response to different chemotherapeutic modalities or detection of recurrence at earlier stages before clinical manifestations is important **[4-9]**.

B-cell lymphoma-2 (Bcl-2) family protein is a critical regulator of apoptosis, whose expression frequently becomes altered in human cancers, including lymphoma. Bcl-2 was the first member to be identified by virtue of its involvement in chromosomal translocation *t* (14:18), commonly found in B-cell non-Hodgkin's lymphoma **[10-12]**.

An important regulator of apoptosis is Fas/APO-1 (CD95) which is a membrane glycoprotein belonging to the tumor necrosis factor/ nerve growth factor receptor family, which can trigger apoptosis in some lymphoid cell lines **[1,13-16]**.

PATIENTS AND METHODS

This study was performed in Clinical Hematology Unit of Internal Medicine Department,

Assiut University Hospitals. The study included thirty patients (22 males and 8 females) with Non Hodgkin's Lymphoma. Their mean age was 42±7.13 years. Those newly diagnosed before receiving radio or chemotherapy were considered as group (I). The same patients after receiving 4 cycles of chemotherapy were considered as group (II). Ten apparently healthy volunteers were chosen as a control group (III) and they were age and sex matched to the patients.

Inclusion criteria for patients with NHL (group I):

Patients newly diagnosed with documented histopathological diagnosis of NHL who were not receiving any radio or chemotherapy.

Exclusion criteria of patients with NHL:

1- Patients with NHL who previously received steroids, chemotherapy or recombinant cytokine therapy.
2- Patients with NHL in relapse.

All patients were subjected to full history taking, thorough clinical evaluation, baseline investigations and specific investigations for detection of Bcl-2 protein and human FAS/APO-1 using ELISA technique.

All patients were treated with (CVP) regimen for low grade and (CHOP) chemotherapy regimen for intermediate and high grade lymphoma. They were subdivided according to the response of treatment into those acquired complete remission (CR): 12 patients, partial remission (PR) 4 patients, no remission (NR) 12 patients according to Banadonna **[17]**. Two patients were defaulters before completing 4 cycles of chemotherapy.

Statistical analysis:

The data of each group are tabulated in the Microsoft Excel 97 program from the master sheet giving a serial number for each subject, then expressed as mean ± SE for all parameters. The data were analyzed by using GraphPad Prism data analysis program (GraphPad Software, Inc., San Diego, CA, USA). For the comparison of statistical significance between patients and normal subjects, Student Newman-Keuls *t*-test for unpaired data was used. For each group paired two tailed Student's "*t*" test was used to compare values of varies parameters tested pre and post chemotherapy, the differences were considered statistically significant when *p* values were less than or equal 0.05.

RESULTS

Clinical features of patients with NHL group I (before start of treatment) and group II (after 4 cycles of chemotherapy) are illustrated in Table (1).

Table (1): Clinical features of patients with NHL group I and II.

Group / Clinical data	Group I (n = 30)		Group II (n = 28)	
	Number	%	Number	%
Lymphadenopathy	26	86.6	12	42.8
Splenomegaly	4	13.3	1	3.6
Hepatosplenomegaly	6	20	5	17.8
B symptoms	20	66.6	17	60.7

B symptoms: include drenching night sweats, unexplained fever more than 38C°, metabolic wasting more than 10% of body weight in the preceding 6 months.

Disease characteristics of patients with NHL group I (before start of treatment) and group II (after 4 cycles of chemotherapy) are presented in Table (2).

Only erythrocytic sedimentation rate (ESR) showed statistically significant increase in the first and the second hours measurements in groups I and II versus control group ($p<0.001$) Table (3). After receiving four cycles of chemotherapy statistical significant reduction ($p<0.001$) was observed in ESR (1st and 2nd hours) in group II when compared to group I (Table 3).

Bcl-2 serum levels in patients with NHL group I and II showed highly statistical significant increase ($p<0.001$) than that in the control group (246.7±6.88 and 206.85±8.1versus 148.62±8.69 ng/ml). Also, mean Bcl-2 serum levels of patients with NHL group II had highly significant reduction ($p<0.001$) comparable to group I (206.85±8.1 ng/ml versus 246.7±6.88) (Table 4).

When outcome was considered patients with NHL who developed complete remission showed significant decrease ($p<0.01$) in serum level of Bcl-2 (201.75±8.68) after receiving four cycles of chemotherapy compared to that before therapy (251.25±10.47) while patients

with NHL who developed PR or NR showed insignificant changes in relation to their levels before therapy (Table 5, Fig. 1). Serum levels of Bcl-2 in patients who developed CR, PR or NR showed insignificant changes when compared to each other either before or after treatment (Table 5, Fig. 1).

Fas/APO-l levels were significantly very high in sera of patients with NHL before treatment (group I) and after treatment (group II) and both showed high statistical significant values (p<0.001) as compared with levels in the control (group III) with mean value ± SE of 9.676±0.53, 8.610±0.90 versus 2.528±0.71 ng/ml respectively (Table 6). Fas/APO-l in patients after treatment showed statistical significant elevation (p<0.01) among those who had CR than in those with PR or NR (16.44± 0.88, 9.52±2.99 and 10.47±1.5 ng/ml respectively), Table (7), Fig. (2).

Table (2): Different disease characteristics in NHL patient's group I and II.

Group / Parameter	Group I (n = 30)		Group II (n = 28)			
	Number	%	CR	PR	NR	Total and % of total
Stage:						
I	9	30	5	1	4	10 (35.7%)
II	11	36.7	4	1	4	9 (32.1%)
III	9	30	3	1	4	8 (28.6%)
IV	1	3.3	0	1	0	1 (3.6%)
Grade:						
Low	4	13.3	2	0	2	4 (14.3)
Intermediate	23	76.7	10	2	10	22 (78.6)
High	3	10	0	2	0	2 (7.1%)
Histopathology:						
Diffuse	20	66.7	10	2	7	19 (67.9%)
Follicular	10	33.3	2	2	5	9 (32.1%)
Presentation:						
Nodal disease	26	86.7	9	4	12	25 (89.3%)
Extra nodal disease	4	13.3	3	0	0	3 (10.7)
Bone marrow infiltration						
Present	3	3.3	0	0	1	1 (3.6%)

CR: Complete remission NR: No remission PR: Partial remission

Table (3): Comparison of the total leucocytic counts, platelet counts, hemoglobin levels and erythrocytic sedimentation rate in patient groups I, II and controls.

Group / Parameter	Group I (before treatment) N = 30	Group II (after treatment) N = 28	Group III (control) N = 10	p-value		
				I vs. II	I vs. III	II vs. III
TLC (x 10^9/L)	6.86±3.25	6.66±2.33	5.83±1.13	NS p=0.366	NS p=0.339	NS p=0.288
Hb (gm/dl)	12 .92±1.38	11.26±2.27	14.74±1.70	NS p=0.286	NS p=0.749	NS p=0.076
Platelets (x 10^9/L)	254.6±123.9	239.4±99.9	240.2±60.96	NS p=0.299	NS p=0.726	NS p=0.982
ESR (1^{st} Hour) mm	36.3±22.97	19.25±13.9	7.1±1.52	*** p<0.001	*** p<0.001	*** p<0.001
ESR (2^{nd} Hour) mm	61.3±34.06	33.25±23	12.5±1.5	*** p<0.001	*** p<0.001	*** p<0.001

NS: Non significant ***: p<0.001 TLC= Total leucocytic count
Hb= Hemoglobin ESR= Erythrocytic sedimentation rate

Table (4): Bcl-2 serum level in NHL before treatment (group I), NHL after treatment (group II) and controls (group III).

Group / BCL-2 (ng/ml)	Group I (before treatment) N = 30	Group II (after treatment) N = 28	Group III (control) N = 10	*p*-value		
				I vs. II	I vs. III	II vs. III
Mean ± SE	246.7± 6.88	206.85± 8.1	148.62± 8.69	***	***	***

***: $p<0.001$

Table (5): Comparison between level of Bcl-2 before and after treatment in NHL patients in relation to response of treatment.

Subject / Response	CR N = 12	PR N = 4	NR N = 12	Significance		
				CR vs. PR	CR vs. NR	PR vs. NR
Bcl-2 (ng/ml) Before treatment (mean ± SE)	251.25±10.47	235±18.38	244.66±10.6	NS	NS	NS
Bcl-2 (ng/ml) After treatment (mean ± SE)	201.75±8.68	203.25±32.63	213.16±14.33	NS	NS	NS

$p>0.050$ is not significant (NS) PR: Partial remission CR: Complete remission NR: No remission

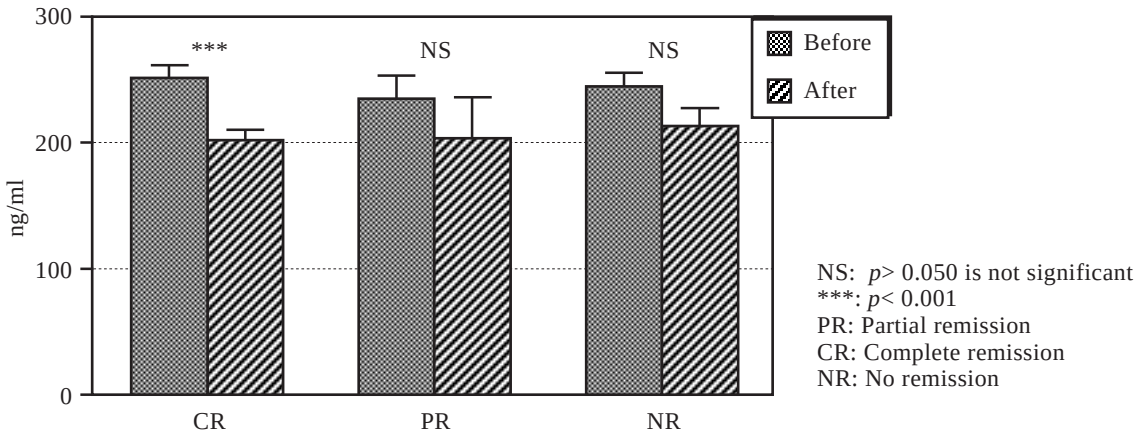

Fig. (1): Mean values ± SE of BCL-2 serum level before and after treatment in NHL patients in relation to response of treatment.

Table (6): Fas/APO-1(CD95) serum levels in patients with NHL before treatment (group I), after treatment (group II) and controls (group III).

Fas/APO-1 (CD95) ng/ml	Group I (before treatment) (n=30)	Group II (after treatment) (n=28)	Group III (control) (n=10)	*p*-value		
				I vs. II	I vs. III	I 1vs. III
Mean ± SE	9.676±0.53	8.610±0.90	2.528±0.71	NS	***	***

***: $p< 0.001$ NS: Non significant

Table (7): Comparison of serum levels of APO-1/FAS before and after treatment in relation to the response to treatment.

Subject / Response	CR N = 12	PR N = 4	NR N = 12	Significance		
				CR vs. PR	CR vs. NR	PR vs. NR
Fas/APO-1 (ng/ml) Group I (Before treatment)	11.23±1.2	8.42±0.13	8.76±0.20	**	**	NS
Fas/APO-1 ng/ml Group II (After treatment)	16.44±1. 88	9.52±2.99	10.47±1.54	**	**	NS

NS= Non significant **= $p< 0.01$ PR: Partial remission CR: Complete remission NR: No remission

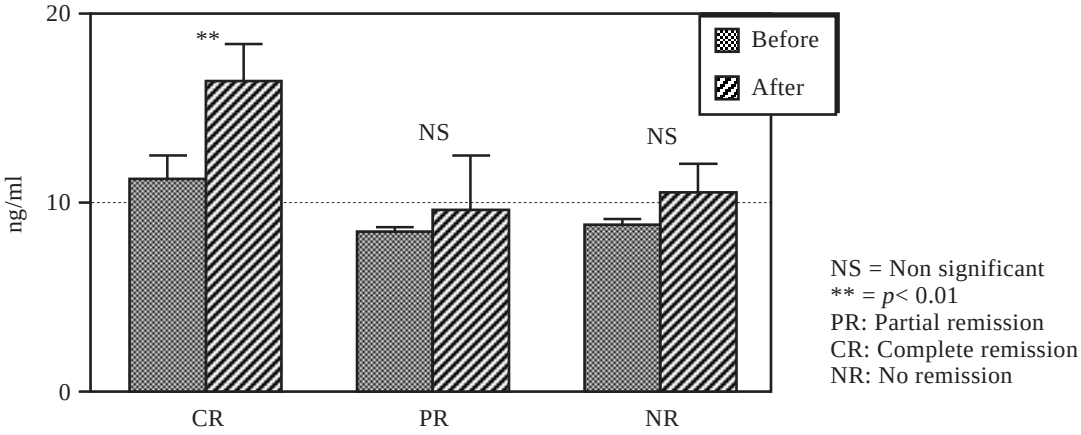

Fig. (2): Mean values of serum Fas/APO-1 in NHL patients before and after treatment with treatment outcome CR, PR and NR.

