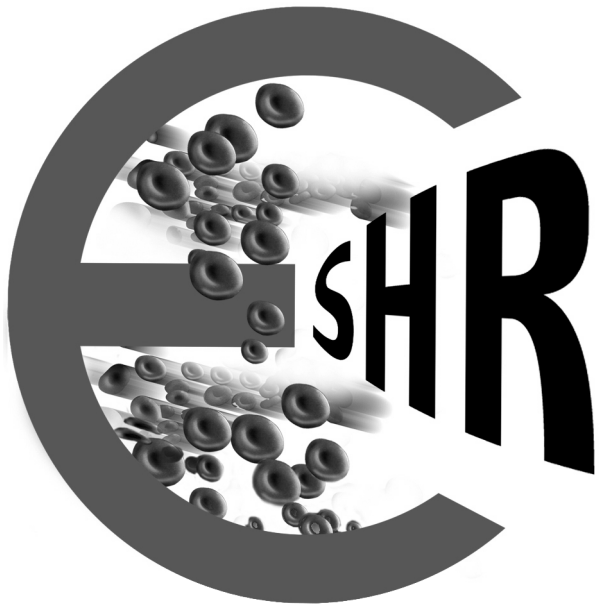
The Journal of the Egyptian Society of Haematology & Research



The Official Journal of the
Egyptian Society of Haematology & Research

Vol. 8, No. 2, September 2012

The Journal of the Egyptian Society of Haematology & Research

The Official Journal of the Egyptian Society of Haematology & Research

MAILING ADDRESS

ESHR (NCI) Fom El-Khalig, Cairo, Egypt

The Journal of the Egyptian Society of Haematology & Research

The Official Journal of the Egyptian Society of Haematology & Research

Volume 8 * Number 2 * September 2012

CONTENTS

Journal of the Egyptian Society of Haematology & Research, Vol. 8, No. 2, September: 35-42, 2012

Improved Detection of Chromosomal Abnormalities in Chronic Lymphocytic Leukemia by Integrating the Results of Conventional Cytogenetics Using CpG-Oligodeoxynucleotide/ Interleuken-2 Mitogen Stimulation and Fluorescence in Situ Hybridization Techniques

EMAN R. RADWAN, M.D.*; MARCO MANCINI, M.D.**; MAURO NANNI, Ph.D.**; RITA BARZOTTI, Ph.D.**; MARIA ZAIRA LIMONGI, Ph.D.**; FRANCESCA ROMANA MAURO, M.D.**; MARIA STEFANIA DE PROPRIS, Ph.D.**; ANNA GUARINI, Ph.D.** and ROBIN FOA`, M.D.**

The Departments of Clinical & Chemical Pathology, Faculty of Medicine, Cairo University, Egypt and Cellular Biotechnologies & Hematology, "Sapienza" University of Rome, Rome, Italy***

ABSTRACT

Background: Cytogenetic abnormalities are important prognostic parameters in chronic lymphocytic leukemia (CLL) patients. Because traditional mitogens are not always effective mitotic stimulants, usually, only interphases Fluorescence in Situ Hybridization (FISH) is done for diagnosis and follow-up of patients. Recently, CpG-oligodeoxynucleotide (ODN) stimulation has shown more effectiveness than standard mitogens in CLL cells.

Objectives: Our study aimed to test the effectiveness of CpG-ODN/IL-2 as a mitotic stimulant for CLL cells in comparison with other traditional ones and verify the consistency of detected cytogenetic abnormalities between different cultures. In addition we aimed to assess if doing interphase FISH analysis in parallel improved the rate of abnormalities detection in our cohort study.

Material and Methods: Our study included 60 CLL patients referred for routine cytogenetic and FISH analysis during disease evolution. Parallel cultures of peripheral blood were done adding either Lipopolysaccharide (LPS), B-cell mitogen 12-O-tetradecanoyl-phorbol-13-acetate (TPA) or a combination of CpG-ODN and interleukin-2 (IL-2). Cytogenetic analysis was done on all cases cultured using CpG-ODN/IL-2, TPA and LPS. Simultaneously FISH was done for 40/60 cases using the following probes: 13q14.3/13q34, ATM/CEP11, IgH14q32, CEP12, 6q21/ SEC63, p53 (17p13)/SE17.

