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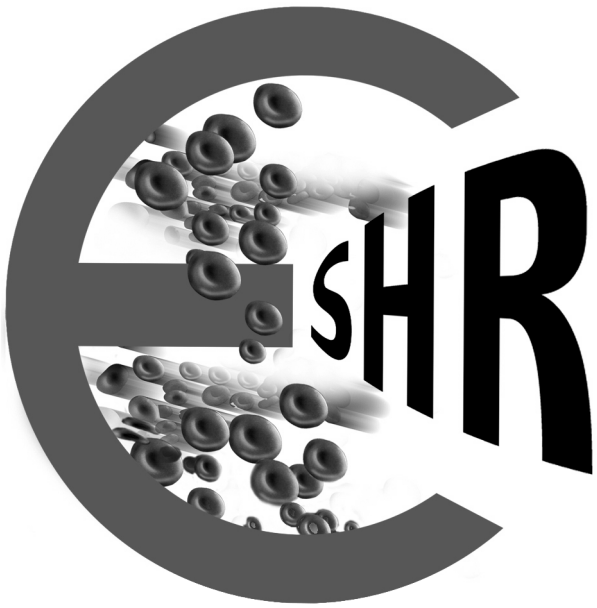

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CLLU1 Gene Levels Quantitation as an Assessment Tool in Predicting the Time to Initiation of Therapy in Egyptian CLL Patients

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ABSTRACT

Background:

Chronic lymphocytic leukemia (CLL) is an incurable disease with a highly variable clinical course. An estimation of survival or time to treatment in patients with CLL may be achieved based on numerous clinical, cell based, and molecular prognostic markers. A recent identified novel CLL-specific gene (CLL up-regulated gene 1, CLLU1), is exclusively up-regulated in CLL cells and its expression levels are important to evaluate the progression of the disease.

Aim of the Study:

To investigate CLLU1 expression in de novo Egyptian CLL patients and to evaluate its relation to the start time of treatment as a potential prognostic parameter.

Patients and Methods:

A cohort of 40 untreated CLL patients was studied. The expression levels of CLLU1 transcript c DNA was determined by quantitative real time-polymerase chain reaction. The relation between CLLU1 expression and time to initiation of therapy was analyzed.

Results:

There was high statistically significant difference between all patients when compared to controls as regards the expression level (p-value <0.001), with patient median expression showing 78.78 fold increase than the median controls levels. The median level of CLLU1 in cases who need treatment is more than those who did not take treatment with 36.75 fold increase and high statistically significant difference (p-value <0.001).

Conclusion:

In conclusion, our findings showed that the level of expression of CLLU1 gene may be used as an estimation of the time to initiation of therapy. Accordingly it may be used for prediction of the clinical course of the disease and hence prognosis.

Key Words: *CLLU1 gene – CLL – QRT-PCR.*

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is one of the most common lymphoid malignancies accounting for approximately 11% of all hematologic neoplasms **[1]**. It is the most common leukemia in the western world with an annual incidence of 5/100000. The clinical course of the disease is heterogeneous, with some patients experiencing rapid disease progression and others living for decades without requiring treatment **[2]**.

The prognosis of patients with B-CLL has long been determined by the clinical staging systems of Binet and Rai **[3]**. Unfortunately, these systems are unable to identify patients in the early stages whose disease will rapidly progress. Neither can this approach clearly predict the response of individual patients to specific therapies **[4]**. A few other prognostic factors have been identified which are independent of clinical stage: Lymphocyte doubling time, pattern of bone marrow infiltration, LDH, serum B2 microglobulin, soluble CD23, serum thymidine kinase, expression of CD38 on CD 19 positive B-cells, genetic defects in specific molecular pathways, involving p53 at 17p13, or ATM at 11q22 and the mutational status of immunoglobulin heavy chain variable region genes (IgVh) **[5-9]**.

Clinical features and molecular/biologic factors such as ZAP-70, and cytogenetic abnormalities on fluorescent in situ hyperdization (FISH) have been found to be robust predictors

of treatment-free survival and overall survival among newly diagnosed patients **[10]**.

While cytogenetics and IgVh mutational status are good predictors of outcome, their determination is confined to specialized centers **[11,12]**. The identification of other markers which can be determined by monoclonal antibodies or by polymerase chain reaction would improve the diagnosis of CLL. Therefore, the major aim in the management algorithm of an indolent disease like CLL is to search for novel predictors of outcome and to develop non-toxic, target-specific therapies **[13]**.

One of the recent discovered genes is chronic lymphocyte leukemia up-regulated gene 1 (CLLU1) which was cloned and identified by Buhl et al., 2006 **[14]**. CLLU1 gene is located at chromosome 12q22, its transcripts have no significant similarity to other human genes and most of transcripts appear to be non-coding while no miRNAs were detected, nevertheless, the non-coding transcripts may have functions that presently have not been described.

The gene is not conserved in other species and even though several of the putative CLLU1 splice variants contain a putative open reading frame of 121 amino acids, researchers have not able to convincingly detect expression of the putative CLLU1 protein in CLL patient samples. Thus, they do not know whether CLLU1 plays a role in the pathogenesis of CLL, or whether its expression is a reflection of other events in the CLL cells **[15,16]**.

The goal of the current study is to detect the prevalence of CLLU1 gene expression among 40 de novo Egyptian CLL patients as a molecular marker that may be useful in the prediction of the time to initiation of therapy and useful in prognosis.

MATERIAL AND METHODS

Patients and methods:

Fourty Egyptian Patients with the diagnosis of Chronic lymphocytic leukemia (CLL) referred to the Kasr El-Aini Center of R adiation, Oncology and Nuclear Medicine (NEMROCK) and Beni Suef University Hospital between January 2007 and December 2008 were included in this study.

Peripheral blood (PB) and bone marrow samples from these patients were collected at diagnosis and centrifuged to obtain mononuclear cells (MNCS).

The diagnosis of CLL was made based on the standard morphologic and immunophenotypic criteria including absolute lymphocytosis, presence of basket cells and positivity for CD5+ and CD19+. Clinical data were obtained from the patients' medical records.

Ten age and sex matched individuals with lymphocytosis were included as controls.

CLLU1 gene was analyzed using real time quantitative reverse transcriptase polymerase chain reaction (RTQ-PCR) to investigate a possible relation, association, or correlation with the clinical features of patients at diagnosis (such as: Gender, age, hemoglobin (Hb), TLC, platelets count) and with treatment outcome and prognosis.

RNA isolation and real-time quantitative RT-PCR:

Mononuclear cells (MNCs) were isolated from 2ml peripheral blood at diagnosis by Ficoll density gradient centrifugation. Total RNA was extracted from MNCs using a QIAamp RNA blood kit (Qiagen,Germany). Complementary DNA (cDNA) was synthesized using (dt) 15-mer primer by superscript III Reverse transcriptase and stored at –20°C till use.

The mRNA expression levels of CLLU-1 gene and β2 microglobulin were measured by quantitative RT-PCR using an ABI PRISM 7000 Sequence Detector System (Applied Biosystems, Foster City, CA, USA). The quantitative RT-PCR amplification was performed using one of the CLLU1 gene transcripts named cDNA1 having the following primer and probes: ´5´-AGCTTGCAGATGGCAGATCA-´3´ (forward primer), ´5´-CATAAAGGGCAGCGAAATGC-´3´ (reverse primer) and ´5´-TATCTCCAGGCC-TTTCATTGGGTCAGGT- ´3´ (FAM-probe).

And for β2-microglobulin: ´5´-TGACTTT-GTCACAGCCCAAGATA-´3´ (forward primer), ´5´-AATCCAAATGCGGCATCTTC-´3´ (reverse primer) and ´5´-TGATGCTGCTTACATGTCTC-GATCCCA-´3´ (FAM-probe). All probes were TaqMan probes obtained from Applied Biosystems.

All reactions were perfomed in triplicate using 50ng c DNA and one-step RT-PCR Mix reagents (AB4309169) as described by the manufacturer. Primer and probe concentrations were 200 and 100nm, respectively. The reaction protocol used involved heating for 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles of amplification (15 seconds at 95°C and 1 minute at 60°C). Analysis was performed using ABI PRISM 7000 Sequence Detection Software (Applied Biosystems).

The expression levels of CLLU-1 gene in tested samples were expressed in the form of CT (cycle threshold) level then normalized copy number (relative quantitation) was calculated using the Δ Δ CT equation. A negative control without template was included in each experiment.

Expression level of CLLU-1 was correlated with the clinical features of the studied patients at diagnosis including: Age, gender, TLC, hemoglobin, platelets, etc.

Differences in expression levels of CLLU-1 with respect to the prognostic factors were analysed using the Mann-Whitney U test. The expression levels were represented as the mean or median values. Results were considered significant at $p<0.05$.

For univariate CLLU1 analysis, time to treatment was also considered an end-point, defined as time from diagnosis to first treatment or end of follow-up.

RESULTS

The clinical characteristics of the study cohort are presented in Table (1). The median age at diagnosis was 50 years, with a mean of 53.5±9.5 and a range of 44-65 years.

Of the fourty patients included in this study 19 (47.5%) were males, while 21 (52.5%) were females.

The mean total leucocytic count (TLC) of the patients at diagnosis was 57±53.6x10^9/L (range 3.4-110x10^9/L) with mean lymphocytic percentage 70.5±22.5% (range 45-90%), the mean hemoglobin 10.7±4gm/dl (range 6.3-14.8 gm/dl), mean platelet count 181.6±130x10^9/L (range 58-520x10^9/L).

Thirty of 40 patients (75%) had hepatosplenomegally (HSM), while 25/40 (62.5%) had lymphadenopathy.

According to Rai staging system which stated that stage 0=lymphocytosis with 40% of BM cells lymphocytes, stage I=stage 0 + enlarged LN, stage II=stage I + enlarged liver, spleen or both, stage III=stage II + Hb<11gm/dl and stage IV=stage III + platelets <100x10^9/L), 21 patients were in stage II, 9 patients were in stage III, and 10 patients were in stage IV.

CLLU1 gene was expressed in 38/40 (95%) patients at diagnosis with a median level of 333.1 and mean of 14179.74±14179.212 (range 0.5285-42642.37). While the median level was 4.2281, with mean level 5.86±5.8593 (range 0.0007-14.7230) in controls.

There was high statistically significant difference between all patients when compared to controls as regards the gene expression level ($p<0.001$) with patients median expression level showing 78.78 fold increase than median controls level.

The follow-up of patients from January 2007 to December 2008 showed that 18/40 cases showed disease progression and started treatment, while 22/40 cases did not show any change in disease activity and needed no treatment. The median CLLU1 level in cases who needed treatment was 8079.21, while the median CLLU1 level of those who did not need treatment was 219.79 with 36.75 fold increase in the treated cases than the untreated cases and with high statistical significant difference ($p< 0.001$).

When correlating the relative quantitation (RQ) level of CLLU1 gene with the lag period before starting treatment (weeks) there was negative correlation ($r=–0.557$) but high statistical significant difference ($p=0.016$).

There was no statistically significant difference when comparing patients (treated or untreated) with different clinical and laboratory findings including age, gender, total leucocytic count, hemoglobin level, platelet count, lymphocytic count, hepatosplenomegally or lymphadenopathy ($p>0.05$).

Table (1): Characteristics of 40 CLL patients.

Characteristic	Value
Gender, no (%)	
Male	19 (47.5%)
Female	21 (52.5%)
Age at diagnosis (years)	53.5±9.5 years (44-65 years)*
Median	50 years
Total leucocytic count x10^9/L	57±53.6x10^9/L (range 3.4-110x10^9/L)
Hemoglobin gm/dl	10.7±4gm/dl (range 6.3-14.8gm/dl)
Plateletsx10^9/L	181.6±130x10^9/L (range 58-520x10^9/L).
Lymphocytic count (%)	70.8% (45-90%)
Absolute Lymphocytic count (Mean ± SD)	13868.3±7.5
Scoring of CLL no (%)	
II	21 (52.5%)
III	9 (17.5%)
IV	10 (25%)
Follow-up	
Needed treatment	18 (45%)
Did not need treatment	22 (55%)

* Mean ± SD (range).

Table (2): CLLU1 gene in 40 CLL patients compared to controls.

CLL U1 gene	Positive cases	Level of CLLU1 gene (median)	Fold increase	*p*-Value
Cases	38/40 (95%)	333.1	78.78	*p*<0.001
Controls	8/10 (80%)	4.2281		
Treated Cases	18/40 (45%)	8079.21	36.75	*p*<0.001
Untreated Cases	22/40 (55%)	219.79		

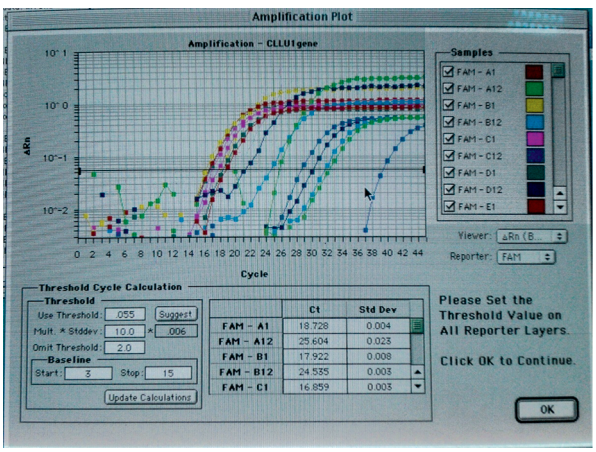

Fig. (1): CLLU1 gene in CLL cases and controls.

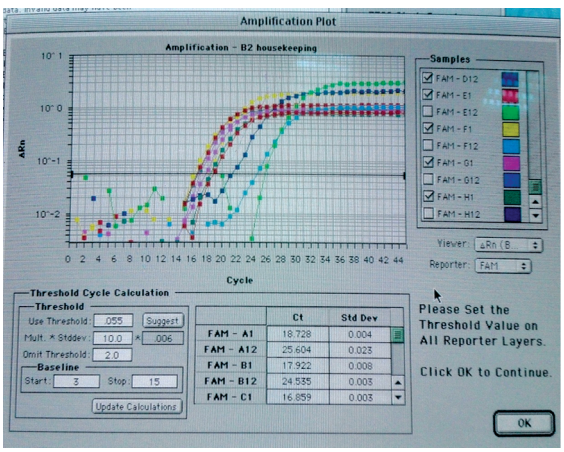

Fig. (2): Housekeeping gene (β2 microglobulin) in CLL cases and controls.