DISCUSSION

Some proteins regulating apoptosis as Bcl-2 and Fas/APO-1 (CD95) had been studied in 30 newly diagnosed patients with NHL. The clinical significance of Bcl-2 and Fas/APO-1 has been the subject of many studies **[10,15,16,18, 19,20]**. To our knowledge no clear-cut conclusions have been drawn regarding the prognostic importance of any of these parameters. This triggers us to conduct this study to clarify the interplay between apoptotic markers as Fas/APO-1 and proliferation markers as Bcl-2 with variable levels in various presentations in NHL, a finding that may be useful in predicting treatment outcomes.

The clinical characteristics of the patients included in this study were the predominance of peripheral lymphadenopathy in 86.6% of group I patients regressed to 42.8% after they completed 4 cycles of chemotherapy, hepatosplenomegaly in 20% reached only to 17.8% in patients group II. Also, 13.3% of patients group I had splenomegaly became only 3.6% after chemotherapy indicating that primary nodal and splenic infiltration responded more frequently to chemotherapy than extra nodal infiltration when the liver was affected. These data were consistent with that documented by Burkhardt et al.; Longo et al.; Yu et al. and Karadeniz et al. **[7,21,22,23]**. Also, B symptoms were present in 66.6% of patients group I reduced to 60.7% of group II patients, which is in agreement with results of Kamienska; Bakermerer et al.; Au et al. and Hwang et al. **[5,24, 25,26]**.

Treatment outcome of our patients after completing 4 cycles of chemotherapy was: CR in 12 patients (40%), PR in 4 patients (13.34), NR in 12 patients (40%) with 2 defaulters (6.66%). This is consistent with the finding of Karadeniz et al.; Reed and Akhmedkhanov et al. **[23,27,28]** as they reported that almost all anticancer drugs available can kill tumor cells by activating endogenous biochemical pathways for cell suicide, known as programmed cell death or apoptosis. However, many malignant cells develop defects in the regulating genes that control the apoptotic pathway, thus rendering such cells more resistant to the used regimen of chemotherapy that may result in partial remission or no remission at all.

In relation to disease staging, there was increase in percentage of patients stage I and decrease in percentage of stages II and III after receiving chemotherapy indicating disappearance of one or more of lymph node groups with therapy without changes in extra nodal infiltration in stage IV. These findings are coinciding with those of Armitage and Weisenburger (1998), Marsden et al.; Salies and Na et al. **[29-32]**.

Peripheral blood counts in the patient's groups were within normal ranges either at the start or after receiving four cycles of chemotherapy, which could indicate absence of bone marrow infiltration, present only in one patient. This disagrees with List et al. **[33]**, who reported anemia in most of their patients at presentation secondary to lymphatic infiltration of bone marrow but coincided with data mentioned by Ansell et al. **[34]**.

Erythrocytic sedimentation rate (ESR) showed highly significant higher values in patients with NHL both before and after receiving chemotherapy compared to control group due to disease effect. After receiving chemotherapy, highly significant reduction in ESR values in patients group II compared with group I indicating good response to treatment. These results matched with that of Banadonna and Ansell et al. **[17,34]**.

The Bcl-2 class of anti-apoptotic protein is an important inhibitor of the mitochondrial mediated pathway of apoptosis by preserving mitochondrial membrane integrity and preventing the release of cytochrome-C and other pro apoptotic molecules from the mitochondria to the cytosol or the nucleus **[30,35]**. Also, Bcl-2 has been suspected to be a major regulator of a homeostatic balance between cell survival and cell death, and can slow cell proliferation in both lymphocytes and myeloid cells **[11,36]**.

In the present study, Bcl-2 protein detected in sera of NHL patients before receiving chemotherapy was heterogeneous due to case to case variability (ranged from 197 to 316 ng/ml). However, the mean value was significantly higher in patients with NHL than that in control group (246.7 versus 148.62 ng/ml). This finding is consistent with that of Salem et al.; Tang et al.; Yunis et al. and Pezzela et al. **[8,10,37,38]** who reported that high levels of Bcl-2 protein was observed in follicular and diffuse non Hodgkin's Lymphoma.

In the current study, the mean values of Bcl-2 protein in sera of patients before treatment (group I) showed very high statistical significant level when compared to that after treatment (group II). This finding may explain the apoptotic effect of used chemotherapeutic agents, which was antagonized by the antiapoptotic Bcl-2 protein. This is consistent with the finding of Schmitt and Lowe **[39]** who found that high levels of initial Bcl-2 suppress apoptosis induced by depletion of survival factor, hypoxia and cytotoxic drugs. Also, Schendel et al. **[40]** suggested that Bcl-2 forms ion channels that allow the transport of an ion or a protein across the mitochondrial membrane in the direction that is presumably cytoprotective (anti-apoptotic). Alternatively Reed **[27]**, suggested that Bcl-2 forms cytotoxic channels and protects cells by nullifying the channel activity and thus promoting cell survival. On the contrary, **[11,12,41-44]** suggested that Bcl-2 has different anti-apoptotic functions depending on the level of Bcl-2 at the pro-apoptotic stages.

So, the function of Bcl-2 is dependent upon which other members of the Bcl-2 family that is dimerzes with or upon the phosphorylation status of Bcl-2 **[45,46]** and upon the upstream and downstream events **[47]**. The net effect of the Bcl-2 family may be pro-apoptotic despite the high level of Bcl-2 due to over expression of other pro-apoptotic members, or high Bcl-2 are a response to the stimulation of pro-apoptotic family members and that the cells need to maintain very high levels to prevent apoptosis.

These cells presumably are living on the edge of apoptosis, and because Bcl-2 expression is near its maximum, it cannot be raised further in response to an apoptotic signal, so the cell dies **[44,47]** in contrast that cell with lower Bcl-2 level do not require Bcl-2 for protection, and there is sufficient room for increases in Bcl-2 expression to occur in response to an apoptotic signal. Another possibility in those cells with lower levels may develop other methods of avoiding apoptosis (i.e. high expression of Bcl-XL), **[48]** or that downstream regulators of apoptosis are modified **[47]**.

In the current study there was no association between Bcl-2 expression and treatment outcome. This result is consistent with the study of Elbordini et al. **[49]**, who reported the same results. Another study by Reed **[50]** stated that elevation of Bcl-2 expression could cause drug resistance to chemotherapeutic agents, thus supporting the anti-apoptotic function of Bcl-2 while decrease in Bcl-2 expression promotes the apoptotic response to anticancer drugs.

An important factor of apoptosis in the immune system is Fas/APO-1 a synonym for CD95, a transmembrane receptor that is a member of the tumor necrosis factor and nerve growth factor receptor super family **[44,51]**.

In the present study Fas/APO-1 levels were significantly very high in sera of both NHL patients before treatment (Group I) and after 4 cycles of chemotherapy (Group II) than in group III (controls). These are consistent with the reports of Beltinger et al. **[52]** who found that Fas/APO-1 was detected in sera of patients with

human T-cell leukemia and was increased in the sera of patients with lymphoid malignancies and also in agreement with Xerri et al. and Zodelava & David **[13,53]** who reported that CD95 can trigger apoptosis in some lymphoid cell lines.

The report of AkhmedKhanov et al.; Kobayashi & Koike; Kono et al.; Kamihira & Yamada and Shimizu et al. **[28,54-57]** suggested that elevated Fas/APO-1 production may promotes tumor genesis and disease progression. In addition, Kondo and colleagues **[57]** reported that high CD95 levels had been detected in NHL patients also by immunohistochemical analysis. As well as the reports of Hara et al.; Reed & Pellecchia; Knipping et al. and Papoff et al. **[14,44,59,60]** who found that sera of patients with different high and low grade malignant B and T cell lymphomas had an increased levels of Fas/APO-1.

In this study, Fas/APO-1 in the sera of patient group II, showed significant statistical elevation among those who had CR than in those with PR or NR. This result is consistent with the study of Elbordini and Co-workers **[49]**, who found that NHL patients who had high CD95 levels tended to be more sensitive to the used drugs and their patients acquired complete response than those with low levels. Also, interesting studies by Hara et al.; Lajmanovich et al.; Hazar et al.; Trauth et al.; Falk et al.; Aftabuddin et al.; Robertson et al. and Schattner et al. **[14-16,61-65]** in NHL patients with high levels of CD95 showed a higher apoptotic cell count than those with low levels of CD95 suggesting that Fas/APO-1 is involved in the apoptotic response of tumor cells to chemotherapy in NHL. In addition they reported that defect in apoptotic effect delivered through this antigen may contribute to the pathogenesis of hematological neoplasm.

So, from the results of this study we can conclude that increased serum levels of Bcl-2 in patients with NHL before treatment may indicate the possible anti-apoptotic role of Bcl-2 in tumor genesis, which antagonizes the effect of chemotherapy as inducer of apoptosis. Also, elevated levels of Fas/APO-1 is an important apoptotic marker in NHL, as it can reduce tumor genesis and tumor progression so it can be considered a good predictor for response to chemotherapy.

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Morphological and Immunophenotypic Heterogeneity of Pediatric Acute Megakaryoblastic Leukemia (M7)

AZZA M. KAMEL, M.D.; HEBA S. MOUSSA, M.D.; YASSER H. EL-NAHASS, M.D.; NAHLA EL-SHARKAWY, M.D.; DINA A. YASSIN, M.D.; KHALED M. SHAABAN, M.B.B.C.H. and IMAN SIDHOM, M.D.*

The Departments of Clinical Pathology, Pediatric Oncology, NCI, Cairo University.*

ABSTRACT

Background: M7 was first recognized by the FAB group as a separate subtype of AML in 1985. Since then, variability in its relative frequency has been reported. A major cause of this variability could be attributed to morphological heterogeneity. Some cases may lack the classical morphology; these may not be tested for the specific megakaryocytic markers and will be misdiagnosed as other FAB subtypes of AML.

Aim of the Work: In this work we aimed to investigate the true relative frequency of M7 within pediatric AML diagnosed at NCI, Cairo University. We also wanted to emphasize the morphologic heterogeneity that might lead to missing a diagnosis of M7.

Patients and Methods: In the years 2003-2005, 280 cases were diagnosed as pediatric AML (age <18 years). The diagnosis was established according to standard methods including clinical picture, complete blood count, bone marrow aspiration, cytochemistry and immunophe notyping. Bone marrow biopsy was performed when the aspiration was not adequate. All M7 cases were revised for clinical, morphological and immunophenotypic findings. Data on trisomy 21 were obtained from the files. Four M7 cases were tested for t (1; 22) and one was tested for BCR/ABL p 210 chimeric gene.