Results: Success rate in cultures was 86.6% using CpG-ODN/IL-2 mitogen, 48.3% using TPA and 40% using LPS. Clonal abnormalities were observed in 33/52 cases (63.46%) cultured using CpG-ODN/IL-2; the aberrant clones were present consistently in TPA and/or LPS cultures when successful. Out of these, 7 cases (21.2%) showed clonal abnormality with normal FISH, and 5 cases (15.15%) showed clonal abnormality with positivity only for 13q14.3 deletion. On the other hand, performing FISH allowed the detection of abnormalities (deletion 13q14.3) invisible by conventional cytogenetic analysis in 8/40 (20%) cases.

Conclusion: our results confirm that CpG-ODN/IL-2 stimulation increases the detection rate of chromosomal abnormalities and complexity in CLL compared with TPA and LPS. Furthermore, applying the used FISH panel, improved abnormalities detection rate. However, neither conventional karyotyping nor FISH detected all aberrations, demonstrating the indispensability of both techniques.

Key Words: *Chronic lymphocytic leukemia – CpG-oligodeoxunucleotide/IL-2 – Fluorescence in situ hybridization – Karyotyping*

INTRODUCTION

Assessment of cytogenetic aberrations has been shown to represent an independent predictor of prognosis in B-cell chronic lymphocytic leukemia (B-CLL) **[1-4]**. However the standard used mitogenic stimulants as 12-O-tetradecanoyl -phorbol-13-acetate (TPA) and Lipopolysaccharides (LPS) reveal cytogenetic abnormalities in only ~50% of cases **[1]** which has limited the application of metaphase cytogenetics in CLL. On the other hand, fluorescence in situ hybridization (FISH) has been widely applied in CLL increasing the rate of detected abnormalities up to 80% **[2,5-9]**. Nonetheless, the results provided are only informative about the genomic regions for which the FISH probes are designed neither detecting other abnormalities nor complex karyotyping. CLL is a clonal disease in which lymphocytes have a low mitotic index. Therefore,

CD40 ligand-induced cell cycle stimulation has been tried increasing the rate of abnormalities detection to 89% **[10]**. However, it was not widely used due to its difficult applicability in routine analysis. More recently CpG–oligodeoxynucleotide (ODN) stimulation has been used in combination with IL-2 increasing the rate of abnormalities detection up to 80% **[3,11-14]**, a percentage nearly equivalent to that obtained in interphase FISH with the added advantage of having a global view of cytogenetic abnormalities and/or complexities. CpG-ODNs are synthetic or bacterial short single strands of DNA, in which the CpG motifs are not methylated. They enter the B-cells and stimulate response to cytokines through Toll-like receptor-9 mediation **[15-18]**. Some studies addressed performing comparative cytogenetic analysis using different mitogenic stimulants: TPA and CpG-ODN/IL-2 **[13,14,19]**. The study conducted by Put et al. **[14]** and Muthusamy et al. **[20]**, addressed the relevance of combining both cytogenetic analysis and interphase FISH although in the latter the mitogenic stimulant was different. Therefore we decided to perform cytogenetic analysis for CLL using these different mitogenic stimulants in parallel with interphase FISH to establish CpG/IL-2 stimulation as a more effective method for conventional Karyotyping and to further confirm the importance of integrating both the results of conventional karyotyping with interphase FISH for more accurate detection of chromosomal abnormalities in CLL.