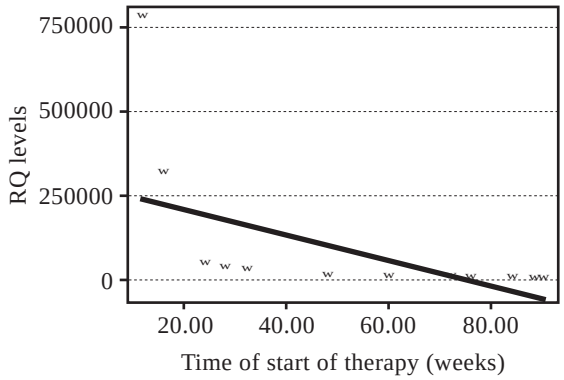

Fig. (3): Correlation curve between relative quantitation (RQ) levels of CLLU1 gene and time of start of therapy.

DISCUSSION

The origin of new genes is extremely important to evolutionary innovation. Most new genes arise from existing genes through duplication or recombination. The origin of genes from non-coding DNA is extremely rare, and very few eukaryotic examples are known in yeast and drosophilia **[16]**.

In this study, CLLU1 gene expression was analyzed in peripheral blood of de novo 40 CLL Egyptian patients in comparison to peripheral blood of 10 healthy controls, by using QRT-PCR. While many of the patients were presenting at the beginning with low grade CLL and are preferentially treated with a [watchful waiting] approach **[17]**, the patients were followed-up for two years, to observe who was in need for early treatment and compare the levels of CLLU1 in the patients who needed early aggressive treatment to those who stayed with stable disease.

Our results are in accordance with the earlier study **[14]**, which showed that the restricted high levels of CLLU1 was found in CLL when compared to normal B lymphocytes and other hematological disorders, also, this was confirmed by a recent study **[18]**.

Another study **[19]** reported a significantly high levels of CLLU1 gene in CLL patients using two QRT-PCR reactions, for c DNA and CDS (the most common splice variants of CLLU1 gene), where they found the median c DNA expression levels was 27.27 fold above the level found in normal B cells of healthy controls and a significant linear relationship between the expression level of c DNA and CDS within the patients was found. However, they recommend to use cDNA variant as a measure for the CLLU1 level which we used in our study, since the c DNA QRT-PCR reaction spans an exon-intron boundary and is thus not affected by contaminating DNA. No correlation was found between the patient's age of diagnosis and CLLU1 gene level, this was in line with our results.

In another work, CLLU1 expression levels represented a continuum ranging from 0.0005- to 10.000-fold up-regulation compared with that of normal B cells with a median of 22.9-fold up-regulation **[20]**.

In fact, the absence of expression in the other tissues raised the possibility that CLLU1 may not be required for normal human function and development and the reason for the high expression in CLL could be the result of accidental activation of a promotor upstream of CLLU1 **[19]**.

The specificity and the sensitivity of the CLLU1 expression as well as its utility in distinguishing B-CLL from other B-cell lymphoproliferative disorders was assessed **[21]**. CLLU1 expression levels were measured by QRT-PCR and the results showed over expression of CLLU1 in more than 85% of the cases of B-CLL in comparison to the healthy controls and other B-lymphoproliferative disorders **[21]**. CLLU1 expression was not detected in the majority of the lymphoma patients and these results were confirmed by Kienle et al. **[22]**, whose results suggested that analysis of CLLU1 expression by RT-PCR is a simple novel diagnostic tool especially in cases where known diagnostic parameters fail to establish the diagnosis.

In our study, there is association between the expression levels of CLLU1 and time to initiation of therapy during the study period which was found to be significantly higher in patients who received treatment early than those who did not need treatment (p-value<0.001).

Buhl et al. **[19]**, revealed that a doubling of c DNA transcript level was associated with an increased risk of initiation of therapy and this association may help to identify how soon a given CLL patient will be in need for therapy.

Low to moderate (≤40 fold increase than controls) CLLU1 expression is associated with an indolent disease that may never require treatment, whereas high (>40 fold increase than controls) CLLU1 expression is associated with a more aggressive clinical course with early therapy onset. Moreover its high expression has strong independent prognostic significance for overall survival in CLL.

The value of CLLU1 level estimation was that it was the first demonstration within the field of dose-response relationship between the expression level of a prognostic gene and time to therapy initiation **[19]**.

A continuous proportional relationship between the expression level of CLLU1 at time of diagnosis and the relative risk of early death was demonstrated **[20]**. Therefore, CLLU1 is a prognostic marker in CLL, as high expression levels are associated with shorter time to treatment and poor overall survival in patients **[19,20,23]**.

In a Chinese study, Chen et al. **[24]**, examined CDS transcript expression instead of c DNA1, CDS transcription was expressed in 50% of patients with CLL where it was negatively expressed in normal B-subpopulation and other hematological disorders as acute leukemia, multiple myeloma and polycythemia vera. They correlated CLLU1 expression to the prognostic factors of CLL as CD 38, IgVH somatic hypermutational status in CLL, ZAP-70 and cytogenetic aberrations with a conclusion that patient stratification according to the prognostic markers demonstrates a significant increase of CLLU1 expression in high risk groups and that the unique exclusive expression of CLLU1 might be an important prognostic factor in CLL.

Hayette and colleagues **[25]** confirmed the prognostic significance of CLLU1 in CLL and its usefulness as a reliable alternative to IgVh gene sequencing. Moreover, its quantification does not require purification of B lymphocyte, a significant advantage in routine practice.

The expression of CLLLU1 might play an important critical role in monitoring minimal residual disease and therefore might be used for follow-up of patients with CLL. In addition evaluation of CLLU1 levels in the blood is highly specific for detecting residual CLL with sensitivity that might preclude the need for marrow assessment of all patients **[26]**.

Buhl et al. **[14]** hypothesis is that if CLLU1 turns out to be involved in CLL pathogenesis, targeting of CLLU1, for example using siRNA **[27,28]** could represent an ideal strategy for development of CLL-specific therapy because such therapy would not affect other tissues and if CLLU1 does not have an important role in CLL development or progression, it may still be useful for targeting cell-suicide gene therapy to CLL cells **[29,30]**.

Buhl et al. **[15]** continued his studies to further investigate the biological properties of the CLLU1 m RNAs; their results suggested that all the splice variants are derived from one primary transcript and regulated by a common promotor upstream from Exon 1 and demonstrated that CLLU1 levels in serial CLL samples of untreated patients were stable over time and with similar levels in peripheral blood and bone marrow. Moreover, the CLL cells of anyone patient have uniform expression as assessed by in situ hybridization. The expression levels are similar in patient samples before treatment and after relapse; hence a high CLLU1 level did not appear to be a feature that is acquired by CLL clone during the course of the disease **[15]**.

The current standard of care of CLL is to initiate treatment when a patient has progressive or symptomatic disease **[31]**.

As of today, only two molecular CLL clone-specific features are known: The IgVh structure and CLLU1 expression level. These two parameters may be considered the fingerprint of a particular CLL clone **[15]**.

In conclusion, our finding further emphasized that the expression level of CLLU1 in newly diagnosed CLL patients give an idea about the time of initiation of therapy, accordingly it could be highly predictive of the prognosis and the clinical course of the disease. Further studies and more clinical trials should help elucidate the important function of this gene and elucidate the potential biological implications of detecting CLLU1 expression levels in CLL patients and its potential use as a therapeutic target.

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Genomic Aberrations in Egyptian Chronic Lymphocytic Leukemia Patients

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ABSTRACT

Background: B-chronic lymphocytic leukemia is a heterogeneous disease with a highly variable clinical course and prognosis. Several clonal chromosomal aberrations which have different prognosis were found in CLL, the most common chromosomal abnormalities include del 13q14.3, del 11q22.3 (ATM gene), trisomy 12, del 17p13.1 (tumor suppressor gene p53) and del 13q34.

Aim of the Work: Was to determine the prevalence of the common recurring genetic defects in an Egyptian cohort of B-CLL patients and to evaluate the correlation of these genetic defects with the clinical presentation, laboratory data, response to induction therapy, disease progression, prognosis as well as overall survival.

Patients and Methods: This study included 30 B-CLL patients as well as 15 age and sex matched normal healthy subjects as a control group. All patients were subjected to Fluorescence In Situ Hybridization (FISH) technique for detection of different genomic aberrations including del 13q14.3, del 11q22.3, trisomy 12, del 17p13.1 and del 13q34.

Results: Our study revealed that the CLL FISH panel detected genomic abnormalities in 28 patients (93%). Twelve CLL patients (40%) had del 13q14.3, 7 patients (23%) had del 11q22.3, 19 patients (63%) had trisomy 12, 12 patients (44%) had del 17p13.1, while 13 patients (43%) had del 13q34. There was a statistically significant difference between CLL patients with positive and negative different genomic aberrations as regards Rai and Binet staging system and immunophenotyping including CD38 and ZAP70 ($p<0.05$) except for del 11q22.3. Also, there was a highly statistically significant difference between CLL patients with positive and negative different genomic aberrations as regards serum LDH, B2-microglobulin, number of chemotherapy cycles, response to chemotherapy and total free survival ($p<0.01$) except for the number of chemotherapy cycles in del 11q22.3 and a significant difference was found as regards overall survival ($p<0.05$). While there was no statistically significant difference as regards BM lymphocyte percent and duration of illness ($p>0.05$) except for del 13q34 which showed a significant difference ($p<0.05$).

Conclusion: Multiple genomic aberrations can play an important role in the clinical presentation, prognosis, disease progression and response to chemotherapy as well as overall survival in an Egyptian cohort of CLL patients as compared to others.

Key Words: *Del 13q14.3 – Del 11q22.3 – Trisomy 12 – Del 17p13.1 – Del 13q34 – FISH – CLL.*

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a monoclonal disorder characterized by a progressive accumulation of functionally incompetent lymphocytes. It is the most common form of leukemia found in adults in Western countries **[1]**. Some patients show an indolent disease and never require treatment, while in others the clinical course is aggressive requiring intensive treatment shortly after diagnosis **[2]**.

The staging systems developed by Rai et al. **[3]** and Binet et al. **[4]** have been recognized as standard methods of assessing the survival and the treatment requirements in B-CLL patients. However, these systems can not identify stable or progressive forms of the disease, especially in the early stages of B-CLL, which include most of the patients at diagnosis. The substantial heterogeneity within clinical stages has prompted for additional prognostic factors **[5]**.

Identifying chromosomal aberrations could pinpoint subgroups of patients with chronic lymphocytic leukemia who have different prognosis. These abnormalities are independent predictors of the disease progression and survival, as well as response to standard chemotherapy and monoclonal antibody therapy **[6]**.

Conventional cytogenetic analysis has been hampered by the low mitotic activity and proliferative index of leukemia cells in vitro, and

even with mitogens; metaphases are difficult to obtain. Fluorescence In Situ Hybridization (FISH) allows the detection of chromosomal aberration not only in dividing cells but also in interphase cells **[7]**. Most commonly, these abnormalities are deletions or aneuploidy, and in contrast to other low-grade lymphoid malignancies, translocations are infrequent **[8]**.

The most common chromosomal abnormalities in CLL are del 13q14.3, del 11q22.3 (ATM gene), trisomy 12, del 17p13.1 (tumor suppressor gene p53) and del 13q34. These findings have implications for the design of risk-adapted treatment strategies **[9]**.

Del 13q14.3 is the most frequent structural abnormality in CLL. Cases with this abnormality usually have mutations of the IgHV gene, classic CLL cell morphology, and good prognosis. Also, patients with del 13q34 had a similar survival to those with normal chromosomes and only one-third requires therapy **[10]**.

The ataxia telangiectasia (ATM) gene is located within the minimal region of loss at 11q22.3, suggesting that alterations in this gene may be involved in the pathogenesis of the disease. This is further supported by the finding that mutations in the ATM gene are associated with poor prognosis **[11]**.

Trisomy 12 occurs as a result of duplication of one homolog. Chromosome 12 contains the murine double minute (mdm-2) gene, and overexpression of mdm-2 could stimulate a p53 mutation, as mdm-2 binds and inactivates p53 **[12]**. While, del 17p13.1 involves the p53 locus at 17p13.1; that deletion and mutations in the p53 gene can contribute to disease progression and drug resistance **[13]**.

CLL cells often exhibit multiple cytogenetic abnormalities that may be involved in the pathogenesis of the disease, prognosis, disease progression and alter the sensitivity of CLL cells to chemotherapy agents. These findings have implications for the design of risk-adapted treatment strategies **[14]**.

Aim of the work:

The aim of this work was to study the prevalence of the common recurring genetic defects in an Egyptian cohort of B-CLL patients including del 13q14.3, del 11q22.3 (ATM gene), trisomy 12, del 17p13.1 (tumor suppressor gene p53) and del 13q34. We also aimed to evaluate the correlation of these genetic defects with the clinical presentation, laboratory data, response to induction therapy, disease progression, prognosis as well as overall survival of these patients.

PATIENTS AND METHODS

Patients:

The present study was conducted on 30 patients with B-chronic lymphocytic leukemia, their ages ranged between 42 and 80 years with a mean of 57.96±10.55 and a median of 56 years. They were 23 male (77%) and 7 female (23%). Patients were diagnosed and selected among cases referred to the Haematology Clinic and the Haematology-Oncology Unit at El-Kasr El-Aini Hospital, Cairo University. Fifteen age and sex matched normal healthy individuals were also included as a control group.

The diagnosis of leukemia was based on complete history taking, clinical examination for organomegaly and lymphadenopathy and laboratory investigations for diagnosis and prognosis of B-CLL including complete blood count, bone marrow aspirate, immunophenotyping of lymphoid cells, serum LDH, B2-microglobulin and special laboratory investigations (for patients and controls) for detection of different genomic aberrations including del 13q-14.3, del 11q22.3 (ATM gene), trisomy 12, del 17p13.1 (tumor suppressor gene p53) and del 13q34 using Fluorescence In Situ Hybridization (FISH) technique according to the method described by Nascimento et al. **[15]**.