Results: M7 was encountered in 17/280 (6%) of the cases. Patients included 12 males and 5 females. They showed an age range of 2 days -14 years, median 3 years; they included 3 infants four of them were associated with Down's syndrome including the 2 days old one. Typical M7 morphology with large blasts showing cytoplasmic blebbing was obtained in 12 cases (70.6%); one of them showed many micromegakaryocytes with platelet budding and thrombocytosis of $750x10^9$/L. This latter case expressed BCR/ABL fusion gene p 210 and proved to be acute crisis on top of CML. The other 5 cases (29.4%) showed morphology suggestive of AML in three and of ALL in 2 cases. Bone marrow blasts were <20% in 2 cases and hence bone marrow trephine was performed. Cytochemistry showed a characteristic pattern of acid phosphatase in all cases with strong cytoplasmic positivity distributed in the whole cell. Diagnosis was confirmed in all cases by expression of CD 61 and/or CD 41. CD 41 and CD 61 were both coexpressed in 8/15 (53.3%) of cases tested for both. Four out of 7 remaining cases showed cyt CD 61 only. One case was positive for CD 41 only (CD 61 was 17%) and two cases were positive for CD 61 alone (CD 41 was 4 & 14%). Cyt CD 61 was the tool for diagnosis in one more case. In the remaining case, CD 41 was 90% and CD 61 was not tested. Coexpression of megakaryocytic antigens with other myeloid antigens namely CD 13 and/or CD 33 was encountered in 9 cases (52.9%). CD 7 was expressed in 2 cases, one of them expressing CD 56 as well, and the other was expressing CD 2. CD 4 was encountered in one case. t (1; 22) was encountered in 1/4 cases tested.

Conclusion: M7 showed marked morphological het erogeneity with 29.4% lacking the typical features. These cases could be missed especially if they are expressing myeloid markers or occasionally markers of other lineages. CD 61 and CD 41 should be tested in all cases of AML as well as in cases lacking other lineage markers. Cy tochemistry especially the characteristic pattern of acid phosphatase could be highly suggestive of M7 and directs attention to testing for CD 61 and CD 41 expression.

Key Words: *M7 – Pediatric leukemia – AML – Immunophenotypic.*

INTRODUCTION

Acute Megakaryoblastic Leukemia (M7) comprises a heterogeneous group of disorders that are considered to represent clonal expan sions of megakaryocytic cells, which have un dergone malignant transformation and are ar rested at distinct stages of differentiation **[1]**. Although M7 was first described in 1931 **[2]**, it was not included in the original classification of acute leukemia (AL) of the French-American-British (FAB) leukemia Cooperative Study Group because of the difficulty in recognizing megakaryocytic cell lineage precursors. For this

reason, the disease was considered for many years to be a rare variant of acute myeloid leukemia (AML). In addition, inaspirable bone marrow has contributed to the difficulty in establishing the diagnosis. However, with the development of ultrastructural cytochemistry, immunocytochemistry and monoclonal antibodies (MoAbs) against platelet specific glycoproteins (Gps), it soon became apparent that M7 occurred more frequently than previously suspected **[3,4]**. This fact prompted the FAB group to establish the criteria for the diagnosis and to add this category as a distinct subtype of AML-M7 in 1985 **[5]**.

The incidence of M7 has been estimated to be 4-7% of pediatric AML in large Cooperative Group studies in developed countries **[6,7,8]**. However, higher incidence rates of 10-15% and 29% were reported **[9,10]**. The peak age for M7 in children is 1-3 years in those with and without Down's syndrome **[10,11]**. A special type of M7 is seen in Down's syndrome (DS) that may present in the neonatal period and remits spontaneously **[12]**; in which case the disorder is termed transient myeloproliferative disorder or transient abnormal myelopoiesis (TAM). Around a quarter of these cases recur later on in life into frank M7, but typically respond well to a modified therapeutic regimen for childhood acute myeloid leukaemia. Recent evidence shows that M7 occurring in a patient with a history of TAM may represent clonal evolution of the same disease **[13]**.

Recently t (1; 22) (p 13; q 13) has been described to be associated with M7 **[14]**. It occurs mainly in infants, but is uncommon in cases with Down's syndrome and accounts for one third of other pediatric M7 cases **[6,14,15,16]**. The genes RBM15 on chromosome 1p13 and MLK1 on 22 q 13 have been identified leading to an RBM15/MLK1 fusion gene and a fusion transcript **[17]**. Cases with detectable RBM15/MLK1 fusion transcripts in the absence of t (1; 22) have been reported as well **[14]**. In the small number of children reported, the presence of the t (1; 22) appears to be associated with poor prognosis, though long-term survivors have been noted following intensive therapy **[15]**.

The aim of this study was to assess the incidence, clinical features at presentation, hematological, immunophenotypic and when possible molecular characteristics of M7 in pediatric AML cases in Egypt. We also wanted to emphasize the morphologic heterogeneity that might lead to missing a diagnosis of M7.

PATIENTS AND METHODS

This study included all cases (17 cases) diagnosed as M7 in the period 2003-2005. All patients presented to the Pediatric Oncology Department at NCI, Cairo University. During the study period, 280 pediatric AML cases were newly diagnosed. Written informed consents were obtained from the patients' guardians to use the data in the records and the protocol was approved by the Institution Research Board. Diagnosis of AML was performed according to standard criteria including clinical, morpholog ical and cytochemical criteria as well as immu nophenotyping. The FAB subtype was deter mined **[5]** and cases with diagnosis of de novo M7 were selected. Details of clinical presenta tion, laboratory findings at the time of diagnosis, therapy received, and outcome were collected from patients' medical records. The diagnosis of M7 was established on the basis of the FAB criteria by studies of morphology (peripheral blood (PB), bone marrow aspirate (BMA), as well as BM biopsy in selected cases), cytochem istry (Sudan Black B (SBB), Acid Phosphatase (AP) and alpha naphthyl acetate esterase (ANAE) **[5]** and was finally confirmed by im munophenotyping (CD 41 & CD 61) **[9]**. Cyto genetics for Down's syndrome was available in patients' files. The 17 M7 cases showed an age range from 2 days to 14 years with a median of 3 years; they included 3 infants they were 12 males and 5 females. Four patients had Down syndrome. They were all males aged 2 days, 1.5 years and two patients 3 years.

Immunophenotypic analysis:

Immunophenotypic analysis was performed using whole blood staining method. Fresh samples were obtained from peripheral blood or bone marrow at the time of diagnosis. A wide panel of monoclonal antibodies (Mo Abs) was used (Table 1). Double and Triple marker labeling was performed, including proper isotype controls. Samples were analyzed on Flow Cytometer (Coulter Epics, XL, Hialeh).

Detection of surface markers by direct staining:

The whole blood staining method was performed as previously described **[18]**. In short,

10µl labeled Mo Ab was added to 100µl whole blood (or BM), incubated in the dark for 20 minutes, hemolyzed (Optilyse® No-wash Lysing solutions, A11894, Beckman Coulter) and washed by PBS then analyzed.

Detection of intracellular markers:

Hundred µl of whole blood was lysed using lysis solution (Becton & Dickinson) for 10 minutes. Cells were washed once and re-suspended in 1mL PBS. A mixture of 500 µl 4% paraformaldehyde as fixative, 500 µl PBS and 5 µl tween 20 as detergent was added to the cells and incubated for 10 min. The cells were washed and 10 µl Mo Ab was added and incubated for 30 min at 4ºC. Cells were washed, resuspended in 500 µl PBS and analyzed **[19]**. Any antigen (Ag) was considered positive when ≥20% of blast cells were stained above the negative control except for CD 34 and CD 10 where ≥10% was considered positive.

DNA index (DI): Was done for 16/17 cases. Bone marrow or blood samples were processed with the DNA-Prep coulter® Reagents Kit. The sample was shaken on an automatic shaker then 100µl of the suspension was taken. 100µl of the DNA-Prep LPR for lysing and permeabi lizing cells was added to the suspension with continuous shaking for 8 to 12 seconds and 2 mL of the DNA-Prep Stain (propidium iodide) was added to the previous suspension with continuous shaking for another 30 seconds **[20]**. The DNA-Prep stains DNA and double stranded RNA with Propidium iodide; ribonuclease is included to digest RNA. Samples were analyzed on the Coulter Epics® XL-MCL flow cytometer.

Molecular characterization:

Molecular detection of t (1; 22) was performed on 4 cases for whom RNA was available.

RNA extraction and reverse transcription:

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 RBCs in 300ul blood/BM were lysed by RBCs lysis solution, the cell pellet was lysed by cell lysis solution and protein and DNA precipitated by protein-DNA precipitation solution. The supernatant containing RNA was collected on 100% isopropanol, 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 use.

One µg RNA was reverse transcribed to cDNA in a final volume of 20 µL containing 2 ul of 10 X reverse transcription-polymerase chain reaction (RT-PCR) buffer, 4ul 25 mM MgCl2, 2ul 10mM dNTPs, 0.5ul RNAsin, 15 U AMV reverse transcriptase enzyme, 1ul random hexamers and nuclease free water to a final volume of 20ul (Promega, Madisson, WI). Cyclic temperatures consisted of 10 min at 25°C, 1hourr at 42°C and 5 min at 95°C.

Detection of t (1; 22):

This part of the work was performed at the Molecular Pathology Department of Saint Jude Children's Research Hospital (SJCRH). PCR reaction mix consisted of 300 ng DNA, 5ul 10x PCR buffer containing 25 mM MgCl2, 3.5 ul of 1.25 mM of each dNTPs, 0.5ul of 75 pmol Forward and Reverse Primers, 2.5 U of Amplitaq Gold DNA polymerase enzyme and 5% DMSO. Nuclease free water was added to a total reaction volume of 50 ul.

Thirty five cycles of amplification were performed in a thermocycler with a step program consisting of an initial denaturation step at 95ºC for 11 min and 30sec, 15 cycles of 94ºC for 30sec, 54ºC for 1min & 72ºC for 1 min followed by 20 cycles of 94ºC for 30sec, 54ºC for 1min & 72ºC for 2min. A final extension step was performed for 5 min at 72ºC. A housekeeping gene, Glyceraldehyde Phosphate Dehydrogenase Enzyme (GAPDH) was run with every PCR reaction to check DNA integrity and exclude any PCR failure. A synthetic positive control was used for confirmation of the translocation. PCR products obtained were separated on a 1.2% ethidium bromide agarose gel for 2 hours at 70 volt **[21]**. Fragments size was determined by running a molecular weight marker of known size and comparing the distance of unknown fragment in relation to the ladder. DNA on gels was denatured, neutralized and transferred by a Southern blot technique **[22]** in a high salt buffer solution by either capillary action or positive pressure from its position on agarose gel to a nitrocellulose or nylon membrane (Nytran; Schleisher & Shuell, Keene, NH, USA).

Denatured single-single stranded DNA was permanently bound to the membrane by UV cross linking (UV stratalinker 2400, Stratagene). Single stranded probes were labeled utilizing a 5' DNA end labeling kit (RPN 1509, Amersham Biosciences, USA). The enzyme T4 polynucleotide kinase was used to specifically transfer the γP32 phosphate from ATP to a 5'OH group of DNA. After hybridization, the membrane was washed and labeled to remove unbound or weakly bound probes and then exposed to an autoradiographic film. Results for fusion gene expression were expressed as positive or negative according to the presence or absence of the specific band on the autoradiographic film.

Treatment:

All patients received the same treatment protocol: Induction therapy consisting of one course of ADE (Cytarabine 100 mg/m^2/d 24 hours infusion d1-2; 100 mg/m^2 IV q12 hours days 3-8, Doxorubicin 25 mg/m^2/d IV days 3-5, Etoposide 100 mg/m^2/d one hour infusion days 6-8) followed by another course as consolidation, then four courses of MIDAC as continuation therapy (Mitoxantrone 10 mg/m^2/d 6 hours infusion days 1-3 and intermediate dose Cytarabine 1g/m^2/d two hours infusion q12 hours days 1-3).

Clinical evaluation and follow-up:

Bone marrow aspirate was performed to evaluate response to chemotherapy (status post induction) following first course of ADE in AML. Evaluable patients were followed up for a period ranging from 14-24 months.

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

RESULTS

During the study period, 2003-2005, 280 consecutive pediatric AML cases presented to the Pediatric Oncology department at NCI, Cairo University.

FAB subtype was available for 242 cases. The 38 cases with missing FAB were not M6 or M7 as confirmed by immunophenotyping. Thus M7 constituted 17/280 (6%) of all cases. Other FAB subtypes included 85/242 M1 cases (35.1%), 66/242 M2 (27.3%), 30/242 M3 (12.4%), 19/242 M4 (7.9%) and 24/242 M5 cases (9.9%). M6 was encountered in one case only (0.3%).

The laboratory features of the 17 M7 patients at the time of diagnosis are presented in Table (2). Twelve patients 12/17 (70.6%) were males and 5/17 (29.4%) were females. Their median age was 3 years with a range from 2 days to 14 years, but most cases 10/17 (58.8%) were children < 36 months and 3/17 were infants. The 4 Down's syndrome cases were all males aged 2 days, 1.5 years and two patients 3 years.

*Clinical and hematological features:*Clinical presentation was similar to those observed in patients with other types of AML including pallor, fever, easy fatigability, headache, hyporexia, bleeding and bone pain. Hepatomegaly was encountered in 11/15 (73.3%), splenomegaly in 10/15 (66.6%) and lymphadenopathy in 9/12 (75%), (Table 3).

Anemia was present in 16/17 cases (94.1%) with haemoglobin (Hb) ranging from 3-15.4 g/dl. White blood cell count ranged from 1.8-190x10^9/L with 5/17 patients only having leukocytosis. Fifteen patients 15/17 (88.2%) had thrombocytopenia.

Morphology: Morphological presentation showed a remarkable degree of pleomorphism. BM cellularity was either normal or decreased with marked reduction in megakaryocytes and marrow platelets in most cases (12/15, 80%). The number of blast cells in the bone marrow aspirates ranged from 3-90%. Diagnosis was based, in all cases, on immunophenotyping by Flow Cytometry. An open gate strategy was used to determine the true percentage of malignant cells that could not be detected by morphology. In 12/17 (70.6%), variable percentage of blasts showed typical morphologic features of megakaryoblasts which differ from other classical myeloblasts by relatively hyperchromatic nuclei and abundant cytoplasm with blebbing and sometimes cytoplasmic granulations (Figs. 1-A, 2). In other cases, blasts could not be distinguished from myeloblasts (Fig. 3). In others, blasts resembled lymphoblasts being small with a high nucleocytoplasmic ratio and chromatin condensation (Fig. 4). One case out of the 17 showed normal platelet count and another one showed thrombocytosis of 750 x

10^9/L. This latter was the one with micromegakaryocytes and proved to be M7 on top of CML (Fig. 5). The peripheral blood of almost all cases (15/16 93.8%) even those with leucopenia showed blast cells. In cases with < 20% blasts in the BM (2 cases), trephine biopsy was performed. Extensive fibrosis was encountered with fibrous tissue entangling blast cells (Fig. 6).

Cytochemical findings: Cytochemistry was performed on PB and/or BM for all 17 cases where they showed myeloperoxidase (MPO) and SBB negativity. Acid Phosphatase showed characteristic pattern in all cases with strong cytoplasmic positivity distributed in the whole cell while ANAE showed moderate positivity partially inhibited by sodium fluoride. Figs. (1-B, 3-B) represent the characteristic pattern of positivity of AP in typical and atypical M7 morphology.

Immunophenotypic features (Table 4): In 7/17 cases (41.1%) blast cells expressed platelet specific markers only while in the remaining 10 cases (58.%) blast cells simultaneously co-expressed megakaryocytic as well as markers of other cell lineages. One case (Nº16) was biphenotypic expressing T, myeloid/ megakaryocytic markers. The coexpression of megakaryocytic, lymphoid and myeloid markers by single cells was confirmed by standard 2 or 3 color flow cytometric analysis using appropriate monoclonal antibodies (MoAbs). CD41 and CD61 were both coexpressed in 8/15 (53.3%) of cases tested for both. Four out of 7 remaining cases showed cyt CD 61 only. One case was positive for CD 41 only (CD 61 was 17%) and two cases were positive for CD 61 alone (CD 41 was 4 & 14%). Cyt CD 61 was the tool for diagnosis in one more case. In the remaining case (nº6), CD41 was 90% and CD61 was not tested. There was no reactivity with platelet specific markers in the remaining cases of AML (M1-M6).

CD 13 and/or CD 33 were expressed in 10/17 (58.8%), both in 3/17(17.7%) and 7/17 (41.1%) had either. As for HLA DR expression, 7/17 (41.1%) cases were positive. CD 33 and HLA-DR were simultaneously expressed on 4/17 (23.5%) of cases. Simultaneous expression of CD 61, CD 41, HLA-DR and CD 33 was detected in 1/17 (5.8%) of cases.

DNA index (DI): Most cases were diploid (13/16 cases). One case was hyperdiploid (DI 1.3) and 2 cases were hypodiploid (DI 0.86, 0.89).

t (1; 22): Molecular detection of t (1; 22) was performed for 4/17. One case showed the gene expression (a 2 years old female).

Two cases deserve elaboration:

The first (Nº4) was a 5 years old girl. PB and BM blast morphology was typical and acid phosphatase showed classical positivity (Figs. 1-A, b). Initial immunophenotyping showed CD 61 and CD 41 expression on 8.7% and 5.5% respectively; the case expressed as well CD 45 and CD 34 and was negative for all other lineage markers. Due to failure to achieve the 20% threshold for the markers together with classical morphology and cytochemistry, immunophenotyping was repeated 5 days later. CD 61 and CD 41 were then expressed on 25% and 26% respectively. The case was documented as M7.

The second (Nº1) was a 6 years old male who presented with huge splenomegaly, anemia (7gm/dl) leukocytosis ($50X10^9$/L) and thrombocytosis ($750X10^9$/L). PB and BM showed a lot of micromegakaryocytes with plentiful platelet production (Fig. 5-A). Immunophenotyping showed expression of DR: 19%, CD 41: 71% and CD 61: 63%. Molecular characterization documented the presence of BCR/ABL p 210 fusion gene (Fig. 5-B) denoting acute crisis on top of CML.

Clinical outcome: In this study, 9/17 cases were non evaluable; either they died early in the course or before the start of treatment, 1/17 lost follow-up after one month of therapy, 1/17 became refractory, 3/17 relapsed and 3 were in CR till end of study (for 12,14 & 24 months).

DISCUSSION

M7 was first recognized as a separate subtype of AML in 1985 **[18]**. Since then, variability in its relative frequency has been reported **[1,6-10,23]**. A major cause of this variability could be attributed to morphological heterogeneity. Some cases may lack the classical morphology would not be tested for the specific megakaryocytic markers, and hence misdiagnosed as other FAB subtypes of AML.

Table (1): 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
CD41	P2-PE	Coulter Hialeah, FL
CD61	SZ21-FITC	Coulter Hialeah, FL
Glycophorin A	11E4B.7.6 (KC16)	Coulter Hialeah, FL
Myeloperoxidase	MPO7 FITC	DAKO
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

*NK: Natural killer.

Table (2): Laboratory features of 17 pediatric M7 cases.

No	FAB	TLC	HB	PLT	Blasts	Initial BM blasts %	DS	Molecular testing
1	M7	50	7.4	750	70	HC, 43%	No	Philadelphia+
2	M7	9	7.1	70	10	HC, 3%	No	NA
3	M7	8.2	3	13	0	NC,16%	Yes	NA
4	M7	7.1	7.1	8	11	DILUTED	No	NEG t (1; 22)
5	M2	83	15.4	124	55	NC, 30%	Yes	NA
6	M7	48	8.4	64	90	HC, 70%	No	NA
7	M7	3	8	150	50	DILUTED	No	NA
8	M7	13	3.5	15	70	NC,63%	Yes	NA
9	M7	190	4	9	80	NC, 90%	No	NA
10	M7	7	4.2	19	17	HC, 70%	Yes	NEG t (1; 22)
11	L2	9.5	7.8	40	NA	NA	No	NA
12	M7	74	5.9	35	28	NOT DONE	No	NA
13	M7	12	5.8	12	6	HC, 22%	No	POSITIVE t (1, 22)
14	L1	1.8	6	22	15	HC, 20%	No	NA
15	M2	2.9	4.4	22	20	NC, 58%	No	NEG t (1; 22)
16	M1	5	10	100	10	HC, 20%	No	NA
17	M7	4	10	100	3	DILUTED	No	NA

HB: Hemoglobin. PLT: Platelets. NA: Not available. HC: Hypocellular. NC: Normocellular.

Table (3): Clinical features of 17 pediatric M7 cases.

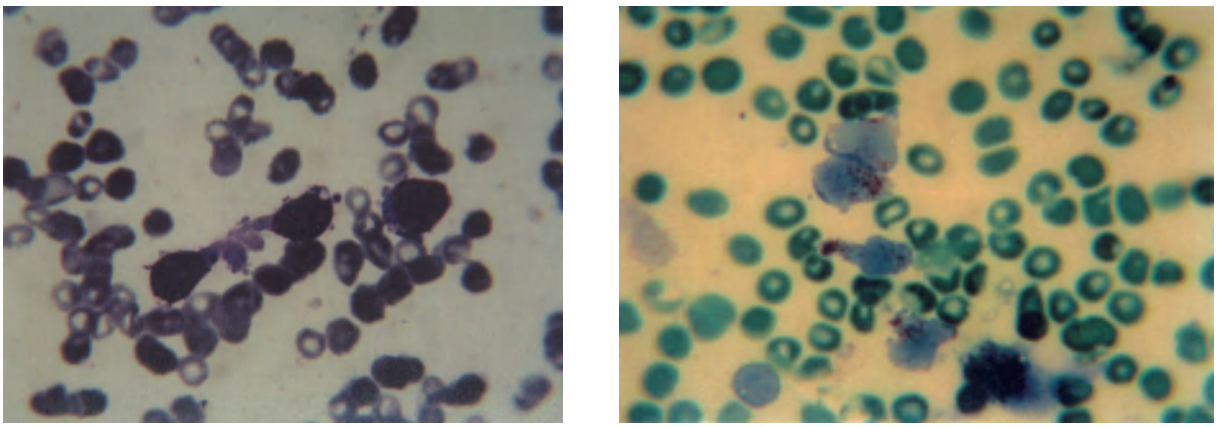
No	AGE (years)	SEX	CXR	CSF	LIVER (in cm)	SPLEEN (in cm)	LNS
1	6	M	free	free	3	huge	–
2	0.25	M	NA	free	huge	huge	–
3	3	M	free	free	3	2	–
4	5	F	NA	NA	no	no	yes
5	2 days	M	NA	NA	yes	yes	no
6	0.9	M	free	free	yes	yes	yes
7	13	M	free	NA	yes	yes	yes
8	1.5	M	free	free	palp	yes	yes
9	2	F	free	free	yes	no	yes
10	3	M	NA	NA	yes	yes	no
11	3	F	NA	free	no	no	yes
12	2	M	NA	NA	yes	yes	yes
13	2	F	NA	free	huge	huge	yes
14	6	M	NA	NA	no	no	no
15	14	F	free	abcess	no	no	yes
16	11	M	NA	NA	NA	NA	NA
17	14	M	NA	NA	NA	NA	NA

CXR : Chest X ray.
LNS : Lymph nodes.
– : No comment on LNS in patient's records.
NA : Not Available.
NE : Non Evaluable.
R: : Relapse.
CR : Complete Remission.
LFU : Lost follow-up.
Palp : palpable.

Table (4): Immunophenotyping details of 17 M7 patients.

No	CD 13	cCD 13	CD 33	CD 41	cCD 61	CD 61	CD 34	DR	MPO	Others	DI
1	2.27	4.8	0.6	71	nd	63	nd	19	1.5	–	0.86
2	2	2	19	67	nd	32	2	10	1	–	0.89
3	7.2	83	5.3	70	nd	40	0	11	23	–	1
4	5	6	8	26	nd	25	37	-ve	-ve	–	1
5	15	23	48	8	47	12	35	4	12	7 (49%) 56 (21%)	1
6	0.7	1	0.5	90	nd	nd	0.5	5	1	–	1
7	0.4	1	1.5	55	nd	67	1.1	4.9	1.2	–	1
8	19	16	30	27	nd	17	0	43	1.5	CD7 (18%)	1
9	3	88	2	65	nd	67	0.2	5.6	8	–	1.13
10	19	19	0	14	57	9	nd	17	1.5	CD7 (19%)	nd
11	1	–	6	4	nd	54	6	29	2	–	1
12	27	32	30	14	nd	28	20	35	8	–	1
13	2	–	76	54	nd	39	41	0	2	–	1
14	75	nd	10	10	70	10	nd	40	5	CD4 (50%)	1
15	7	72	47	2	58	12	nd	31	7	–	1
16	81	nd	0.3	0.0	79	nd	71	56	0.5	CD7 (80%), CD2 (97%)	1
17	2.5	2.5	40	25	nd	76	1	20	9	–	1

c: cytoplasmic. DI: DNA index. nd: not done.