Patients were studied prior to chemotherapy and followed up after induction chemotherapy. Patients were treated by one of the following lines of chemotherapy depending on age, performance status and stage of disease; chlorambucil (CIb) and prednisone: CIb was given orally at dose of 0.2mg/kg/day and predinisone 20mg/m^2/day. Cyclophosphmide, vincristine and prednisone (CVP): Cyclophosphmide 400 mg/m^2 IV on days 1-3, vincristine 1.4mg/m^2 IV on days 1 and oral prednisone 400mg/m^2 on days 1-5. Fludarabine, rituximab and cyclophosphamide: Fludarabine 25mg/m^2 IV, rituximab 375mg/m^2 IV and cyclophosphmide 250mg/m^2 IV on days 1-3. Response to induction chemotherapy was assessed according to the criteria proposed by the National Cancer Institute (NCI)-

sponsored working group prior to study SWOG-9108 **[16]**.

Evaluation of response to chemotherapy had been made according to the following criteria; Complete remission (CR): Asymptomatic patients with no organomegaly or lymphadenopathy. Lymphocyte count <4x10^3/µl, neutrophils >1.5x10^3/µl, hemoglobin >11gm/dl, platelets count >100x10^6/µl and bone marrow lymphocytes <30%. Partial remission (PR): More than 50% decrease in organomegaly or lymphadenopathy plus one of the following: Neutroplils >1.5x10^3/µl, hemoglobin >11gm/dl and platelets count >100x10^6/µl. Progressive disease (PD): New lesion or >50% increase in organomegaly or lymphadenopathy, circulating lymphocytes revealing >50% increase.

Methods:

1- Sample collection:

Seven milliliters of venous blood were collected from each patient and each individual of the control group by sterile venipuncture under aseptic precautions and divided as follows: 2ml on ethylene diamine tetra-acetic acid (EDTA) sterile vacutainer for performing a complete blood picture and immunophenotyping, the remaining 5ml on sodium-heparin sterile vacutainer for the study of different genomic aberrations by FISH technique.

2- Detection of different genomic aberrations using FISH technique:

I- Cell culturing and fixation:

Under the laminar flow, peripheral blood lymphocytes were cultered. The culture media (Gibcobrl) comprised of 5ml RPMI culture medium, 50µl pencillin-streptomycin (1%), 1ml fetal calf serum (20%), 1ml patient's cell-rich plasma. After 2 hours, 100µl colcemide (Boehringer) was added followed by 5ml hypotonic solution then 4 times wash with methanol-acetic acid.

II- *FISH technique for the study of different genomic aberrations:*

Cytogenetic abnormalities using FISH technique was detected using five DNA probes in two sets (Abott Vysis, Downers Grove, IL, USA):

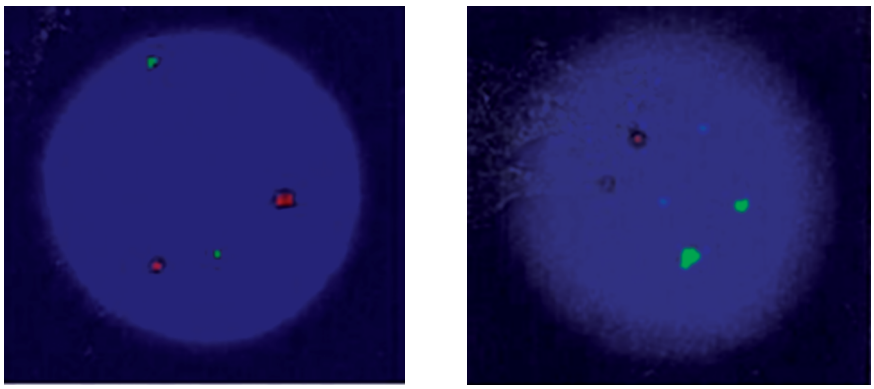
- Probe set 1: Allows assessment of the following chromosomal regions; LSI p53 (17p13.1) and LSI ATM (11q22.3) in two orange and two green signals respectively.
- Probe set 2: Allows assessment of the following regions; LSI D13S319 (13q14.3), LSI 13q34 (13q34), CEP12 (12p11.1-q11) in one mix as two orange, two aqua and two green signals respectively.
- The two probes were set up separately on 2 different slides prepared from fixed cell pellets for each patient. Hybridization was done using Vysis hybrit TM according to the manufacturer's protocols and rapid wash procedure was performed, then 10µl 4-6-diaidino-2-phenylindole dihydrochloride (DAPH II) counterstain were added to target areas and covers applied to slides.

III- *Detection of hybridization signals:*

Detection of hybridization signals were performed using epi-thrombin fluorescence Olympus microscope equipped with filters capable of simultaneously passing DAPI/FITC/Texas red and Quips spectra Vysis hardware and software. The hybridized probes fluoresce with bright intensity both in interphase nuclei and on metaphase chromosomes. Two hundred interphase cells were counted and the results taken according to the signals detected in a score system in which the number of bright distinct fluorescent spots in each nucleus was recorded as 0, 1, 2, 3, 4 signals.

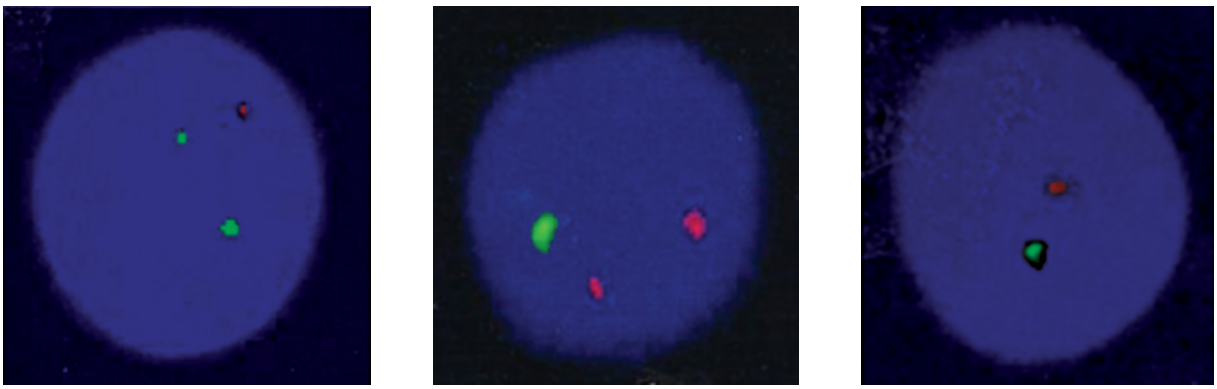
IV- *Interpretation of results:*

- Normal: In interphase nuclei of normal cells, the probe set 1 generally appears as two distinct orange signals and two green signals for p53 (17p13.1) and ATM (11q22.3) respectively as shown in Fig. (1a), Probe set 2 appears as two distinct orange signals, two aqua and two green signals for D13S319 (13q14.3), 13q34 (13q34), CEP12 (12p11.1-q11) respectively as shown in Fig. (1b). The signals may appear split or diffused. Occasionally, the probe may show extra-signals (diffuse or split) depending upon the condensation of the DNA and the relative distance between chromatids.
- Deletion: p53 (17p13.1), ATM (11q22.3), D13S319 (13q14.3) and 13q34 (13q34) areas appear as single orange, green, orange and aqua signal respectively as shown in Figs. (2,3) using the same technique.
- Trisomy: CEP12 (12p11.1-q11) area appears as 3 green signals as shown in Fig. (3b,c).



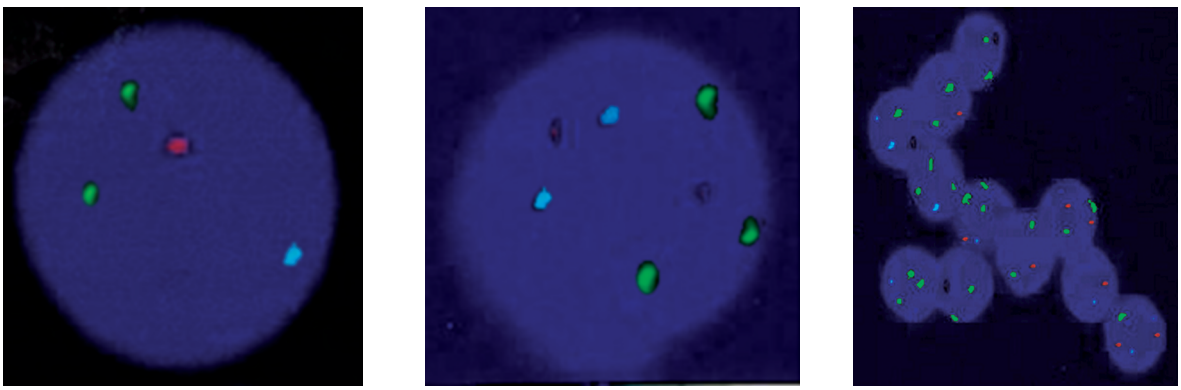
(A) **(B)**

Fig. (1): FISH technique using: (a) Probe set 1 (LSI P53 and LSI ATM) showing normal chromosome 17p13.1 and chromosome 11q22.3 in two orange and two green signals respectively. (b) Probe set 2 (LSI D13S319, LSI 13q34 and CEP12) showing normal chromosome 13q14.3, chromosome 13q34 and chromosome 12 in two orange, two aqua and two green signals respectively.



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

Fig. (2): FISH technique using probe set 1 (LSI P53 and LSI ATM) showing: (a) Del 17p13.1 and normal chromosome 11q22.3 in one orange and two green signals respectively. (b) Normal chromosome 17p13.1 and del 11q22.3 in two orange and one green signals respectively. (c) Del 17p13.1 and del 11q22.3 in one orange and one green signals respectively.



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

Fig. (3): FISH technique using probe set 2 (LSI D13S319, LSI 13q34 and CEP12) showing: (a) Del 13q14.3, del 13q34 and normal chromosome 12 in one orange, one aqua and two green signals respectively. (b) Normal chromosome 13q14.3, chromosome 13q34 and trisomy 12 in two orange, two aqua and three green signals respectively. (c) Del 13q14.3, del 13q34 and trisomy 12 in one orange, one aqua and three green signals respectively.

For each probe, two hundred interphase cells of the control subjects were counted. Means and standard deviations (SD) of the percentage of the nuclei with hybridization signals were calculated. Results were considered abnormal, if the percent of nuclei with the abnormal hybridization signal >2SD from the mean.

Our cut off values were as follows:

10% for del 13q14.3 (D13S319), 7% for del 11q22.3 (ATM gene), 5% for trisomy 12 (12p11.1-q11), 10% for del 17p13.1 (p53) and 9.5% for del 13q34.

Statistical analysis:

Data was analyzed using SPSS win statistical package version 15. Numerical data were expressed as a mean, standard deviation (SD) and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test or Fisher's Exact test was used to examine the relation between qualitative variables. For quantitative data, comparison between two groups was done using the Mann-Whitney test (non-parametric *t*-test). Relation between numerical variables was tested using Pearson product-moment correlation coefficient. A *p*-value less than 0.05 was considered significant and a *p*-value less than 0.01 was considered highly significant.

RESULTS

The patients' characteristics are displayed in Tables (1,2).

Different genomic aberrations detected in B-CLL patients are shown in Table (3).

Table (4) demonstrates the correlation of different genomic aberrations with clinical, laboratory, staging systems and prognostic parameters in B-CLL patients.

Table (1): Clinical and laboratory data of 30 B-CLL patients.

Item	B-CLL Patients (No. 30)
Age (years)	57.96±10.55 (42-80)*
Gender	
Male	23 (77%)**
Female	7 (23%)
Clinical data	
Hepatomegaly	18 (60%)
Splenomegaly	21 (70%)
Lymphadenopathy	7 (90%)
Laboratory data	
Hb gm/dl	9.42±2.49 (5.50-14.20)
TLC x 10^9/L	66.39±48.78 (9.80-212.30)
Platelets x 10^9/L	156.33±84.24 (30.0-373.0)
PB lymphocytes %	78.33±16.66 (40-98)
PB absolute lymphocytic count x10^9/L	62.84±51.90 (5.60-191.07)
Immunophenotyping	
CD38	12 (40%)
ZAP-70	13 (43%)

* Mean ± SD (range).
** No. (%)

Table (2): Different staging systems, prognostic parameters and treatment outcome in 30 B-CLL patients.

Item	B-CLL Patients (No. 30)
Different staging systems	
Rai staging system:	
Stage Modified stage	
0 Low risk	1 (3%)*
I Intermediate risk	8 (27%)
II Intermediate risk	3 (10%)
III High risk	8 (27%)
IV High risk	10 (33%)
Binet staging system:	
A	7 (23%)
B	10 (33%)
C	13 (44%)
Different prognostic factors	
Serum LDH U/L	612.45±215.27 (258.00-952.00)**
B2-microglobulin mg/dl	3.13±0.98 (1.80-4.80)
BM lymphocyte %	48.16±16.75 (35.00-75.00)
Duration of illness in years	5.28±2.16 (2.00-12.00)
Number of chemotherapy cycles	3.92±2.80 (0.00-9.00)
Treatment outcome	
Initial response to chemotherapy	
Complete remission (CR)	14 (47%)
Partial remission (PR)	10 (33%)
Progressive disease (PD)	6 (20%)
Total free survival (TFS) (months)	41.85±27.20 (6.00-120.00)
Overall survival (months)	45.73±29.38 (7.00-120.00)
Alive	26 (87%)
Death	4 (13%)

* No. (%).
** Mean ± SD (range).

Table (3): Different genomic aberrations detected in 30 B-CLL patients by FISH technique.

Item	B-CLL Patients (No. 30)
Cytogenetic abnormalities	28 (93%)*
Del 13q14.3	12 (40%)
Del 11q22.3	7 (23%)
Trisomy 12	19 (63%)
Del 17p13.1	12 (40%)
Del 13q34	13 (43%)

* No. (%).

Table (4): Correlation of different genomic aberrations with clinical, laboratory, staging systems and prognostic parameters in 30 B-CLL patients.

Item	Del 13q14.3	Del 11q22.3	Trisomy 12	Del 17q13.1	Del 13q34
Age	0.412 (0.04)*	0.036 (NS)	–0.469 (0.02)	–0.147 (NS)	0.395 (0.03)
Gender	0.216 (NS)	0.051 (NS)	0.187 (NS)	0.182 (NS)	0.171 (NS)
Clinical data					
Hepatomegaly	–0.182 (NS)	0.125 (NS)	0.503 (0.03)	–0.158 (NS)	–0.018 (NS)
Splenomegaly	–0.471 (0.01)	0.396 (0.03)	0.014 (NS)	0.091 (NS)	–0.192 (NS)
Lymphadenopathy	–0.506 (0.009)	0.597 (0.008)	0.562 (0.03)	0.124 (NS)	–0.494 (0.04)
Laboratory data					
Hb gm/dl	0.115 (NS)	–0.055 (NS)	–0.374 (0.01)	–0.016 (NS)	0.392 (0.02)
TLC x 10^9/L	–0.032 (NS)	0.181 (NS)	0.432 (0.04)	0.157 (NS)	–0.466 (0.009)
Platelets x 10^9/L	0.205 (NS)	–0.018 (NS)	–0.355 (0.03)	–0.369 (0.02)	0.137 (NS)
PB lymphocytes %	–0.417 (0.03)	0.182 (NS)	0.186 (NS)	0.040 (NS)	–0.042 (NS)
Absolute lymphocytic count	–0.482 (0.01)	0.229 (NS)	0.092 (NS)	0.129 (NS)	–0.002 (NS)
Immunophenotyping					
CD38	–0.650 (0.003)	0.031 (NS)	0.794 (0.001)	0.438 (0.02)	–0.634 (0.004)
ZAP-70	–0.789 (0.001)	0.135 (NS)	0.635 (0.004)	0.399 (0.01)	–0.739 (0.001)
Different staging systems					
Rai staging system	–0.482 (0.004)	0.339 (0.03)	0.625 (0.001)	0.475 (0.04)	–0.617 (0.001)
Binet staging system	–0.597 (0.001)	0.350 (0.01)	0.537 (0.002)	0.428 (0.02)	–0.540 (0.002)
Different prognostic factors					
Serum LDH U/L	–0.483 (0.002)	0.425 (0.001)	0.689 (0.002)	0.746 (0.003)	–0.726 (0.002)
B2-microglobulin mg/dl	–0.495 (0.007)	0.384 (0.002)	0.562 (0.003)	0.524 (0.005)	–0.503 (0.001)
BM lymphocyte %	–0.162 (NS)	0.124 (NS)	0.219 (NS)	0.203 (NS)	–0.619 (0.02)
Duration of illness in years	–0.369 (0.04)	0.281 (NS)	0.324 (0.03)	0.189 (NS)	–0.918 (0.008)
No. of chemotherapy cycles	–0.571 (0.005)	0.237 (NS)	0.867 (0.001)	0.817 (0.003)	–0.723 (0.004)
Total free survival	0.625 (0.002)	–0.576 (0.01)	–0.426 (0.02)	–0.682 (0.002)	0.553 (0.009)
Overall survival	0.446 (0.01)	–0.407 (0.02)	–0.365 (0.04)	–0.435 (0.03)	0.487 (0.02)

* *r*-value (*p*-value). NS: Non-significant.

Statistical comparison between CLL patients with positive and negative different genomic aberrations as regards age, gender and clinical data was studied. Comparison revealed no significant difference as regards age, gender and clinical data ($p>0.05$) except for age which showed significant difference in del 13q14.3, trisomy 12 and del 13q34 positive and negative patients ($p=0.04$, 0.03 and 0.04 respectively), while hepatomegaly showed significant difference in trisomy 12 only ($p=0.04$), and splenomegaly showed significant difference in del 13q14.3 and del 11q22.3 ($p=0.02$ and 0.03 respectively), and lastly, lymphadenopathy showed significant difference in del 13q14.3, del 11q-22.3, trisomy 12 and del 13q34 ($p=0.03$, 0.02, 0.04 and 0.04 respectively).

Regarding laboratory data, comparison between CLL patients with positive and negative different genomic aberrations revealed no significant difference between different groups ($p>0.05$) except for P.B. absolute lymphocytic count in del 13q14.3 which showed significant difference ($p=0.03$), haemoglobin level in trisomy 12 which showed highly significant difference ($p=0.008$), platelets count in del 17q13.1 which showed significant difference ($p=0.04$)

and lastly, haemoglobin level and total leucocyte count in del 13q34 which showed significant difference (p=0.03 and 0.02 respectively). While regarding immunophenotyping, comparison revealed a significant difference between CLL patients with positive and negative different genomic aberrations as regards CD38 and ZAP-70 (p<0.05) except for del 11q22.3, in which CD38 and ZAP-70 expression was almost absent in del 13q14.3 and del 13q34 positive cases but increased in trisomy 12 and del 17q13.1 positive cases. No significant difference was found as regards other immunophenotyping markers (p>0.05).

Table (5) displays statistical comparison between CLL patients with positive and negative different genomic aberrations as regards different staging systems. A significant difference was found as regards Rai and Binet staging system (p<0.05), in which del 13q14.3 and del 13q34 were common with early Rai and Binet stages while del 11q22.3, trisomy 12 and del 17q13.1 were common in late stages.

Table (5): Comparison between 30 B-CLL patients with positive and negative different genomic aberrations as regards different staging systems.

Item	Del 13q14.3		Del 11q22.3		Trisomy 12		Del 17q13.1		Del 13q34	
	+ve (No.12)	–ve (No.18)	+ve (No.7)	–ve (No.23)	+ve (No.19)	–ve (No.11)	+ve (No.12)	–ve (No.18)	+ve (No.13)	–ve (No.17)
Rai staging system:										
0	1 (100%)*	0 (0%)	0 (0%)	1 (100%)	0 (0%)	1 (100%)	0 (0%)	1 (100%)	1 (100%)	0 (0%)
I	7 (87.5%)	1 (12.5%)	0 (0%)	8 (100%)	1 (12.5%)	7 (87.5%)	1 (12.5%)	7 (87.5%)	8 (100%)	0 (0%)
II	1 (33%)	2 (67%)	1 (33%)	2 (67%)	3 (100%)	0 (0%)	1 (33%)	2 (67%)	2 (67%)	1 (33%)
III	2 (25%)	6 (75%)	3 (37.5%)	5 (62.5%)	5 (62.5%)	3 (37.5%)	4 (50%)	4 (50%)	1 (12.5%)	7 (87.5%)
IV	1 (10%)	9 (90%)	3 (30%)	7 (70%)	10 (100%)	0 (0%)	6 (60%)	4 (40%)	1 (10%)	9 (90%)
p-value	0.03		0.02		0.001		0.03		0.001	
Binet staging system:										
A	6 (86%)	1 (14%)	1 (14%)	6 (86%)	2 (28.5%)	5 (71.5%)	0 (0%)	7 (100%)	7 (100%)	0 (0%)
B	4 (40%)	6 (60%)	1 (10%)	9 (90%)	7 (70%)	3 (30%)	3 (30%)	7 (70%)	5 (50%)	5 (50%)
C	2 (15%)	11 (85%)	5 (38.5%)	8 (61.5%)	10 (77%)	3 (23%)	9 (69%)	4 (31%)	1 (7%)	12 (92%)
p-value	0.002		0.009		0.001		0.02		0.001	

* No. (%).

Statistical comparison between CLL patients with positive and negative different genomic aberrations as regards prognostic factors is shown in Table (6). Comparison revealed a highly significant difference as regards serum LDH, B2-microglobulin and number of chemotherapy cycles (p<0.01) except for number of chemotherapy cycles in del 11q22.3 which showed insignificant difference (p>0.05). Del 13q14.3 and del 13q34 positive cases showed lower serum LDH, B2-microglobulin levels and decreased number of chemotherapy cycles, while del 11q22.3, trisomy 12 and del 17q13.1 positive cases showed higher serum LDH, B2-microglobulin levels and increased number of chemotherapy cycles. While no significant difference was found between CLL patients with positive and negative different genomic aberrations as regards BM lymphocyte percent and duration of illness (p>0.05) except for del 13q34 which showed significant difference (p=0.04 and 0.02 respectively), as decreased BM lymphocyte percent and duration of illness was found in positive del 13q34 cases.

Statistical comparison between CLL patients with positive and negative different genomic aberrations as regards treatment outcome (initial response to chemotherapy), total free survival and overall survival was studied. There was a highly significant difference as regards treatment outcome and total free survival (p<0.01), as del 13q14.3 and del 13q34 positive cases had a higher incidence of favorable outcome (complete remission) and higher total free survival, while del 11q22.3, trisomy 12 and 17q13.1 positive cases had a higher incidence of unfavorable outcome (partial remission and progressive disease) and lower total free survival. Lastly, a significant difference was found as regards overall survival (p<0.05), in which del 13q14.3 and del 13q34 positive cases showed longer survival and lower death rate than del 11q22.3, trisomy 12 and del 17q13.1 positive cases.

Table (6): Comparison between 30 B-CLL patients with positive and negative different genomic aberrations as regards prognostic factors.

Item	Serum LDH U/L	B2-microglobulin mg/dl	BM lymphocyte %	Duration of illness in years	Number of chemotherapy cycles
Del 13q14.3					
+ve (No.12)	331.21±54.39*	2.02±0.89	39.42±3.84	5.21±3.11	3.21±2.77
–ve (No.18)	680.22±116.19	4.13±0.35	57.00±18.81	6.35±1.68	6.50±1.84
p-value	0.003	0.004	0.34	0.08	0.002
Del 11q22.3					
+ve (No.7)	744.78±174.02	3.84±0.46	53.00±19.13	6.73±1.67	5.10±2.60
–ve (No.23)	362.09±61.79	1.98±0.18	38.50±4.94	4.13±3.19	2.09±1.46
p-value	0.001	0.001	0.09	0.23	0.14
Trisomy 12					
+ve (No.19)	784.62±174.03	4.10±0.75	57.66±20.52	5.62±1.66	5.87±2.09
–ve (No.11)	506.50±164.87	2.18±0.95	38.66±3.05	4.69±2.35	2.73±1.52
p-value	0.001	0.001	0.25	0.06	0.001
Del 17q13.1					
+ve (No.12)	763.00±180.97	3.81±0.70	51.21±18.23	5.15±1.18	6.38±1.94
–ve (No.18)	557.07±208.70	1.84±0.95	35.98±3.01	4.34±2.50	3.42±2.24
p-value	0.007	0.003	0.38	0.35	0.002
Del 13q34					
+ve (No.13)	352.44±58.06	1.98±0.62	38.50±4.94	4.72±1.25	2.55±1.50
–ve (No.17)	600.00±172.02	3.53±0.35	58.01±19.97	6.10±3.57	6.50±1.84
p-value	0.001	0.001	0.04	0.02	0.005

* Mean ± SD.

DISCUSSION

B-cell chronic lymphocytic leukemia (B-CLL) is a B-cell neoplasm characterized by an indolent course with progressive splenic and lymph node enlargement associated with chronic lymphocytosis **[17]**. Patients with B-CLL follow heterogeneous clinical courses. Some survive for a long time without therapy, while others die rapidly despite aggressive treatment **[18]**.

Chromosomal abnormalities play a major role in the pathogenesis of CLL, the study of leukemia specific cytogenetic abnormalities has contributed greatly to the clinical diagnosis and identification of specific chromosomal abnormalities are important for predication of the disease progression and survival, as well as response to chemo and monoclonal antibody therapy and for stratification of patients into the appropriate treatment protocols **[17]**.

The most important numerical and structural abnormalities found in CLL include trisomy 12 and deletions in several chromosome regions, such as 13q14.3, 13q34, 11q22.3 and 17p13.1 as well as other less frequently occurring aberrations. They have been reported to be of significant prognostic value in B-CLL **[19]**. A correlation between high-risk cytogenetic aberrations and unmutated IgVH genes has been found **[10]**.

This study included 30 B-CLL patients, their ages ranged between 42 and 80 years. They were 23 male (77%) and 7 female (23%). Fifteen age and sex matched normal healthy subjects were included as a control group. They were all subjected to FISH technique for detection of different genomic aberrations including del 13q14.3, del 11q22.3, trisomy 12, del 17p13.1 and del 13q34.

Our study detected genomic abnormalities in 28/30 patients (93%). This result was nearly similar to the results of other reasearchers who have reported the frequency of abnormalities as 81%, 82% and 80% respectively **[7,20,21]**. While other investigators detected the frequency as 52%, 68% and 75% respectively **[15,22,23]**. Their results were varied and lower than our results, this may be due to the difference of their cut off values or the difference of the number of patients they have studied.

In the present study, the most frequent abnormality was trisomy 12 (63%) in 19/30 patients which is higher than what was reported

by other investigators who detected chromosome 12 abnormalities in 19%, 23%, 23.3% and 25.5 % of CLL patients respectively **[21,23-25]**. The difference may be due to the higher number of their patients.

The second most frequent abnormality was involving chromosome 13 either of the loci 13q14.3 or 13q34. It was detected in 12/30 patients (40%) having del 13q14.3 and 13/30 patients (43%) having del 13q34. In concordance with our study, other researchers have detected this abnormality in 41%, 42% 43% and 45% of patients respectively **[6,21,24,25]**. Also, abnormalities of chromosome 17p13.1 (p53) was detected in almost equal percent to del 13q14.3 and del 13q34, it was detected in 12/30 patients (40%). Lower percentage of this abnormality 12%, 20%, 10% and 16% respectively were previously detected by other investigators **[6,15, 24,25]**. While others almost did not find p53 abnormality except in 3% and 2% of patients respectively **[19,23]**.

Finally, the least frequent abnormality was involving chromosome 11q22.3 (ATM locus). We found deletion in only 7/30 CLL patients (23%). This result is in accordance with previous studies which recorded this abnormality in 25%, 18%, 13% and 10.5% of their cases respectively **[19,21,24,25]**. Also, recent studies found ATM deletion but at a lower percent of 5% and 7% respectively **[9,15]**.

In the present study, our results showed a significantly higher age with del 13q14.3 and del 13q34 and lower age with trisomy 12 and insignificant correlation regarding age with other cytogenetic aberrations. This comes in agreement with recent studies which could not elicit a significant correlation regarding age **[15,24]**, while other researchers reported that patients with positive genomic aberrations showed significant difference when compared to negative patients regarding their age and found that favorable alterations (del 13q14.3, del 13q34) were seen more frequently among older patients, while unfavorable alterations (del 11q22.3, trisomy 12, del 17p13.1) were more frequently observed among younger patients and this matched our results in del 13q14.3, trisomy 12 and del 13q34 **[20,22]**.