(A) **(B)**

Fig. (1): BM picture of an M7 patient showing (a) typical M7 morphology (b) characteristic acid phosphatase positivity.

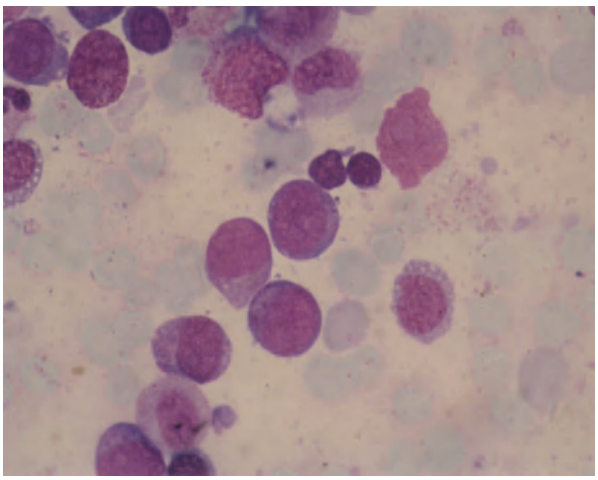
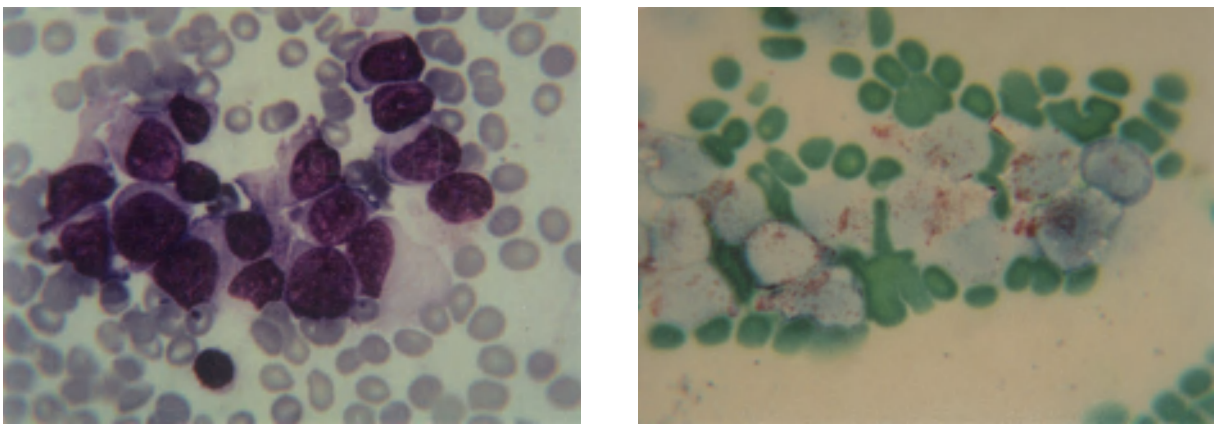
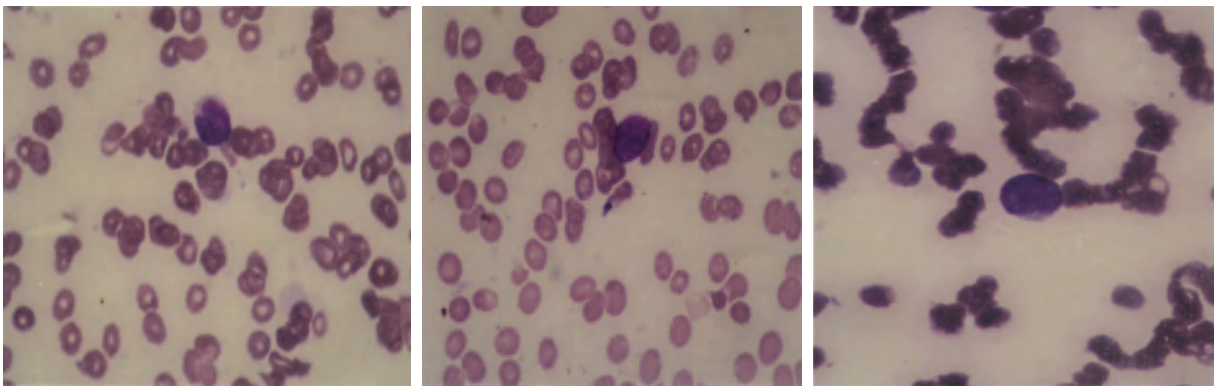


Fig. (2): BM picture of typical M7 patient showing megakaryoblasts with cytoplasmic granulations.



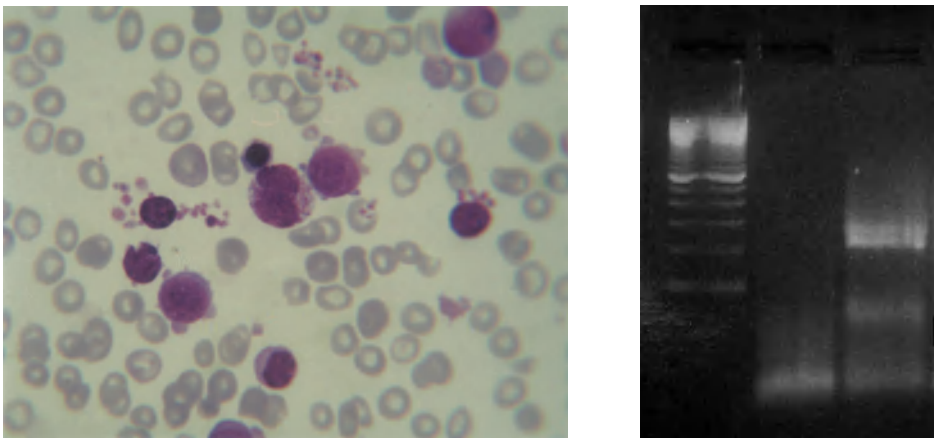
(A) **(B)**

Fig. (3): BM picture of an M7 patient showing (a) blasts resembling myeloblasts (b) characteristic acid phosphatase positivity.



(A) **(B)** **(C)**

Fig. (4): BM aspirate showing megakaryoblasts morpholically similar to lymphoblasts.



(A) **(B)**

Lane 1: 100 bp marker Lane 2: -ve control Lane 3: p210 (patient)

Fig. (5): (a) BM showing micromegakaryocytes in an M7 on top of CML (b) positive bcr/abl amplification band of the same case.

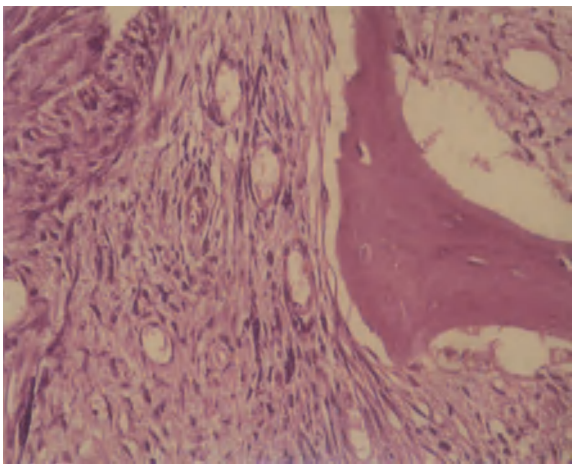


Fig. (6): BM trephine picture of an M7 patient showing marked fibrosis and ectatic sinusoids.

In this work, we aimed to investigate the true relative frequency of M7 within pediatric AML diagnosed at NCI, Cairo University. We also wanted to emphasize the morphologic heterogeneity that might lead to missing a diagnosis of M7.

In the present study, 17/280 (6%) of AML cases were M7 including 4 cases with DS. This is comparable to the 4-7% incidence reported in large Cooperative Group studies in developed countries **[6-10]**. The male to female ratio was 2.4:1. Others reported ratios between 1.6:1 and 1.16:1 **[1,10]**. The age range of our cases was from 2 days to 14 years with a median of 36 months; 10/16 (62.5%) of our cases were <3

years including all cases with DS (4/10, 40%) and 7/16 (43.75%) of cases were <2 years.

The median age in our series was slightly higher than that recorded by the St Jude's study (23.9 months) **[10]**. Our data differ from others **[9]** who reported that 79.2% of the patients in their series were <3 years with 58.3% <2 years and also differ from the Mexican group who reported 24% of the cases to be ≤2 years old **[1]**. In our ≤2 years group, 2/16 cases (12.5%) were DS which is an incidence higher than that of the Mexican group who reported 3.45% of their cases with DS **[1]**.

M7 is the most common form of AML in children with DS with a 400-500 fold increased risk **[24-27]**. Several collaborative pediatric group studies have found that approximately half of all pediatric cases of M7 occur in patients with DS **[28,29]** while others reported only 17% of their patients with de novo M7 to have DS. This disparity may reflect a variable rate of referral of patients with DS to different institutions **[10]**.

In our study, 23.5% of cases were DS. Their median age was 26 months which is comparable to the median age recorded (25.8 months) by St Jude's study **[10]** but higher than that reported by others to be <2 years **[30]**. Only 2 of our 4 M7 cases with DS lied in this latter age group (2 days and 1.5 years). All our DS cases were boys in contrast to the reported higher female to male ratio published by other groups **[14]**.

In this work, clinical presentation of M7 was similar in many respects to those observed in patients with other types of acute leukemias.

A study done at St Jude's Hospital proposed a combination of 2 findings to be highly suggestive of M7 **[10]**; typical morphologic features of leukemic cells isolated from the BM together with multifocal punctate cytoplasmic alpha naphthyl acetate esterase cytochemical staining that is incompletely inhibited by sodium fluoride. In our experience, as shown in the current study, characteristic positivity of Acid Phosphatase is highly suggestive of M7, even if the percentage positivity of CD 61 and/or CD 41 did not reach the 20% cutoff value. The diagnosis should always be confirmed by immunophenotyping or immunohistochemistry. In this study, 70.6% of our pediatric patients showed the typical M7 morphology. The other cases (29.4%), displayed heterogeneity in blast morphology at presentation that was very difficult to distinguish from myeloblasts or lymphoblasts and the diagnosis was only possible by immunophenotyping.

In the St Jude's experience, the percentage of blast cells in an aspirated BM specimen was not reliable as a single diagnostic indicator of acute leukemia since 20% of their cases had less than 30% leukemic cells in their aspirates (N.B. the study was performed in the era that required 30% blasts to diagnose acute leukemia). In our study, the blast percent in the bone marrow aspirates was over 20% in 10/12 (83.3%) cases while 2/12 (16.6%) cases had 3 and 16% BM blasts, as judged by morphology; however, the diagnosis was favored by BM trephine findings and the megakaryocytic origin of the blasts was established by immunophenotyping.

In this study the MoAb to platelet GpIIIa (CD 61) was detected in 15/16 cases (93.8%) with 10/15 surface & 5/5 cytoplasmic expression. CD 41 was detected in 10/17 (58.8%) cases and showed a coexpression with CD 61 in 8/15 cases (53.3%). This is in concordance with the observations that GpIIIa is the earliest Gp to be expressed during megakaryocytic maturation **[31]**. Previous studies indicated that the MoAb against GpIIIa should be the marker of choice for the diagnosis of M7 **[32]** however, one case (1/17) expressed CD 41 only. HLA-DR is not normally expressed on circulating platelets or normal megakaryocytes possibly because this Ag appears early in the differentiation of this lineage and is subsequently lost **[33]**. Several studies reported that the blast cells reacted with the anti-HLA-DR MoAb in the majority of their cases **[31,33]** however, only 7/17 (41.1%) of our cases expressed HLA-DR. Some studies viewed HLA-DR expression as an intermediate stage of megakaryocytic differentiation **[34]** but no particular comment was made on its significance. As regards CD 33, it has been reported to appear on megakaryoblasts already expressing CD 41 when HLA-DR Ags were lost **[1]**. In our cases, 5/7 HLA-DR positive cases coexpressed CD 33 and/or CD 13. Coexpression of CD 61, CD 41, HLA-DR and CD 33 was detected in 1/17 (5.8%) of cases. M7 seems to arise from various stages of megakaryocytic differentiation. The expression of HLA-

DR together with CD 13 and/or CD 33 might lead to erroneous diagnosis of AML especially in the absence of the characteristic morphological features. Such a situation is not infrequent and the diagnosis of M7 could be missed. Accordingly MoAb panel should be extended to include CD 61 and CD 41 with both surface and cytoplasmic staining in all AML cases as well as in cases lacking all lineage markers. This will guard against missing an M7 case for a case of AML or undifferentiated leukemia.