Earlier studies found a significant correlation with gender and stated that there was a prevalence of female in the worse prognostic group (del 11q22.3, trisomy 12, del 17p13.1) and prevalence of male in the group with a better prognosis (del 13q14.3, del 13q34) **[15]**, but this is the contrary of our results as we could not find a significant correlation with gender, while our results are in agreement with what was previously reported in other studies **[24,25]**.

As regards clinical data, Patients with trisomy 12 were significantly presented almost all with hepatomegaly, and patients with del 13q14.3 were significantly less prone to present with splenomegaly while patients with del 11q22.3 were more prone to present with huge splenomegaly. and lastly, patients with del 13q14.3 and del 13q34 were less likely to have multiple lymphadenopathy while patients with del 11q22.3 and trisomy 12 were more likely to have multiple generalized lymphadenopathy. Other studies found a significant difference between the CLL patients with positive and negative different genomic aberrations regarding splenomegaly and lymphadenopathy as we detected and showed that del 11q22.3 and trisomy 12 were more likely to have splenomegaly and multiple bulky lymphadenopathy **[20,26]**.

Our findings demonstrated no significant difference between CLL patients with positive and negative different genomic aberrations regarding laboratory data except for a significant lower P.B. absolute lymphocytic count in patients with del 13q14.3, lower haemoglobin level in patients with trisomy 12, lower platelets count in patients with del 17q13.1 and lastly, higher haemoglobin level and lower total leucocyte count in patients with del 13q34. These results are in concordance with that recorded by previous studies which reported lower haemoglobin level, platelets count and higher total leucocyte count in del 11q22.3, trisomy 12, del 17p13.1 and higher haemoglobin level, platelets count and lower total leucocyte count in del 13q14.3, del 13q34 **[20]**. Also, recent studies reported no significant correlation between cytogenetic aberrations and P.B. absolute lymphocyte count and this comes in agreement with our results except for del 13q14.3 **[24]**.

In the current study, a significant difference was found between CLL patients with positive and negative different genomic aberrations regarding CD38 and ZAP-70 except for del 11q22.3. CD38 and ZAP-70 expression is almost

absent in cases with del 13q14.3 and del 13q34 but increased in cases with trisomy 12 and del 17q13.1. In aggreement with these results, recently reported researches could elicit as well a significant correlation regarding CD38 and reported that trisomy 12, del 17p13.1 and del 11q22.3 tended to present with high CD38 expression in comparison to the other aberrations. But, on the contrary, they could not elicit a significant correlation regarding ZAP-70 as we detected **[15,24,27]**, while earlier studies reported a significant correlation **[27]**, this may be attributed due to technical factors as different monoclonal antibodies and different gating strategies used in flowcytometry detection of ZAP-70.

Our findings showed a significant difference between CLL patients with positive and negative different genomic aberrations regarding Rai and Binet staging system, as patients with del 13q14.3 and del 13q34 tended to be A Binet stage and 0, I Rai stages, while in patients with del 11q22.3, trisomy 12, del 17p13.1 B, C Binet and II, III, IV Rai stages prevailed. This is in agreement with a number of previous studies **[13,15,19,20]**. In contrast to our results, other investigators showed insignificant difference **[6,24,25]**. This may be due to their large sample size compared with smaller sample size in our study.

As regards prognostic factors, our results demonstrated that cases with del 13q14.3 and del 13q34 showed a highly significant lower serum LDH, B2-microglobulin levels and decreased number of chemotherapy cycles, while cases with del 11q22.3, trisomy 12 and del 17q13.1 showed a highly significant higher serum LDH, B2-microglobulin levels and increased number of chemotherapy cycles. These results are in accordance with that of other researchers **[13,20]**. On the other hand, recently reported researches found insignificant difference **[24]**. This may be due to the difference of their normal values or the number of patients they have studied. Also, recent studies stated no significant differences between patients with positive and negative different cytogenetic aberrations regarding B.M. lymphocyte percent and duration of illness. This comes in consistency with our results, except for del 13q34 which showed a significant lower BM lymphocyte percent and duration of illness **[24]**.

Earlier studies reported that the percentage of cells with del 11q22.3, trisomy 12 and del 17q13.1 increased during follow-up period in CLL patients with signs of progressive disease, drug resistance and require more chemotherapy cycles and had shorter treatment-free intervals, while del 13q14.3 and del 13q34 do not carry an adverse prognostic significance or disease progression and require less therapy and had longer treatment-free intervals **[19,27-29]**. This comes in agreement with our results, as we found that cases with del 13q14.3 and del 13q34 showed a significant decreased number of chemotherapy cycles, good prognosis, favorable treatment outcome and better response to chemotherapy as well as longer total free survival, while cases with del 11q22.3, trisomy 12 and del 17q13.1 showed increased number of chemotherapy cycles, poor prognosis, unfavorable outcome and poor response to chemotherapy as well as shorter total free survival.

Lastly, a significant difference was found between CLL patients with positive and negative different genomic aberrations regarding overall survival ($p<0.05$), as cases with del 13q14.3 and del 13q34 showed longer survival and lower death rate than cases with del 11q22.3, trisomy 12q11.1 and del 17q13.1. In concordance with these results, previous studies have reported that the survival time was significantly shorter in patients with trisomy 12, p53 or ATM gene deletion, while patients with deletion at chromosome 13 had longer survival time **[19,20,25,29]**.

In conclusion, our study categorized chromosomal aberrations into two groups; first with favorable prognosis alterations with no adverse disease progression, better therapy response and longer survival which are 13q deletion whether 13q14.3 or 13q34, and second the unfavorable alterations with signs of disease progression, drug resistance and shorter survival were more frequently with del 11q22.3 (ATM), trisomy 12 and del 17q13.1 (p53). Additionally, chromosomal aberrations with poor prognosis tended to express CD38 and ZAP-70 proteins. Finally, multiple genomic aberrations can play an important role in the clinical presentation, prognosis, disease progression and response to chemotherapy as well as overall survival in an Egyptian cohort of CLL patients as compared to others.

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Polymorphism of NAD (P) H: Quinone Oxidoreductase 1 and Susceptibility to Acute Myeloid Leukemia in Egyptian Patients

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ABSTRACT

Background: Polymorphic variations of several genes associated with dietary effects and exposure to environmental carcinogens may influence susceptibility to leukemia development.

Quinone-Oxidoreductas, NQO1, is one of these genes, it is a two-electron reducing enzyme that is important for the detoxification of quinones compounds, some chemotherapy metabolites and is an activator of bioreductive antitumor agents, such as mitomycin C. It is indicated that NQO1 (C609T) polymorphism has been associated with susceptibility to several malignancies.

Aim of Work: To study the main genetic polymorphism of NQO1 (C609T) and its influence on the risk of acute myeloid leukemia in Egyptian individuals.

Methods: NQO1 (C609T) polymorphism was genotyped in 75 de novo AML patients together with 107 normal age and sex matched healthy controls using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism.

Results: A significant high prevalence of polymorphic variant NQO1 (C609T) was found in AML cases compared to controls (*p* value-0.002). The presence NQO1 polymorphism increases the risk of AML. The odds ratio for heterozygous CT (Pro/Ser) was 2.4, 95% CI 1.0-5.4, for homozygous TT (Ser/Ser) was 5.4, 95% CI 2.1-13.5.

Conclusion: Our results suggest that NQO1 C609T (Pro187Ser) polymorphism seems to be associated with a higher risk of acute myeloid leukemia development in Egyptian people. Further studies are recommended to correlate with different cytogenetic abnormalities.

Key Words: *Quinone oxidoreductase – Acute myeloid Leukemia – PCR-RFLP.*

INTRODUCTION

Clues to the etiology of leukemia may be gained through the study of genetic susceptibility in candidate genes. The carcinogenic effect of xenobiotics is influenced by a series of genes codifying enzymes involved in oxidation/activation [phase l] and conjugation/detoxification [phase ll] of these compounds. Polymorphisms of these genes resulting in functional allelic variants of the corresponding enzymes, have been shown to influence the risk of developing solid tumors, hematologic malignancies and to modify response to cytotoxic treatment **[1]**.

Quinone Oxidoreductase is generally considered as a detoxification enzyme. In 1955, nicotinamide nucleotide-dependent oxidoreductase had been identified in rat liver and named as diphtheria toxin [DT] now known as NAD[P] H; quinone oxidoreductase 1 **[2]**. NQO1 is located on chromosome 16q22, is 20kb in length and has 6 exons and 5 introns. NQO1 is a flavoprotein which functions as a homodimer. The physiological dimer has one catalytic site per monomer. Each monomer consists of 237 amino acids. NQO1 is mainly a cytosolic enzyme although it has also been localized in smaller amounts to mitochondria, endoplasmic reticulum and nucleus **[3]**.

As the name of the enzyme suggests, a common group of substrates are quinones (a large class of aromatic compounds found commonly in plants, benzene metabolites and chemotherapies) which are reduced via a hydride transfer mechanism to generate the corresponding hydroquinone derivative by its unique ability to use either NADH or NADPH as reducing cofactors **[4,5]**. Reduction of these quinone compounds, helps in prevention of the generation of semi-quinone free radicals and reactive oxygen species, thus protecting the cells from oxidative damage. Paradoxically, NQO1 catalyzes the bioactivation of antitumor quinones which

exert their toxicity through direct DNA damage, thereby increasing their antitumor efficacy. Exploitation of this activity is under consideration as therapeutic strategy in treatment cancers. In contrast, the reductive activation of environmental carcinogens such as nitrosamines, heterocyclic amines and dinitropyrines by NQO1 may contribute to carcinogenesis **[6]**.

NQO1 is constitutively expressed in most tissues including human epithelial and endothelial tissues as well as in the bone marrow, where expression is thought to be highly inducible by xenobiotics with quinone moieties and up-regulation occurs during times of oxidative or electrophilic stress **[7]**. NQO1 expression is present at high levels in many human solid tumors as a result of hypoxia compared with normal tissues of the same origin **[3,8]**. Increased expression of NQO1 in tumors suggests that NQO1 may be a marker of neoplasia **[5]**.

There have been more than 93 single nucleotide polymorphisms [SNPs] identified in the NQO1 gene. The most widely studied SNP is a C to T change at nucleotide position 609, also known as NQO1*2. This results in a proline to serine amino acid change at codon 187 that is associated with a loss of enzyme activity due instability of protein product **[9]**. Thus, the enzyme activity of the homozygous variant genotype [NQO1*2/*2] is almost undetectable and the enzyme activity of the heterozygous genotype [NQO1*1/*2] is intermediate between the homozygous variant genotype and wild type [NQO1*1/*1] **[7]**.

Different studies reported that NQO1 C609T genotype is associated with increased risk of therapy related leukemia **[10,11]**, MDS syndrome **[10]**, myeloid leukemia with abnormalities of chromosome 5 & 7 **[10]** and pediatric leukemia with MLL fusion gene **[12]**.

As ethnic variation in risk susceptibility is well documented **[13]**, it is essential to carry such studies in each population. Results from one ethnic group cannot be extrapolated to other.

Therefore, this cases-controls study was carried out to determine if this NQO1 polymorphism is associated with an altered risk of developing acute myeloid leukemia in our Egyptian population or not.

PATIENTS AND METHODS

The study included 75 newly diagnosed Pediatric AML patients who presented to the Pediatric and Adult Oncology Department, NCI, Cairo University, in the period between January 2008 and January 2009. They included 36 males and 39 females with an age range of 1.2 to 74 years, mean of age was 39.1±18.5 and median of age was 41. Diagnosis was performed according to clinical, morphological, cytochemical and immunophenotyping examination. The criteria for inclusion in this group were:

1- Egyptian origin as judged by their names, language and place of birth;

2- Availability of biological material.

A general population control group composed of 107 individual comprising 89 males and 57 females with an age range of 18 to 53 years, mean of age was 30.8±8.9 and median of age was 29. All of them were randomly selected from blood donors. The criteria for inclusion in the control group were:

1- Anonymous, healthy, and unrelated individuals.

2- Egyptian origin as judged by their names, language and place of birth. The study was approved by the IRB of National Cancer Institute. Informed consent was obtained from all participates involved in the study and/or their parents.

Genotyping:

DNA isolation: DNA was isolated from peripheral blood at diagnosis, as described by Bye et al., 1992 **[14]**.

DNA concentration and quality was determined by measuring absorption at 260 and 280 nm; a ratio of 1.6-1.8 was accepted.

NQO1 C609T Polymorphism:

PCR was performed in 20µL reaction mix containing 20ng of genomic DNA, 0.5µmol/L of each primer (NQO 1 Forward AGT GGC ATT CTG CAT TTC TGT G, and NQO 1 Reverse GAT GGA CTT GCC CAA GTG ATG), 200µmol/L of each dNTPs, 10mmol/L Tris-HCl (pH 8.3), 50mmol/L KCl, 1.5mmol/L MgCl2, and 0.5U of ampliTaq DNA polymerase (Hoff-

man-LaRoche, Branchburg, NJ). After initial denaturation for 10 minutes at 95°C, amplification was performed for 35 cycles of 1 minute at 95°C, 1 minute at 59°C, and 2 minutes at 72°C. The last elongation step was extended to 7 minutes. Overnight incubation of 6U HinF1at 37° with 10ul of PCR product was performed. The resulting restricted fragments were evaluated on a 4% agarose gel at 100 volt for 30min. Larson et al., 1999 **[15]**. The C609T substitution creates another restriction site Hinf1 in 188bp fragment. The homozygous polymorphism gives 151 bp, 85 bp and 37 bp; the wild type gives 188 bp, 85 bp (Fig. 1).

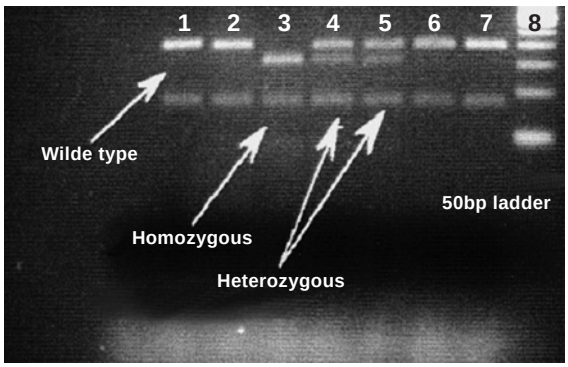

Fig. (1): Pattern of NQO1 C609T Polymorphisms by Hinf1 digest.

Lane 1, 2, 6, 7: Wild (188, 85bp),
Lane 3 : Homo (151, 85, 37bp),
Lane 4 & 5 : Hetero (188, 151, 85, 37 bp),
Lane 8 : 50bp marker.

Statistcal analysis:

Data was coded and entered using statistical package SPSS version 15. Data was summarized using mean ± SD, and median for quantitative data and number and % for qualitative data.

Comparisons between groups were done using Chi-Square test or Fisher's exact test for qualitative data. While Non-parametrically Mann-Whitney test were used for quantitative variable which are not normally distributed. Risk estimates were done using Odds ratio and 95% CI. Logistic regression analysis to test for significant predictor of leukemia, *p*-value of ≤0.05 was considered statistically significant.

RESULTS

The patients' and control group characteristics are shown in (Table 1).

Statistical comparison between AML patients and control groups subjects as regards NQO1 genotype expression and association with AML risk (Table 2).

Table (1): Characteristics of 75 de novo AML Patients.

Variables	AML group (No; 75)
Age	*39.1±18.5
Gender no. (%)	
Male	36 (48%)
Female	39 (52%)
Laboratory data	
Hg. gm/dl	6.6±2.4 (range 1.7-13.9)
TLC x 10^9/L	83.2±94.3 (range 1.3-740)
Platelets x10^9/L	56±44 (range 3.4-300)
BM Blasts %	52.7±28.4 (1-99)
FAB Subtypes no. (%)	
M0	1 (1.3%)
M1	15 (20%)
M2	32 (42.7%)
M3	9 (12%)
M4	14 (18.7%)
M5a	1 (1.3%)
M5b	2 (2.7%)
M7	1 (1.3%)

*Mean ± SD.

Table (2): Statistical comparison between AML patients and Control subjects as regards NQO1 genotype expression and association with AML risk.

NQO1 (C609T) polymorphism	Wild type (CC) (Reference group)	Heterozygous (CT)	Homozygous (TT)	Hetero+Homo (CT+TT)
AML group (n=75)	54 (72%)	16 (21.3%)	5 (6.7%)	21 (28%)
Control group (n=107)	96 (89.7%)	11 (10.3%)	0	11 (10.3%)
p. value		0.04	0.007	0.002
O.R		2.4	5.4	3.4
95% C.I		1.05-5.4	2.1-13.6	1.5-7.6

NQO1 comparisons revealed highly statistically significant difference between the two groups *p*-value 0.002.

NQO1 mutant types (CT, TT and carrier of the variant allele T of the NQO1 gene) were associated with increased risk (odds ratio 2.4 and 5.4 & 3.4 & 95% CI 1.0-5.4 & 2.1-13.6 & 1.5-7.6), respectively.

Genotype frequencies of NQO1 in AML patients and controls:

The wild type was more frequently encountered in the control group. The heterozygous was more frequently represented in AML group. The homozygous was encountered only in the AML; none in the control is homozygous. Carriers of the heterozygous genotype are at 2.4 folds risk of developing AML while those with the homozygous genotype have a risk of 5.4 folds.

The presence of NQO1 (C609T) polymorphism was analyzed in relation to age, gender, and laboratory data in AML patients:

There was no statistical significant correlation between NQO1 genotypes and age, gender, hemoglobin level, total leucocytic count, platelet count, bone marrow blasts or FAB subtypes *p*-value >0.05.

DISCUSSION

Drug or xenobiotic metabolizing enzymes (DME) include several enzyme families involved in the metabolism, bio-transformation and detoxification of xenobiotics such as chemotherapeutic drug as alkylating agents, intercalating agents and anthracyclins **[16]**.

Detoxification enzymes protect DNA from damage due to both endogenous and exogenous sources. When detoxification is ineffective, DNA damage can cause chromosomal instability leading to severe failure of cell function and either apoptosis or oncogenesis. Genetic differences defined by polymorphisms altering the enzymatic activities in detoxification pathways are prime candidates for studies to explain variation in susceptibility to develop acute myeloid leukemia **[17]**.

Quinone oxidoreductase (NQO1) is considered to be one of the phase II DME which are involved in the detoxification of numerous endogenous, foreign compounds and drugs that contain hydroxyl (OH) functional groups, highly reactive, either present on the parent molecules and/or after biotransformation by the Phase I DME which consists of the cytochrome P450 (CYP) superfamily **[16,18]**.

Lack of NQO1 activity might increase the risk of certain types of toxicity and cancer **[19]**. A number of different clinical studies had been carried on NQO1 genotype, most of which have shown an increased frequency of the NQO1 TT allele in patients with esophageal, gastric, breast cancer **[20,21]**, and both pediatric and adult leukemia **[22,23]**. Frequency of homozygous individuals having T-allele was reported to range from 1.5 to 20.3% among different ethnic groups **[24]**. Several reports suggested that NQO1 Pro 187 Ser polymorphism is associated with cancer risk **[25,26]**.

It was reported that acute and chronic side effects of cancer treatment might be involved in causing genetic variations of NQO1. There are many documented cases of cancer patients receiving chemotherapy with alkylating agents who develop secondary myeloid leukemia **[10, 16,27]**.

In this molecular epidemiological study, NQO1 C609T polymorphism was investigated in 75 de novo patients with AML and 107 age and sex matched healthy controls using PCR-RFLP. The present work aimed to clarify whether there is an association between mutant NQO1 polymorphism and increased risk of AML development. The results showed that the heterozygous variant CT (Pro/Ser) increased the risk of AML by 2.4-fold, homozygous variant TT alone increase the risk by 5.4-fold while the presence of either TT or CT increased the risk by 3.4.

NQO1 polymorphism had been investigated in several studies, but the results are not consistent. Guha et al. **[13]** explained that heterogeneity between studies may be due to differences in population exposures to NQO1 substrates and small sample sizes, as well as potential population stratification in non-family-based studies.

In a recent study, using RFLP-PCR, Yamaguti et al. **[28]** showed that NQO1 609 CT+TT genotypes were higher in patients than in controls, with carriers of the variant allele T was 1.92-fold increased risk of AML.

Smith et al. **[23]**, reported that AML case subjects exhibited a higher frequency of low or null NQO1 genotypes than controls with relatively increased risk 1.47 folds and this builds upon earlier findings that NQO1 polymorphism is associated with an enhanced risk of myeloid leukemias including de novo AML, myelodysplastic syndromes [MDS] and therapy related AML **[15]**. They stated that NQO1 protein expression in peripheral blood and bone marrow progenitors is normally very low, but is highly inducible **[29]**.

The increased risk of leukemia associated with a deficit in NQO1 levels due to the NQO1 polymorphism may reflect impaired quinone detoxification and an increased susceptibility of endothelial cells in the bone marrow to environmental insults **[30]**.

In agreement with our results, Yang et al. **[31]** reported similar high frequencies of NQO1 (C609T) C/T and T/T genotypes among AML patients significantly higher than that in the normal controls (53.1% and 25% respectively) and the relative risk of t(8;21) was 4.487 for the subjects with NQO1 (C609T) C/T genotype and was 6.293 for the subjects with NQO1 (C609T) T/T genotype while the relative risk of t(15;17) was 2.53 for the subjects with NQO1 (C609T) C/T genotype and was 4.149 for the subjects with NQO1(C609T) T/T genotype. They stated that determination of the NQO1 C609T genotyping may be used as a stratification marker to predict high risk individuals for AML.

It has been also indicated that NQO1*2 polymorphism seems to increase the risk of AML and reduces survival probabilities in children with AML on the basis of drug-associated toxicity **[22,32]**.

In the current study, no association was found in the distribution of NQO1 polymorphism with respect to the clinical characteristics at diagnosis and this is in agreement with other two studies **[23,33]**.

In contrast to our findings, Voso et al. **[17]** reported no difference in the frequency of NQO1, Pro 187 Ser polymorphism between AML and controls although there was a trend for NQO1 Ser/Ser variants to be overexpressed in therapy related AML, when compared with de novo AML in line with other studies **[10]**.

Eyada et al. **[34]** explored the relation of NQO1 polymorphism and acute leukemia, but their studied group (acute leukemia and controls) expressed only Pro/Pro genotype and therefore no linkage to leukemia susceptibility or prognostic output could be made, however, their studied group is very small in number with only 19 cases of AML.

Also, in disagreement with our results, Malik et al. **[35]** demonstrated that there are significant differences in NQO1 genotypes between Arabs and Jewish individuals and this polymorphism did not predispose to AML in either of these ethnic groups. They related this lack of association to a number of factors, including possible lack of NQO1 substrates in their environment and that other genetic factors are more important. The study carried out by Bolufer et al. **[36]**, did not find any statistical difference of NQO1*2 between AML patients and the control group, as well.

The divergent results in the different studies may be attributed to the racial heterogeneity of the populations and to the variation in AML pathogenesis in different countries **[28]**.

We compared the frequencies of NQO1 polymorphism (mutant homozygous T/T) in healthy Egyptian people examined in this study to the frequencies among different healthy ethnic groups from other studies:

Table (3): Frequency of NQO1 TT in different ethnic groups.

Ethnic groups	Egyptians	Arabs	Chinese	Koreans	Japanese	African American	Native American	Mexican Hispanics	Jewish Caucasian & Ethiopian
NQO1 Varient frequencies	None	7.4%	22.4%	18.8%	12.2%	5.2%	17.9%	15.5%	3.2%
References	Current study	35	37	38	13	38	39,13	38	35

Therefore, it was important to study NQO1 Polymorphism as a risk factor in Egyptian AML patients. The present study shows that AML patients expressing NQO1 Pro 187 Ser polymorphism are at high risk of developing AML. Future studies should be conducted in large number of patients. In addition, other SNPs in NQO1-such as the less studied C465T variant (NQO1*3), should be evaluated to comprehensively assess the importance of NQO1 in the development of acute myeloid leukemia in the Egyptian population.

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Does Isoform IL-10δ3 Expression Have a Protective Role in Pediatric Precursor B-ALL?

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ABSTRACT

Background: Precursor B-acute lymphoblastic leukemia (precursor B-ALL) is the most common form of cancer in children.

The prognostic factors in pediatric precursor B-ALL mostly include clinical and biological characteristics that are assessable at diagnosis as well as early treatment response and outcome. Full-length IL-10 expression and the splicing-derived IL-10 variant (termed IL-10δ3) were detected in relapsed ALL samples and had an impact on disease outcome.

Aim: The purpose of our study was to evaluate whether the expression of IL-10 and IL-10 isoform, IL-10δ3, could be of prognostic relevance in childhood precursor B-ALL.

Patients and Methods: Fifty eight children (age 0.9 to 21 years) with precursor B-ALL were included in the study. They presented to the pediatric oncology department at NCI, Cairo University, during the time period from the January 2003 to December 2005. Reverse-transcription polymerase chain reaction was done on bone marrow and/or peripheral blood samples to detect IL-10 expression and/or its isoform IL-10δ3.

Results: Within the total number of 58 patients, full-length IL-10 and/or IL-10δ3 isoform transcripts were expressed in 34/58 patients (58.6%), 23/58 cases (39.6%) expressed isoform IL-10δ3, 19 cases had full length IL-10 and IL-10δ3 isoform simultaneously while 4 patients had Isoform IL-10δ3 as the sole variant. Patients expressing IL-10δ3 had a significantly longer EFS (p=0.005).

Conclusion: IL-10 isoform IL-10δ3, expression was associated with a favorable prognosis, decreased incidence of relapse in first complete remission and significantly better event-free survival in pediatric precursor B-ALL.

Key Words: *IL-10 – IL-10δ3 – Precursor B-ALL.*

INTRODUCTION

Interleukin-10 (IL-10) is a pleiotropic; homodimeric cytokine produced by a large variety of cells, including monocytes/macrophages, B and T lymphocytes, and resident brain cell populations such as microglia and neurons **[1]** and may act as a cancer-promoting agent **[2]**. Classically, IL-10 is considered a cytokine with a wide range of immunosuppressive and anti-inflammatory activities via the inhibition of lymphocyte and monocyte function and the secretion of inflammatory cytokines **[3]**.

The IL-10 gene comprises 5 exons, spans ~5.2KB, and is located on chromosome 1 at 1q31-1q32 **[4]**. In addition to the full-length IL-10, there is a splicing-derived IL-10 variant that lacks the entire exon 3, termed IL-10δ3. The in-frame splice variant resulting from skipping of exon 3 does not cause alteration of the translational reading frame **[5]**. The exact function of IL-10 isoform, IL-10δ3, is largely unknown. Although most splicing derived isoforms have not been functionally defined, some have been shown to possess antagonistic activities and to act competitively with the native cytokines and receptors **[6-8]**.

Several studies have demonstrated that IL-10 was associated with therapy outcome in haematological and non hematological malignancies **[9-16]**.

IL-10 has been detected in the leukemic cells of most ALL and AML cases and was reported to suppress the immune reactions, suggesting that IL-10 could be associated with escape of leukemia cells from immune surveillance **[5,17-19]**.

Precursor B-acute lymphoblastic leukemia (precursor B-ALL) is the most common form of cancer in children **[20]**.

The prognostic factors in pediatric precursor B-ALL mostly include clinical and biological characteristics that are assessable at diagnosis

as well as early treatment response and outcome. IL-10 might correlate with clinical outcome in childhood acute lymphoblastic leukaemia (ALL). Full-length IL-10 expression and the splicing-derived IL-10 variant (termed IL-10δ3) were detected in relapsed ALL samples and had an impact on disease outcome **[5]**.

In this study we evaluated the expression of IL-10 and its IL-10 isoform IL-10δ3 in 60 children with precursor B ALL to verify its potential prognostic relevance.

PATIENTS AND METHODS

This was a retrospective study done on samples available for pediatric patients. Patients were children with precursor B-ALL presenting to the Pediatric Oncology Department at NCI, Cairo University, during the time period from the January 2003 to December 2005. The study included 58 newly diagnosed patients. They were 36 male and 22 female with age ranging from 11 months to 21 years with a median of 6 years including 2 infants 11 months each. Written informed consent was obtained from the patients' parents and the protocol was approved by the Institution Research Board of the NCI, Cairo University.

All patients were subjected to full clinical, radiological and laboratory investigations including chest X-Ray, abdominal Ultrasound, CSF examination and a full chemistry profile including liver and kidney function tests, complete blood count (CBC) and bone marrow aspiration.

Patients were diagnosed according to standard methods including blood picture, bone marrow, cytochemistry and immunophenotyping.