In agreement with a previous study **[1]**, we reported a subset of patients (3/17, 29.4%) showing a simultaneous expression of surface membrane Ags of multiple hematopoietic lineages, namely CD 2, CD 4, CD 7 and CD 56. The presence of lymphoid markers especially in the absence of typical M7 morphology may also lead to missing the diagnosis.

In this study, one out of 4 cases tested showed t (1; 22) in a 2 years old female. The close association of 2 specific chromosome aberrations, trisomy 21 and t (1; 22) (p13; q13) with M7, both characterized by early onset of the disease, could account for the younger age at diagnosis reported in several series **[6,9,35]**. This strong association of infant M7 with p13 and q13 breakpoints in chromosomes 1 and 22 suggests that prenatal genetic factors are involved in leukemogenesis and that alteration in genes (N-ras or platelet derived growth factor PDGF-B) at or near these sites participate in malignant transformation and proliferation of megakaryoblasts **[1]**.

The prognosis of children who have de novo M7 in the absence of DS has been difficult to determine. Findings increasingly suggest that these patients have a poorer prognosis than do patients with other FAB subtypes of AML. Studies show that, patients with de novo M7 have significantly lower 5-year survival estimates than do patients treated on similar protocols for other FAB subtypes of de novo AML. **[26]**. Apparently, standard therapies used for AML are not always optimal for patients with M7 **[26,36,37,38]**. The Berlin-Frankfurt-Münster (BFM) group recently reported that M7 independently predicts poor prognosis in children with AML **[38]**. Appropriate treatment for patients with DS, who have a good prognosis and a relatively low tolerance for high-dose chemotherapy, has been described in the literature **[28,39]**. In our series the small number of patients makes it difficult to draw any conclusions with regards to treatment outcome. The high incidence of early deaths might indicate that the standard doses of chemotherapy were not well tolerated. This might necessitate reevaluation of the treatment protocol and drug dosage.

Conclusion:

M7 constitutes 6% of Egyptian pediatric AML as encountered at the NCI, Cairo University. Marked morphological heterogeneity was encountered in 29.4% of the cases lacking the typical morphology and could have been missed for other types of AML or even ALL.

Cytochemistry could be a helpful tool in suspecting the diagnosis of M7 especially Acid Phosphatase. CD 61 and CD 41 membrane and/or cytoplasmic expression are the whole mark for diagnosis and should be tested in all AML cases as well as in cases lacking all other lineage markers. Even if the percentage positivity at diagnosis does not reach the cutoff threshold, repetition is recommended; the diagnosis is still very likely to be M7 especially in the presence of the characteristic Acid Phosphatase positivity.

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Prognostic Relevance of Telomerase and Bcl-2 in Acute Myeloid Leukemia

NASR M.A. EL-LAHLOUBY, M.D.[1]; IBRAHIM M. ABD EL-SALAM, M.D.[2]; NAHED ABD EL-WAHAB, M.D.[3]; HUSSEIN E. GABALLA, M.D.[1]; OSMAN M. MANSOUR, M.D.[1]; HANAN R. NASSAR, M.D.[1]; TAREK DARWISH, M.D.[1]; FAYEK M. GHALEB, M.D.[4] and TAMER M. FOUAD, M.Sc.[1]

The Departments of Medical Oncology[1], Molecular Biology[2] NCI, Cairo University; Clinical Pathology, Research Institute of Ophthalmology[3] and Clinical Pathology[4], NCI.

ABSTRACT

Background: Prognosis of AML patients is influenced by both clinical and genetic markers. As therapy and supportive care improves, the intrinsic biologic characteristics of the patient's leukemia become the dominant factor in determining prognosis.

Aim of Work: Is to evaluate of the prognostic relevance of Telomerase activity and serum level of Bcl-2 in AML patients.

Patients and Methods: The study included 63 newly diagnosed cases of acute myeloid leukemia below the age of 60 years. Telomerase activity and Bcl-2 levels were assessed in patients as well as in ten healthy age and sex matched controls. Assessment of telomerase activity was done using PCR-ELISA technique and evaluation of Bcl-2 serum level was done using ELISA. Patients were followed up for 3 years. Assessment of prognostic factors in the present study included three main parameters: cytogenetic abnormalities (20 cases), immunophenotyping (63 cases) and hyperleukocytosis (63 cases). Patients were grouped according to the presence of independent prognostic factors into a poor prognosis group and a non-poor prognosis group. Using this classification half the patients (29 patients: 46%) were categorized in the poor prognosis group.

Results: Thirty six percent of patients had hyperleukocytosis (TLC ≥ 100,000/µl), 38% expressed unfavorable immunophenotypic markers (CD34 positivity and or biphenotypic leukemia's markers), while 3 patients had a poor karyotypic profile (11q 23, t (9; 22), del 5q). The complete remission rate was 57% and the overall median time to CR was 31 days. The 2-year and 3-year overall survival rates were 32.5% and 23.5% respectively. While the 2 and 3-year disease-free survival rates were 21.6% and 18% respectively.

Patients in the poor prognosis group showed an inferior 2-year disease-free survival (12% versus 34%; p=0.02). The median level of telomerase activity for AML patients was 0.40 U was significantly higher than controls. Higher telomerase values were significantly associated with response rate (p 0.01). Lower levels of telomerase activity were associated with a significantly better disease free survival at 1 year when compared to higher levels (34% versus 10%; p=0.012). There was also a highly significant association between higher telomerase activity and the poor prognosis group (p=0.0001).

The median serum level of Bcl-2 for patients in the present study was 204 U/mL which was significantly higher compared with controls (p 0.05). Higher levels showed significant correlation with bad prognostic group (p=0.0001), bad immunophentypic profile (p=0.011) and hyper-leucocytosis (p=0.000l). Higher levels were associated with lower response rates (p=0.06). Lower levels were significantly associated with better DFS at 1 year (p=0.005) as well as better 2 year survival, although in the latter the difference did not reach significance (p 0.07). Patients with more than one poor prognostic criterion had a tendency for lower overall survival rate at 1 year (24% versus 42%; p=0.094) and significantly lower disease-free survival rate at 1 year (24% versus 50%; p=0.036). There was a significant correlation between telomerase activity levels and Bcl-2 level in the serum of AML patients (r=0.623, p<0.0001).

Conclusion: Telomerase activity and Bcl-2 levels correlate significantly with disease-free survival in AML patients. Prospective studies with more numbers of patients will be required to confirm their prognostic values especially in specific subsets of patients as those with normal cytogenetics where there is still debate about the role of intensive therapy with stem cell transplant.

Key Words: *AML – Telomerase – Bcl-2.*

INTRODUCTION

The development of acute myeloid leukemia (AML) is associated with accumulation of acquired genetic alterations and epigenetic changes

in hematopoietic progenitor cells that alter normal mechanisms of cell growth, proliferation, and differentiation with consequent uncontrolled proliferation of clonal neoplastic hematopoietic precursor cells and impaired production of normal hematopoiesis **[1]**. 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. However, overall, only 20% to 60% of patients enjoy long-term disease-free survival (DFS) depending on risk factors at diagnosis. The majority of patients die of their disease, primarily because of persistent or relapsed AML **[2]**.

Post-remission therapy is a sine qua non for curing AML as the median DFS for patients who receive no additional therapy is only 4-8 months, however, 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. Stem cell transplantation is anther option in high risk patients **[3-6]**.

Various factors affect the prognosis of AML including age, Immunophenotype, WBCs count at diagnosis, response to induction therapy and chromosomal abnormalities which are considered the most important prognostic factors currently used for choice of post-remission treatment **[6-11]**.

Chemotherapy kills cancer cells primarily by inducing apoptosis. Therefore, modulation of the key elements of apoptosis signaling directly influences therapy-induced apoptosis. Telomerase is a specialized ribonucleoprotein complex that is responsible for the synthesis and maintenance of telomere repeats. The latter are DNA-protein complexes at the ends of linear eukaryotic chromosomes which maintain chromosomal stability and integrity and protect chromosomal ends against fusion, degradation by exonucleases and recombination events. Unlike embryonic cells, telomerase expression is low or absent in most human somatic tissues and in adult cells. The catalytic protein subunit of TERT is the key determinant of the enzymatic activity of human telomerase **[12]**. The clinical relevance of telomeres is that a cancer cell, unlike a normal cell, can repair eroded telomeres. Telomerase activity is expressed in varying degrees in most primary tumors. The overall importance of telomerase in the pathogenesis of AML has recently been confirmed by the demonstration that hTERT is necessary for growth of primary AML cells in a mouse model **[13]**. However, considerable work has been undertaken to determine whether telomerase activity can further refine these prognostic data.

The pathways responsible for adult tissue homeostasis are governed significantly but not exclusively by Bcl-2-family proteins (pro and anti apoptotic) **[14]**. The Bcl-2 regulation pathway is also called the intrinsic or mitochondrial pathway of caspase dependent apoptosis. As such Bcl-2 genes are regarded as potential oncogenes in view of their function in apoptotic cell death and the consequence of perturbation of the delicate homeostasis of cell population growth. In this case the effect is not achieved by increasing the rate of cell proliferation but by reducing the rate of cell death **[15,16]**. Bcl-2 overexpression in haematopoietic lineages yields excess B, T and myeloid cells that are refractory to diverse cytotoxic insults **[17-19]**.

The aim of this study is to estimate levels of telomerase and Bcl-2 in patients with de novo AML and to correlate it with clinical and biological factors which are known to influence prognosis.

PATIENTS AND METHODS

This study comprised a total of 63 newly diagnosed cases of acute myeloid leukemia who presented to the Medical Oncology department at the National Cancer Institute, Cairo University between February 2002 and April 2004. We followed up the patients for 3 years. Pretreatment assessment included:

Full history and clinical examination:

Hematological studies: Complete blood count with differential, Bone marrow examination with cytochemical stains needed for proper diagnosis (eg; Sudan black, myeloperoxidase, non specific estrases).

Routine biochemical investigations that included hepatic and renal profiles (bilirubin, ALT, AST, alkaline phosphatase, urea and cre-

atinine in addition to tumor lysis syndrome panel including uric acid, Ca, K, Mg and Po4.

Immunophenotyping: Was done using Flow-cytometry Partec III from DAKO. A wide panel of monoclonal antibodies was used as part of the diagnostic procedure and for the documentation of surface or cytoplasmic marker expression by leukemic cells. CD34, CD33, CD11, CD13, CD14, CD15, MPO, HLA-DR, CD10, CD19, CD22, CD7, CD41, CD16, CD56. All monoclonal antibodies were obtained from DAKO (Denmark).

Cytogenetic studies: Conventional cytogenetic analyses were 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. For cytogenetic analysis an automated karyotyping system (Quips, Vysis, USA) was used.

Imaging studies: Chest X-ray, abdominal ultrasonography and CT scan when appropriate in addition to ECG and Echocardiography.

Telomerase activity was assessed by Telomerase PCR ELISA utilizing the telomeric repeat amplification protocol (TRAP), developed by Boehringer Mannheim (Germany) which is an extension of the original method described by Kim et al. **[20]**. TRAP assay is a 2-step process in which the telomerase-mediated elongation products are subsequently amplified by PCR to allow highly sensitive detection of telomerase activity.

Serum Bcl-2 level was assayed using ELISA method (Oncogene Research Products, Cambridge, MA).