Immunophenotyping was done using monoclonal antibodies; stained cells were analyzed on Coulter XL. The Panel included CD1, CD2, CD3, CD4,CD5, CD7, CD8, CD10, CD19, CD22, Cytoplasmic μ, anti κ, anti λ, CD13, CD33, anti class II MHC and TdT **[21]**.

RNA extraction, reverse transcriptase polymerase chain reaction (RT-PCR):

Peripheral blood and/or Bone marrow samples collected on EDTA for RNA preparation were obtained at the time of diagnosis for the 58 newly diagnosed patients.

Total RNA was extracted from patients' samples using total RNA isolation kit, (Purescript, Gentra Minneapolis USA) according to the manufacturer's instructions. RNA was stored at –80°C until tested.

Quantification and purity of RNA were determined by measuring the absorbance at 260nm (A260) and (A280) using a spectrophotometer (nanodrop). Pure RNA of an A260/A280 ratio of 1.9-2.1 was used. The integrity and size distribution of total RNA purified (18S and 28S) was checked by running on 1% agarose gel electrophoresis for 20 minutes at 80 volts and ethidium bromide staining. The GeneAmpGold RNA PCR Reagent Kit (P/N 4308206 Applied Biosystems) was used for the reverse transcription (RT) and polymerase chain reaction (PCR) amplification from total RNA was performed according to the manufacturer's instructions.

First-strand cDNA was synthesized from 2μg of RNA using RT reaction. The integrity of cDNA for all cases was tested by amplifying the house keeping gene, β actin.

For the detection of IL-10 and its splice variant, the following primer pair was used **[5]**, IL-10 sense primer: 5'ATGCACAGCTCAGCACTGCT 3'; IL-10 antisense primer: 5'TCAGTTTCGTATCTTCATTGTCAT3'. Amplification was done in the Thermal Cycler, cycle conditions were as follows: Initial denaturation at 94°C for 10 minutes, followed by 35 cycles at 94°C for 45 seconds, 62°C for 30 seconds, 72°C for 1.25 minutes, and a final elongation at 72°C for 10 minutes. The amplification products were separated on 2% agarose gel electrophoresis at 100 volt for 20 minutes and visualized by ethidium bromide staining (Fig. 1). All assays were done in duplicate. BM samples negative for IL-10δ3 expression were confirmed by nested PCR.

Treatment:

All enrolled patients were treated according to NCI protocols modified from the St. Jude Total XIII protocols. Infants (≤12 months) were treated according to infantile protocol (interfant 99).

Follow-up time:

Patients were followed-up for a median follow-up time of 35 months (range 6 month to 72 months).

Statistical analysis:

Frequencies were calculated for descriptive purposes. Differences in the distribution of categorical variables were analyzed by χ^2 or Fisher's exact test if the frequencies were small (<5) and by Mann-Whitney U for non-paired data test. To verify the hypothesis of differences in mean values among the independent groups the ANOVA analysis of variance was used. Analysis of the probabilities of event-free survival (pEFS) was performed using the Kaplan-Meier method and the groups were compared with the log-rank test. Statistical analysis was done by the SPSS Software for Windows (version 18; portable SPSS). Significance was set to $p<0.05$ and highly significant to $p<0.01$.

RESULTS

IL-10 was detected at approximately 540bp, while isoform IL-10δ3 at 385bp.

The phenotype of the 58 cases was 4 pro B, 28 CALL and 26 pre B.

Full-length IL-10 and/or IL-10δ3 isoform transcripts were expressed in 34/58 patients (58.6%), 23/58 cases (39.6%) expressed isoform IL-10δ3 (Table 1); 19 cases had full length IL-10 and IL-10δ3 isoform simultaneously while 4 patients had Isoform IL-10δ3 as the sole variant (Table 2).

There was no statistically significant correlation between IL-10 expression and any of the tested parameters, age, sex, TLC, Immunophenotype, liver and/or spleen size or response to therapy.

Although not statistically significant, cases expressing IL-10δ3 showed less incidence of relapse compared to patients expressing only the full length IL-10 transcript and/or patients not expressing IL-10 at detection limit (p= 0.267). A total of 11/58 patients relapsed after achieving complete remission. They were 6/24 (25%) IL-10 negative, 3/11 (27.3%) expressing the full length IL-10 and 2/23 (8.7%) patients expressing the IL-10 and the IL-10δ3 variant. None of the 4 patients expressing IL-10δ3 as the sole variant relapsed.

Event free survival (EFS):

Patients expressing IL-10δ3 had a significantly longer EFS (p=0.005) (Table 1). The 4 cases expressing IL-10δ3 as a sole variant, all had an event free course (range 27-70 months) (Table 2).

In survival curve analysis of predicted event free survival (pEFS) (Fig. 2), patients with IL-10δ3 expression had a significantly better pEFS at five years (p=0.047) compared to patients expressing only the full length IL-10 transcript or patients expressing IL-10 below detection limit (IL-10 negative) (p=0.034 and 0.026, respectively). Also, when comparing non expressing IL-10δ3 to IL-10δ3 expressing cases, pEFS significance became more marked (p= 0.014) at five years (Fig. 3).

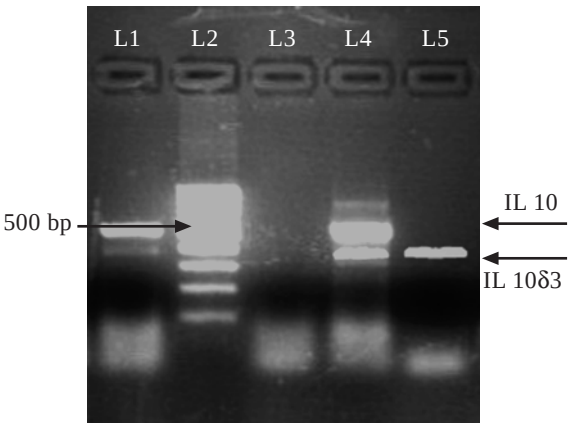

Fig. (1): Expression of IL-10 and isoform IL-10δ3 in precursor B-ALL.

Lane 1 : IL 10 positive case (540bp).
Lane 2 : 100 bp molecular weight marker (reference band 500bp).
Lane 3 : Negative control.
Lane 4 : IL 10 positive and IL 10δ3 positive case (540bp and 385bp).
Lane 5 : IL 10δ3 only positive cases (385bp).

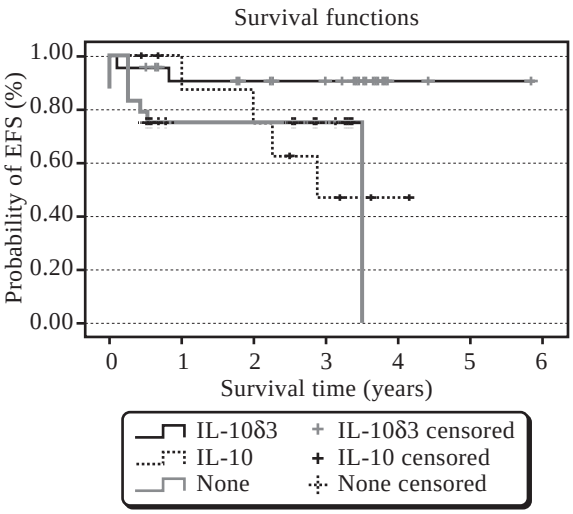

Fig. (2): Correlation between IL-10 expression and pEFS in pediatric precursor B-ALL patients.

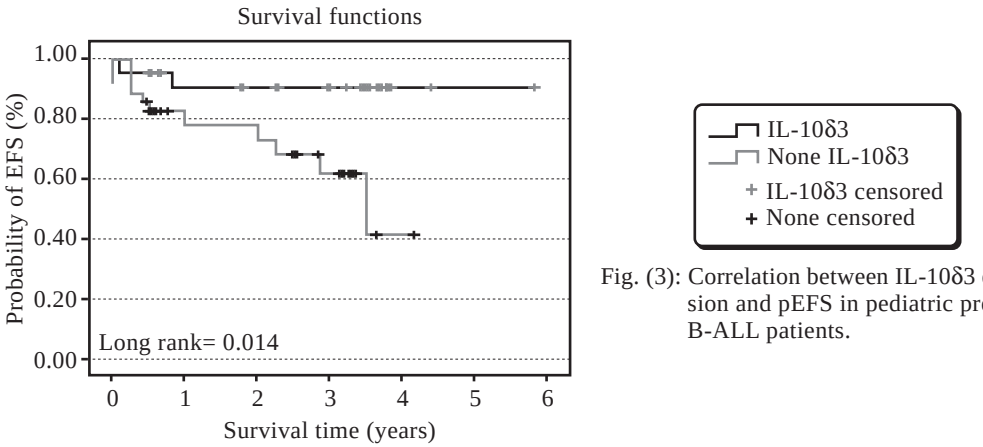

Fig. (3): Correlation between IL-10δ3 expression and pEFS in pediatric precursor B-ALL patients.

Table (1): Correlation between event free survival and IL-10 expression in precursor B-ALL patients.

	IL-10 negative	IL-10 full length	IL-10δ3 isoform	Total	*p* value
Number (%)	24(41.4%)	11 (18.9%)	23 (39.7%)	58 (100%)	
EFS (months)					
median	8	27	41		0.005*
Range	(0-32)	(5.4-30.5)	(6-70)		

IL: Interleukin,
* : Statistical significance: $p \leq 0.05$.

Table (2): Features of the 4 precursor B-ALL patients expressing IL-10δ3.

	IL-10δ3			
	Case 1	Case 2	Case 3	Case 4
Age (years)	10	8	11	6
Sex	F	M	M	F
TLC ($x10^9$/L)	6.77	101	67	57.47
Liver below costal margin (cm)	2	Free	2	Free
Spleen below costal margin (cm)	3	Free	6	Free
CD34 positivity	Yes	Yes	Yes	Yes
CR	Yes	Yes	Yes	Yes
Relapse	No	No	No	No
Status	alive	alive	alive	alive
EFS (months)	27	44.13	43.66	70
OS (months)	27	44.13	43.66	70
Others	t(4;11)	none	none	none

IL : Interleukin. EFS: Event free survival.
CR: Complete remission. OS : Overall survival.

DISCUSSION

IL-10 is an immunoregulatory cytokine and its main biological function is limitation and termination of inflammatory responses. IL-10 also regulates differentiation and proliferation of several immune cells **[22]**. Antiangiogenic properties of IL-10 have also been described **[23]**. Thus, its dual role as immunosuppressive and antiangiogenic cytokine may have both promoting and inhibiting effect on tumor development and progression **[24]**.

Studies confirmed the secretion of IL-10 by leukemic cells in bone marrow specimens of children with relapsed ALL **[18]**. Also, pretreatment serum levels of IL-10, IL-12 and IL-10/IL-12 balance in children with Soft Tissue Sarcoma, Hodgkin's Lymphoma and ALL might be of value as additional prognostic tools to

predict the response to therapy and probability of event free survival (EFS) and overall survival (OS) **[25]**.

Results in AML were contradictory. A report proposed that IL-10 contributed to T-regulatory (Treg) mediated suppression; Treg were reported to control peripheral immune tolerance and their accumulation in the peripheral circulation of AML patients mediate vigorous suppression. Hence, patients with lower Treg frequency at diagnosis were shown to have a better response to induction chemotherapy **[16]**, while in the other, IL-10 was not found to be correlated with CR, survival, or EFS **[14]**.

The involvement of IL-10 expression in childhood ALL has been suggested in various studies **[5,26,27]**. Also, published reports connected genotype of IL-10 with sensitivity for steroid therapy and proposed to include this feature into prognostic factors, especially in children with disease relapse **[26,27]**. IL-10 gene polymorphism studies suggested a correlation between the IL-10 genotype and prednisone response in childhood ALL. Patients displaying the IL-10 G/G genotype might have a lower risk for poor prednisone response **[12]**.

Similar to our findings other reports suggested that the splicing-derived IL-10 isoforms may modulate IL-10-mediated biologic effects and therapeutic efficacy in lymphatic disease and expression of IL-10δ3 is a positive prognostic feature in childhood ALL **[27]**. Our data showed a possible modulatory role of IL-10δ3 particularly in the decreased incidence of relapse. Cases expressing IL-10δ3 showed less incidence of relapse compared to patients expressing only the normal IL-10 transcript and/or patients expressing IL-10 below detection limit. In our study, none of the 4 patients expressing IL-10δ3 as a sole variant relapsed. Interestingly, one of the cases was a pro B, harboring t(4;11) rearrangement, a situation that is known to be of poor prognosis, yet the patient had an event free course, EFS and OS of 27 months. However, the follow-up period is not long enough to exclude the possibility of relapse at a later point of time.

In conclusion, we report that in childhood precursor B-ALL, IL-10 isoform, IL-10δ3, expression might be associated with a favorable prognosis, decreased incidence of relapse in first complete remission and statistically better event-free survival. Screening of a larger group of patients is needed to confirm, the unfavorable impact of the lack IL-10 isoform IL-10δ3 expressions and if its presence might have a protective role, so therapy and management of patients would be modified accordingly.

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Study of Vascular Endothelial Growth Factor Gene Polymorphism in Acute Myeloid Leukemia Patients

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ABSTRACT

Introduction: Acute myeloblastic leukemia (AML) represents a group of clonal hematopoietic stem cells. Angiogenesis is a prerequisite for the growth and progression of malignancies. Vascular endothelial growth factor (VEGF) is a potent angiogenic peptide. Several polymorphisms have been described in the VEGF gene, some of these variants are in the promoter region (Locus-2578c>A), 5' untranslated region (Loci-1154 G>A,–634G>A) and 3' untranslated region (+936 c>T) were found to be associated with variations in VEGF protein production.

Aim of the Work: To evaluate the ability of VEGF polymorphisms to predict prognosis in AML patients.

Patients and Methods: The study was performed on 45 newly diagnosed AML patients. Genotypes of VEGF were determined using a polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) method. ELISA was used for quantitative assay of VEGF in serum. Patients were re-evaluated post induction.

Results: Patients with 936CC genotype were associated with significant worse response to induction than those with CT or TT genotypes (p=0.021). Patients with 634 CG genotype had significant bad response (p=0.035). Patients with-2578 AA/CC genotypes were associated with significant better CR in comparison to patients with –2578 CA genotype (p=0.0001). Regarding –1154 AA, GA, GG different polymorphisms, no significant correlation with CR. Hardy-Weinberg equilibrium was observed for all polymorphisms. The association of genotype AA, GG, CT for loci –2578/–634/936 in the same patient, as well as CC/CC/TT were associated significantly with CR (p=0.036 & 0.02). Combination polymorphisms which were associated with significantly wase CR were CA/CC/CC for loci –2578/–634/936 (p=0.021), CA/CG/CC (p=0.012), CA/CG/CT (p=0.036).

Conclusion: We found that certain combination polymorphisms are associated significantly with remission while others with worse response to induction chemotherapy.

Key Words: *AML – VEGFA – Genotype – Polymorphism.*

INTRODUCTION

Acute myeloblastic leukemia (AML) represents a group of clonal hematopoietic stem cells disorder that results from genetic alterations in normal hematopoietic stem cells **[1]**. A number of clinical and biologic features are used to predict clinical outcome **[2]**.

Constitutive genetic characteristics of the patient may play an important role in the prognosis, yet this has scarily been investigated in AML **[3]**.

In this regard, it is well recognized that most drugs exhibit wide interpatient variability in their efficacy and toxicity **[3,4]**.

Moreover, recent studies have shown that genetic polymorphisms can be used to predict the clinical outcome of malignancies **[5,6]**.

Angiogenesis, the process of new blood vessel formation from endothelial precursors, is a prerequisite for the growth and progression of malignancies. Vascular endothelial growth factor (VEGF) a soluble, 34-46 KDa, heparin binding glycoprotein is a potent angiogenic peptide with diverse biological activities including angiogenesis **[7,8]**. VEGF is located on chromosome 6p21.3 and composed of eight exons and seven introns **[8]**. Dysregulation of VEGF production was suggested to have a major impact on leukaemic growth and constitutes an important step in the progression of AML **[9]**.

Several polymorphisms have been described in the VEGF gene, some of these variants are in the promoter region (Locus-2578c>A), 5' untranslated region (Loci-1154 G> A,–634G>A) and 3' untranslated region (+936 C>T) were

found to be associated with variations in VEGF protein production **[10,11]**.

As the prognosis of AML patients may correlate with the degree of angiogenesis and VEGF, the current study evaluated the ability of VEGF polymorphisms to predict prognosis in AML patients.

PATIENTS AND SAMPLES

The study was performed on 45 newly diagnosed AML patients from Haematology division Faculty of Medicine, Alexandria University, with age range of 16-60 years, median 24 years (mean ± SD 25.63±10.45). Patients were treated with standard cytarabine and daunorubicin protocol. (Daunorubicin 45mg/m^2/day for 3 consecutive days and cytarabine 100mg/m^2/day continuous infusion for 7 consecutive days). For patients over 50 years the anthracycline dose was reduced by one third. Patients diagnosed as AML M3, received all trans-retinoic acid (ATRA) 45mg/m^2/day and idarubicin 12 mg/m^2/day on day 1 through 4.

Genotyping of the VEGF gene polymorphisms:

Genomic deoxyribonucleic acid (DNA) was isolated from peripheral blood by a standard extraction method using blood DNA extraction kit (OMEGA BIO TEK, USA). The nucleotide sequence of four VEGF gene polymorphisms which were in the promoter region at –2578 and –1154, in the 5' untranslated region (UTR) at –634 and in the 3'-UTR at 936 was amplified by polymerase chain reaction (PCR).

Genotypes were determined using a polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) method as previously described **[12-14]**.

Briefly, the PCR primers for –2578C/A, –1154G/A, –634C/G and 936C/T were 5'-GGCCTTAGGACACCATACC-3' (forward) and 5'-CACAGCT TCTCCCCTATCC-3' (reverse); 5'-TCCTGCTCCCTCCTC GCCAATG-3' (forward) and 5'-GGCGGGGA CAGGCGAGCCTC-3' (reverse); 5'-CGACGGCTTGGGGAGATTGC-3' (forward) and 5'-GGGCGGTGTC TGTCTGTCTG-3' (reverse); and 5'-AGGGTTTCGGG AACCAGATC-3' (forward) and 5'-CTCGGTGATTT AGCAGCAAG-3' (reverse), respectively. PCR was performed in a final volume of 25µl, using 2 x Dream Tag Green PCR Master Mix (Fermentas, EU) containing, 20pmol/ul of each primer, and 50-100ng of genomic DNA. After the initial denaturation step at 95°C for 10min, 35 cycles consisted of denaturation at 95°C for 45s, annealing at 62°C for 45s, extension at 72°C for 30s, followed by final extension lasting 10min at 72°C. Genotypes were determined by restriction fragment length polymorphism (RFLP). The restriction enzymes which detect –2578C/A, –1154G/A, –634C/G and 936C/T are BstYI, MnlI, BsmFI and NlaIII respectively. Amplified DNA was digested with 1-3U of endonucleases for overnight at 37°C as indicated by the manufacturer (Fermentas, EU), and then electrophoresed on 3% agarose gel. The restriced DNA size of each polymorphism type was as follow (Fig. A,B):

VEGF –2578: CC (438bp), CA (438, 231, 207bp), AA (231, 207bp).

VEGF –634: CC (274bp), CG (274, 156, 118bp), GG (156, 118bp).

VEGF 936: CC (326bp), CT (326, 271, 55bp), TT (271, 55bp).

VEGF-1154; GG (206bp), GA (206,184, 22bp), AA (184,22bp).

ELISA assay:

Serum samples were collected and centrifuged at 1000g for 10min within 30min from collection. Serum was aliquoted and stored at –20°C until VEGF evaluation. VEGF-A (Platinum ELISA eBioscience) concentrations were determined in serum according to the manufacturer's instructions. Concentrations are reported as picograms per milliliter. Normal range value of VEGF is up to 42.6pg/ml for serum.

Statistical analysis:

The Data was collected and entered into the personal computer. Statistical analysis was done using Statistical Package for Social Sciences (SPSS/version 17) software. Arithmetic mean and standard deviation were used for categorized parameters, Chai square test was used while for numerical data, *t*-test was used to compare two groups while for more than two groups ANOVA test was used. The level of significance was considered 0.05.

Hardy-Weinberg: Hardy-Weinberg describes genetic balance within a studied group. The law is used to determine whether the number of harmful mutations in patients is increased.

RESULTS

We evaluated VEGF polymorphism at different loci among 45 AML patients (6 were FAB M0 11.1%, 10 M2 22.2%, 5 M3 11.1%, 11M4 24.4%, 11 M5 24.4%, 3 M7 6.7%).

Among 45 patients 20 (44.4%) achieved complete remission (CR was defined as the presence of no more than 5% blast cells in the bone marrow aspirate). Table (1) presents a CR status in relation to different polymorphisms of VEGF.

Patients with 936 CC genotype were associated with significant worse response to induction chemotherapy than those with CT or TT genotypes (p=0.021); (among 28 patients with CC genotype, 20 did not achieve CR).

Patients with –634 CG genotype had significant worse response (p=0.035); (among 25 patients who had this polymorphism 17 [68%] did not achieve CR) while all patients with -634 GG genotype achieved CR. Patients with –2578 AA/CC genotypes were associated with significant better CR in comparison to patients with –2578 CA genotype (p=0.0001). Regarding –1154 AA, GA, GG different polymorphisms, we did not find significant correlation with remission.

VEGFA polymorphism and disease characteristics:

Disease characteristics including clinical (splenomegaly, hepatomegaly, lymphadenopathy, gum hypertrophy), peripheral WBCs mean ± SD 68.457±80.538, sex and age at diagnosis did not show any significant correlation with the four genotypes of VEGFA polymorphisms. While level of VEGFA mean ± SD 626.933± 577.980 was significantly higher in 936CC genotype in comparison with CT, TT genotypes.

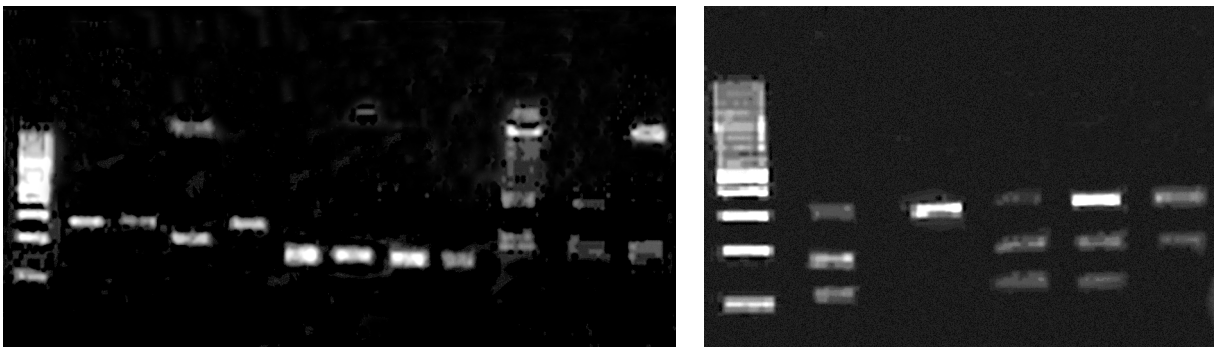
Hardy-Weinberg equilibrium was observed for all polymorphisms. Accordingly, we analysed VEGFA polymorphism based on three genotypes at loci 936, –634, –2578 and we correlated them with achievement of CR (Table 2). The combination of genotype AA, GG, CT for loci –2578/ –634/936 in the same patient, and CC/CC/TT were associated with significant CR (p=0.036). Combination polymorphisms which were associated with significant wase CR were CA/CC/ CC for loci –2578/–634/936 (p=0.021), CA/CG/ CC (p= 0.012), CA/CG/CT (p=0.036).

Table (1): VEGFA polymorphism and achievement of CR after first cycle of induction chemotherapy.

	Response to induction chemotherapy				
	Remission		No Remission		*p*
	No.	%	No.	%	
936 C>T					
C/C	8	40.0	20	80.0	
C/T	6	30.0	3	12.0	
T/T	6	30.0	2	8.0	.021
–634 C>G					
C/C	9	45.0	8	32.0	
C/G	8	40.0	17	68.0	
G/G	3	15.0	0	0.0	.035
–1154 G>A					
A/A	5	25.0	8	32.0	
G/A	0	0.0	3	12.0	
G/G	15	75.0	14	56.0	.201
–2578 C>A					
A/A	3	15.0	0	0.0	
C/A	2	10.0	22	88.0	
C/C	15	75.0	3	12.0	.0001

Table (2): Associations of different VEGFA polymorphisms in relation to CR.

–2578 C>A	–634 C>G	936 C>T	Response to induction chemotherapy				Total	*p*
			Remission		No Remission			
			No.	%	No.	%		
A/A	G/G	C/T	3	100	0	0.0	3	0.036
C/A	C/C	C/C	0	0.0	5	100	5	0.021
C/A	C/G	C/C	2	14.3	12	85.7	14	0.012
C/A	C/G	C/T	0	0.0	3	100.0	3	0.036
C/A	C/G	T/T	0	0.0	2	100.0	2	–
C/C	C/C	C/C	3	50.0	3	50.0	6	0.85
C/C	C/C	T/T	6	100.0	0	0.0	6	0.020
C/C	C/G	C/C	3	100.0	0	0.0	3	0.85
C/C	C/G	C/T	3	100.0	0	0.0	3	0.85



(A) **(B)**

Fig. (1): Polymorphisms of the VEGF. (A) Lane 1: 100-bp DNA ladder. Lanes 2, 3 & 5: VEGF-936 CC genotype (326-bp bands), lane 4: VEGF 936 TT genotype (271-bp band), lanes 6-9: VEGF-1154G/G genotype (206-bp bands), lanes 10 & 11: VEGF 2578C/A genotype (438, 231 & 207-bp bands), and lane 12: A/A genotype of the same SNP. (B) Lane 1: a 100-bp DNA ladder. Lanes 2, 5 & 6: VEGF-634 C/G genotype (274, 156, and 118-bp bands), lane 3: C/C genotype (274-bp band) of the same SNP, and lane 4: G/G genotype (118 & 156-bp bands).

DISCUSSION

Data suggest that VEGF is an important pathogenetic factor in myeloid leukemia. VEGFA has been described as a mediator of leukemia-dependent angiogenesis and as an autocrine growth regulator of AML cells **[15]**.

Our study demonstrated the importance of VEGFA polymorphism for predicting the prognosis in patients with AML. The VEGFA polymorphisms, 936 CT/TT were associated with better CR than 936 CC which was associated with significant worse response to induction chemotherapy. This finding coincide with previous data which found that 936 CT or TT alleles had favourable prognosis in AML, while 936 CC had unfavourable outcome **[16]**.

In the present work, –2578 AA or CC genotypes have been shown to be associated with significant good response to treatment. Others found that the wild and polymorphic types of VEGFA at position –2578 were associated with the development of certain diseases while carriers of wild type-polymorphic type were not **[13]**. Previous data found that –2578CC genotype was associated with shortened OS in patients with ovarian cancer **[17]**.

The current study assessed polymorphism of –1154 locus and did not find correlation with prognosis in AML in contrast to other researches who found that GG allele was associated with risk of RA **[13]**.

Our data showed that CG polymorphism was associated with significant worse response to induction therapy. In contrast to Soo-Han Hun et al who found that –634 CC or CG polymorphisms were associated with better OS and PFS in patients with colorectal cancer compared with GG genotype **[18]**.

It has been demonstrated that the pathogenesis of acute leukemia involves complex inter-

actions between host susceptibility, chromosomal damage and possibly the incorporation of genetic informations into susceptible progenitor cells **[19,20].**

We reported that the level of VEGF was significantly higher in 936 CC genotype in comparison with CT and TT genotypes; this is comparable to previous data which found that higher levels of VEGF are associated with poor outcome in patients with Hodgkin and Hodgkin diseases **[21-23].**

We correlated the association of certain polymorphisms at different loci for VEGFA with prognosis; we found that certain association polymorphisms were associated with significant favorable response while others with worse outcome. Young Park et al. studied the haplotypes of certain loci on VEGFA and found that CTG haplotype on loci 25781/405/460 was associated with worse prognosis **[16].**

In conclusion, further studies integrating VEGF polymorphism with other prognostic parameters are needed to verify if it would stand a multivariate analysis as independent prognostic parameter to refine therapeutic decision.

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