Treatment plan:

Remission induction:

Patients received the conventional 7 and 3 regimen, consisting of cytarabine 100 mg/m^2 continuous infusion over 24 hours for 7 days and doxorubicin 45 mg/m^2 IV shot for 3 days. If 14 days after induction therapy, the result of bone marrow aspirate revealed partial remission or no response to therapy, a second induction course was given consisting of the same regimen used in the induction course.

Consolidation/ early intensification therapy:

Patients who achieved complete remission received consolidation therapy consisting of:

- Two cycles of cytarabine 100 mg/m^2 continuous infusion over 24 hours for 5 days and Doxorubicin 45 mg/m^2 IV shots for 2 days.
- Followed by two cycles of cytarabine 1 gm/m^2 infusion over 2 hours for the first 3 days and Mitoxontrone 12 mg/m^2 intravenous infusion over 2 hours on days 3, 4, 5.
- Patients who achieved complete remission and were eligible for bone marrow transplantation were referred for Allogenec HCT.

Maintenance therapy:

All patients were kept under follow-up without maintenance therapy.

Statistical analysis:

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.
- 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 less than 0.05 was used in all statistical tests.

RESULTS

This study involved 63 patients with age range of 16-63 Y and a mean of 33.9±11.7 year. Ten age and sex matched healthy individuals were included as a control group. Patient characteristics are illustrated in Table (1).

Overall 24 cases expressed immunophenotypic markers of poor prognosis. In all cases at least 2 myeloid markers were expressed. Twenty one cases were found to be CD34 positive while 5 cases of biphenotypic leukemia. All biphenotypic cases co-expressed B lineage markers (CD22 in all, cytoplasmic Ig in one case). Two

cases were both CD34 positive and expressed CD22. Immunophenotypic characteristics of AML patients studied are shown in Table (2). FAB classification and cytogenetics are shown in Tables (3,4).

Toxicity:

Scoring of treatment toxicity was done according to WHO criteria. Overall the most common non hematological toxicity was stomatitis, which was observed in almost 60% of patients. Stomatitis was also the most common form of GIII-IV toxicity (23.5%). Mild to moderate nausea and vomiting and alopecia were present in 47% patients. Twenty five percent of patients experienced mild diarrhea, whereas III-IV was recorded in 2 patients Furthermore, 21 patients (33%) presented with liver toxicity with 6 of them who experienced severe liver toxicity grade III/IV. Moreover, Six patients (10%) experienced sudden cardiac collapse (3 cases of acute heart failure and 3 cases of sudden death due cardiac arrhythmias), Table (5).

Neutropenia and supportive therapy:

The maximum nadir was reached after a mean of 14.86±4.40 days. The mean duration of neutropenia (neutrophils < 1000/cmm) was 13.56±8.68 days.

Fifty nine patients (93%) received antibiotics during induction chemotherapy, while 46 patients (73%) required antifungal therapy. The mean duration of antibiotics was 22.92±10.14 days while the mean duration of antifungal therapy was 14.43±9.13 days. The duration of neutropenia correlated significantly with the duration of antibiotics (p=0.003) but did not achieve statistical significance when correlated with the duration of antifungal therapy (p= 0.32).

Causes of death:

The main cause of death was related to uncontrolled infection (36% of patients). This was followed by cerebral hemorrhage (13%), while 6 patients (10%) died of disease progression with one patient dying from CNS infiltration (Table 6). Ten percent of cases died from cardiac and circulatory collapse, while 3 patients died from documented heart failure, another 3 due to arrhythmia (a result of electrolyte imbalance from toxicity) and one from hemorrhagic shock. Two patients died from liver failure (hepatic coma).

There were 16 cases of early death (death prior to evaluation of response). Six cases died from febrile neutropenia, 5 cases died of cerebral hemorrhage and 1 patient suffered toxic death as a result of GIII diarrhea leading to circulatory failure.

Response rates:

Complete remission (CR) was achieved in 32 patients after receiving the 1st cycle of induction. Two patients died immediately after achieving CR. Four patients required a second cycle of induction to achieve CR bringing the overall CR rate to 36 patients (57%). Eleven patients achieved partial response after their first cycle while 4 patients showed disease progression. Relapse rate was 30% (11 cases out of 36).

Time to first CR:

Overall median time to first CR was 31 days (14 to 75). There was no difference in the time to first CR between the group of the patients classified as poor prognosis and the non-poor group, (34 Vs. 40 days; p=0.437). No correlation was found between time to first CR and the individual prognostic factors, hyperleukocytosis or poor prognosis immunophenotypic markers (p=0.606 and p=0.254, respectively).

Disease free survival and overall survival:

The 2 and 3-year disease-free survival were 21.6% and 18% respectively for the entire group of patients enrolled in the study.

The 2 and 3-year overall survival rates were 32.5% and 23.25% respectively for the entire group of patients enrolled in the study.

When we compared the outcome of the poor prognosis group to non-poor prognosis group we found no statistical differences in overall survival at 2 years (29% versus 34%; p<0.094). As regards disease-free survival, however, patients classified in the poor prognosis group showed an inferior 2-year disease-free survival (12% versus 34%). This difference was found to be statistically significant (p=0.02).

Telomerase activity:

Telomerase level:

The mean telomerase activity level in leukemic cells of the present study was found to be 0.41 U±0.04 with a median of 0.40 U (0.38 to 0.56). There was a statistically significant difference between the median telomerase ac-

tivity level in the control group and the AML group ($p<0.05$), Table (6).

Correlation between telomerase activity and different prognostic factors:

Telomerase level was inversely correlated with age of patients but statistical significance was not achieved ($p=0.157$). In addition, direct correlation with total leukocytic count was shown but the correlation failed to achieve statistical significance ($p=0.238$). Moreover, there was no statistically significant correlation between telomerase levels and hyperleukocytosis (> 100,000/cmm) or immunephenotypic characteristics of poor prognosis, (CD34 positivity, biphenotypic leukemia), ($p=0.238$, 0.275) respectively. We were unable to correlate telomerase with cytogenetic abnormalities due to an inadequate number of patients; however, all patients with poor cytogenetic profile (3) had elevated telomerase activity levels.

There was a statistically significant correlation between telomerase and the prognostic grouping of the patient. Twenty five patients out of 26 in the poor prognosis category expressed higher telomerase level while only one patients out of 34 in non-poor prognosis group expressed higher telomerase level of activity ($p=0.0001$).

Correlation between telomerase response, DFS and OS:

Although there was a significant correlation between higher telomerase values and achieving CR ($p=0.019$), there were no correlation between the telomerase level and time needed for CR ($r=0.200$) Fig. (1). Lower levels of telomerase were associated with better DFS at 1 year when compared to higher levels (34% Vs. 10%; $p=0.012$) Fig. (2). The 2-year overall survival was higher in patients with levels of telomerase below 0.4 (42%) than those patients with telomerase level higher than 0.4 (34%). The difference was not statistically significant ($p=0.134$) (Fig. 3 and Table 7).

Bcl-2:

Level:

The mean Bcl-2 level in the serum was 232.8±124.4 SD while the median was 204 (50 to 44.0). There was a statistically significant difference between Bcl-2 serum levels in the control group (70±21 U/mL) and patients ($p<0.05$).

Correlation between Bcl-2 level and prognostic factors:

Although a strong inverse association between Bcl-2 level and the age could be detected, it did not reach statistical significance ($p=0.078$). No statistically significant association was found between telomerase activity and TLC ($p=0.238$. Bcl-2 levels were correlated with hyperleukocytosis and the results were highly significant ($p<0.0001$). It also correlated significantly with poor prognosis immunophenotypic profile ($p=0.011$). We were unable to correlate Bcl-2 with cytogenetic abnormalities due to an inadequate number of patients; however, all patients with poor prognosis karyotype (3) had elevated Bcl-2 levels.

There was a statistically significant correlation between Bcl-2 level and the prognostic grouping of the patient. All patients in the poor prognosis group expressed higher levels of Bcl-2, ($p=0.0001$) while only 3 patients from the non-poor prognosis group had higher Bcl-2 levels.

Correlation between Bcl-2 and response, DFS and OS:

The inverse association between higher levels Bcl-2 and CR rate almost achieved statistical significance ($p=0.06$). There was no significant association between Bcl-2 level and time needed to achieve complete remission. Lower levels of Bcl-2 were associated with significantly higher DFS at 1 year when compared to patients with higher levels (37% Vs. 11%; $p=0.005$). The 2-year overall survival was 44% in patients with Bcl-2 levels < 200 U/mL and 24% in those presented with higher Bcl-2 levels. The difference, although almost double, did not achieve statistical significance ($p=0.078$) (Table 8).

Correlation between telomerase and Bcl-2:

There was a significant correlation between telomerase level and Bcl-2 ($r=0.623$ highly significant; $p<0.0001$). We also examined whether the cumulative assessment of telomerase activity in conjunction with Bcl-2 levels and the presence of adverse prognostic factors would correlate better with disease outcome. We found that patients with more than one poor prognostic criterion had a tendency for lower overall survival rate at 1 year (24% versus 42%; $p=0.094$). The disease-free survival was significantly associated with the number of poor

prognostic criteria with a 1-year disease-free survival of 23% in patients with more than one poor prognostic criterion and 50% in those who showed only one criterion (p=0.036).

Table (1): Patient's characteristics.

Character	No (%)
Age (Y):	
Range	16-63
Mean	33.9±11.7
Sex:	
Male	27 (42.9%)
Female	36 (57.1%)
Ratio	1:1.3
WBCs x10 ^{9}/l:	
< 25 x10^9/l	23 (36.5%)
25-100 x10^9/l	17 (27.0%)
> 100 x10^9/l	23 (36.5%)
HB gm/dl:	
< 8	44 (69.8%)
≥8	19 (30.2%)
Platelets x10^9/l:	
< 25	18 (28.6%)
≥25	45 (71.4%)
Bone Marrow Blasts %:	
< 75%	42 (67%)
> 75%	21 (33%)
Symptoms:	
Fatigue	48 (76.2%)
Fever	42 (66.7%)
Bony aches	38 (61.3%)
Bleeding	13 (21.0%)
Signs:	
Splenomegaly	27 (43.6%)
FUO*	25 (39.7%)
Hepatomegaly	18 (28.6%)
Mucositis	11 (17.7%)
Lymphadenopathy	8 (12.9%)

*FUO= Fever of unknown origin.

Table (2): Immunophenotypic character of AML patients.

Cluster of Designation	No (%) of positive cases
CD34	21 (33%)
MPO	34 (58.6%)
CD33	42 (66.7%)
CD13	45 (72.6%)
CD14	10 (15.9%)
CD11	1 (1.6%)
CD15	3 (4.8%)
CD45	13 (20.9%)
CD7	8 (12.6%)
CD10	4 (6.4%)
CD19	6 (9.5%)
CD22	5 (7.9%)
CD41	1 (1.7%)
CD16	2 (32.2%)
CD56	3 (4.7%)
HLA-DR	34 (53.9%)
Biphenotypic	5 (7%)
Total patients with poor IPT profile	24 (38%)

Table (3): French-american-british (FAB) classification.

FAB	Number of patients (%)
M1	23 (36.5%)
M2	22 (35%)
M4	11 (17.4%)
M5	7 (11%)

Table (4): Chromosomal analysis.

Cytogenetic	Number of patients
Good prognosis:	
t (8; 21)	2
inv (16)	none
Poor prognosis:	
11q 23	none
t (9; 22)	2
del 5q	1
Intermediate prognosis:	
Normal karyotype	10
Other abnormalities	5

Table (5): Non-hematological treatment related toxicity.

Toxicity	Grade I-II Number of patients (%)	Grade III-IV Number of patients (%)
Alopecia	30 (47%)	0 (0%)
Stomatitis	23 (36%)	15 (23.5%)
Nausea/vomiting	30 (47%)	0 (0%)
Diarrhea	16 (25%)	2 (3%)
Hepatic	15 (23.8%)	6 (9.6%)
Cardiac	0 (0%)	6 (9.6%)

Table (6): Median levels of telomerase activity.

Telomerase Mean: 0.41± 0.04 Median: 0.4 (0.38-0.56)	No of patients (%) 63 (100)
< 0.40	37 (58.7%)
≥ 0.40	26 (41.3%)

Table (7): Correlation between telomerase and prognostic category.

Telomerase activity/ number of patients	Non-poor	Poor	p	2-year OS	p	1-year DFS	p
< 0.4 (37)	33	4		42%		34%	
			0.0001		0.134		0.012
≥ 0.4 (26)	1	25		23%		10%	

Table (8): Correlation between serum BCl-2 level and prognostic groups at 2-year OS and 1 year DFS.

Bcl-2 (U/mL)	Poor prognosis group	Non poor prognosis group	*p*	2 year OS	*p*	1 year DFS	*p*
<200	0	31 (100)		44%		37%	
≥200	29 (90.6)	3 (9.4)	0.0001	24%	0.078	11%	0.005

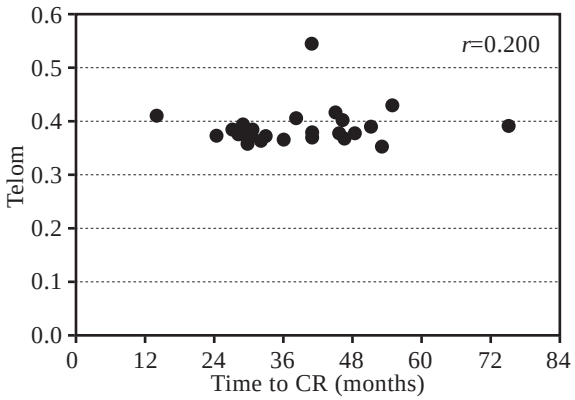

Fig. (1): Telomerase activity and response rate.

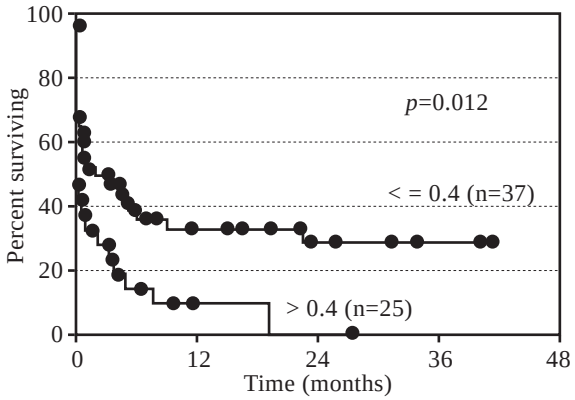

Fig. (2): Correlation between telomerase and disease-free survival.

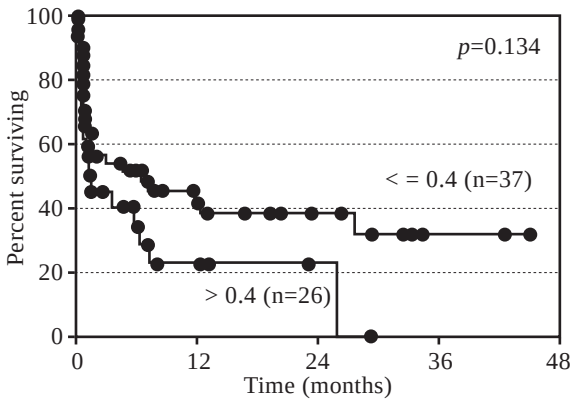

Fig. (3): Correlation between telomerase and overall survival.

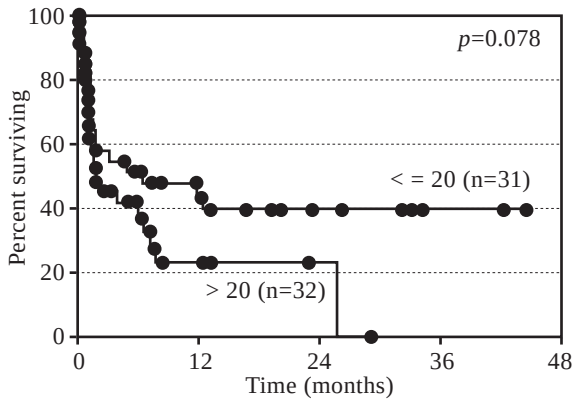

Fig. (4): Correlation between Bcl-2 and overall survival.

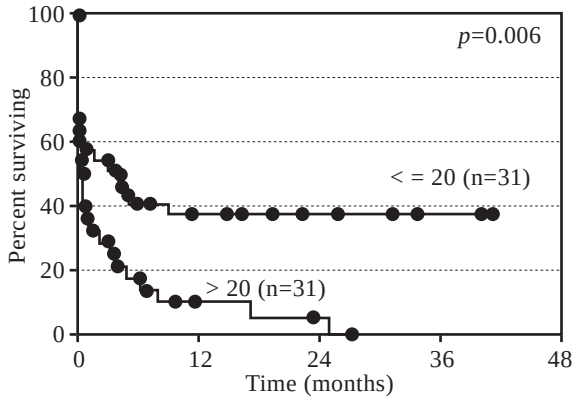

Fig. (5): Correlation between Bcl-2 and disease-free survival.

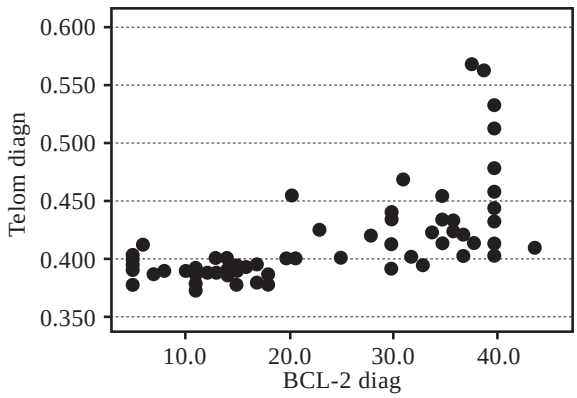

Fig. (6): Correlation between telomerase activity levels and Bcl-2

DISCUSSION

Over the past decade, the application of novel cytogenetic and molecular techniques has markedly improved our knowledge of the pathophysiology of acute myeloid leukemia, resulting in new potential therapies as well as better use of existing ones **[21]**. As therapy and supportive care improves, the intrinsic biologic characteristics of the patient's leukemia became the dominant factor in determining prognosis. Prog-

nosis of AML is mainly affected by cytogenetic risk groupings in addition to total leucocytic count at diagnosis and age **[6]**.

In this study, 63 patients with newly diagnosed acute myeloid leukemia were induced with the standard anthracycline and continuous infusion cytosine arabinoside. Complete remission was achieved in 36 patients (56%) with a 2 Y DFS of 18% and 3-y overall survival of 23%.

Telomerase activity was measured using TRAP assay on peripheral blood samples and it was significantly higher in AML patients compared with normal controls (p<0.05). Patients in the poor prognostic group expressed significant higher levels of telomerase compared with good prognosis group. There was a significant correlation between higher telomerase values and lower CR rates (p=0.019). Lower levels of telomerase were associated with better DFS at 1 year when compared to higher levels (34% Vs. 10%; p=0.012). The 2-year overall survival was higher in patients with levels of telomerase below 0.4 (42%) than those patients with telomerase level higher than 0.4 (34%). The difference was not statistically significant (p=0.134).

Increased telomerase expression in AML was reported by other investigators **[22-28]**. Concurring with our study also is the one conducted by Seol etal., 1998 **[22]** and Huh et al., 2005 **[27]**, who showed no correlation with CD34+, blast counts, white blood cell counts. In the later study, low remission rates were associated with higher telomerase activity with no correlation with time to remission. However, this had not been uniform in all studies as Xu et al., 1998 **[26]**, Verestovesk et al., 2003 **[28]**, did not demonstrate association with response rate or disease free survival. Conversely, Seol et al., 1998 **[22]**, reported that higher response rate were associated with higher telomerase activities. This heterogeneity of results might be explained by variation in the methods used to assess telomerase. Verstovsek et al., 2003 **[28]**, used a modified chain reaction-based, TRAP assay and measured telomerase activity in bone marrow samples. Another study assessed telomerase activity using the TRAP assay with an automatic DNA sequencer to detect and quantitate telomerase activity in peripheral blood samples **[29]**. There were also differences in the samples used with some studies using peripheral blood Xu et al., 1998 **[26]**, others using bone marrow or reporting on cell lines Seol et al., 1998 **[22]** some authors included secondary or relapse leukemia which might have a different biology. Furthermore, differences in the chemotherapy used as well as differences in the median age of patients involved with consequent differences in treatment might explain the inconsistent prognostic results.

Our study also demonstrated higher serum level of Bcl-2 compared with the control group (p<0.05). Significant higher levels were demonstrated in those with hyperleukocytosis (p< 0.0001) and poor prognosis immunophenotypic profile (p=0.011). Several early studies have examined the relation between Bcl-2 expression and individual prognostic factors in AML **[30-32]**. However, an unexpected observation was that among patients with unfavorable prognosis, the prognosis improved as the Bcl-2 level increased **[33]**. This might be explained that the ration of Bcl-2 to other family members which are pro-apoptotic might be more important than Bcl-2only. Overexpression of Bcl-2 was found to correlate with CD34 positivity in many studies of AML patients **[31,34,35]**.

Higher levels of Bcl-2 showed inverse association with CR rate; the results were almost statistically significant (p=0.06). This is in concordance with a number of studies that demonstrated that higher levels of Bcl-2 were associated with low CR rate in a number of studies **[31,32,34-37]**. Lower levels of Bcl-2 were associated with significantly higher 1 Y DFS (p 0.005). There was a trend towards a better survival for lower levels but it did not reach statistical significance (p=0.078). Campos et al., 1993 **[30]**, reported a significantly shorter overall survival (32% v 15% at 2 years; p< 0.005) in patients with high expression of Bcl-2. In this study the percentage of Bcl-2 positive cells, age and the percentage of CD34+ cells were independently associated with poor survival. In another study the three-year overall survival was 10% for patients with high expression levels of Bcl-2 and similar significant differences were observed for the disease free survival **[32]**. The results of these prognostic associations should be interpreted with caution as Bcl-2 has a more complex relationship regarding outcome and prognosis. Some studies

have reported that higher Bcl-2 levels correlate with better prognosis while other studies have reported the reverse **[33,36]**.

A possible explanation for this controversy is that most studies consider Bcl-2 in isolation. However the function of Bcl-2 may depend upon other members of the Bcl-2 family which has the upper hand on the phosphorylation status of Bcl-2. Hence despite the high levels of Bcl-2, the net effect may be pro-apoptotic due to overexpression of the other pro-apoptotic members **[38]**.

In a recent study a high expression of both anti- and pro-apoptotic genes in AML blasts at diagnosis have adverse prognostic impact **[39]**. The authors explained that their finding fits in with the recently defined concept of oncogenic addiction and the primed to death status of some tumor cells **[40]**. This can also explain the higher apoptosis related gene expression variance in AML.

There was a significant correlation between telomerase level and Bcl-2 (r=0.623; p<0.0001). Several studies explored the relation between telomerase and Bcl-2 **[41-44]**. Evaluating a possible association between telomerase activity and Bcl-2 level, we found a significant correlation between telomerase activity levels and Bcl-2 levels (r=0.623 & p<0.0001).

In one of the earliest studies exploring the relationship between Bcl-2 and telomerase activity, Abdel Salam et al. **[45]**, reported that the mean level of Bcl-2 was higher in telomerase positive breast cancer cases than in telomerase negative ones suggesting a possible association between the two markers. A later study confirmed that telomerase and Bcl-2 were independent prognostic factors in Egyptian breast cancer patients and failed to detect a significant association between Bcl-2 and Telomerase **[41]**. As our understanding of the role played by telomerase in tumor progression improves as well as the interplay between various members in the Bcl-2 family of genes, an association between telomerase activity and the anti-apoptotic pathway may emerge.

The important prognostic relevance arise from the fact that they may add to the molecular armentarium including nucleophosmin, FLT-3 and others currently tested to allow for choosing which patient with normal cytogentics will require bone marrow transplantation Beside prognosis two important roles could be potentially useful for these markers either separately or combined which are their use for follow-up of cases to detect early relapse and their potential use as therapeutic targets. Anti Bcl-2 anti sense oligonucleotide is currently under trials. Larger prospective studies with standardization of the techniques and treatment protocols are currently required before definite conclusions could be withdrawn.

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