MATERIAL AND METHODS

Patients:

The study was performed on 60 patients including 43 males and 17 females with an age range of 36-83 and a median of 58.5 years at the time of presentation. The protocol was approved by the IRB of "Sapienza" University of Rome and all patients gave their written informed consent to the blood collection and to the biologic analyses included in the present study according to the Declaration of Helsinki. The diagnosis of CLL was based on the presence of more than 5000 lymphocytes/μL in the peripheral blood that expressed a conventional CLL immunophenotype (CD5/CD20$^+$, CD23$^+$, CD79a$^+$, FMC7$^-$, weak CD22$^+$, weak sIg$^+$, CD79b weak/$^-$, CD10$^-$) **[21]**.

Conventional cytogenetic analysis:

Cell separation:

Peripheral blood samples were drawn on heparin. Samples were separated on Ficoll-Hypaque (CEDARLANE CL5015), and centrifuged. Mononuclear layer was retrieved, washed, centrifuged and cell pellet was obtained. Cell count was adjusted to 10-20X10^6 cells in 5-10ml of medium according to the mitogen added (as explained below).

Mitogens:

For every sample 3 separate cultures were done with LPS, TPA, and CpG-ODN/IL-2 added as follows:

- Culture 1: 20X10^6 cells/10ml of culture medium with 400ul LPS [40ug/ml] (Sigma-Aldrich # L 2654).
- Culture 2: 20X10^6 cells/10ml of culture medium with 50ul TPA [50ng/ml] (Sigma # P8 139).
- Culture 3: 10X10^6 cells/5ml of culture medium with 100ul of CpG-ODN DSP30 [2nmol/ml] (Roche Diagnostics # 232622 DSP30) and 100ul IL-2 [100U/ml] (Roche # 10 799 068 001).

Samples were incubated for 72 hr at 37ºC and 5% CO2 in a fully humidified atmosphere. Colchicin 50ul was added [10ug/ml] (Gibco, Invitrogen Corporation #15210-40) then incubated for 3 hrs in the same conditions. The cells were treated with hypotonic solution, centrifuged, and the resulting pellet was fixed and washed in methanol/acetic acid (3:1). Cells were re-suspended in fixative and dropped on slides. Karyotypes were examined after the G banding technique and described according to International System for Human Cytogenetic Nomenclature (ISCN 2009) **[22]**.

Interphase FISH analysis:

Interphase FISH was performed on nuclei preparations of CpG-ODN/IL-2, TPA, or LPS cultures depending on quality of nuclei on slides preparation. The following commercially available probes were used to investigate loci commonly involved in CLL: 6q21 [SEC63] (Kreatech # KBI-10105), 11q22-23 [LSI ATM, CEP 11 Probe] (Vysis/Abbott # 30-231059), centromere 12 [CEP 12] (Vysis/Abbott # 30-

170012), 13q14.3/13q34 [LSI D13S319 (13q14.3) Spectrum Orange/ LSI 13q34 Spectrum Green Probe] (Vysis/Abbott # 30-231061), 14q32 [LSI IGH Dual Color, Break Apart Rearrangement Probe] (Vysis/Abbott # 30-191019), and p53 (17p13) /SE 17 (Kreatech # KBI-1-112). FISH analyses were performed according to the manufacturer's protocols and as previously reported **[23]**. In all of the analyzed CLL cases, at least two hundred interphase round nuclei with well-delineated fluorescent spots were counted; positive cases were defined as having ≥% the cut-off of nuclei displaying the investigated abnormality which differed with the probe used: ≥5% for 6q21, ≥10% for 11q22-23 and 13q14, ≥0% for centromere 12, ≥4.6% for 14q32, and ≥20% for p53 (17p13).

RESULTS

Success rate in cultures using CpG-ODN/IL-2 mitogen was 86.6%. Failure occurred in 8 cases. A minimum of 20 metaphases were cytogenetically analyzed in them. Failure occurred in 31 cases after TPA (success rate of 48.3%) and in 36 cases after LPS cultures (success rate of 40%). Failure was defined as less than 10 metaphases in the culture under consideration, whereas success was defined as 10 or more metaphases or the presence of a clonal abnormality. Clonal abnormalities were observed in 33/52 cases (63.46%) cultured using CpG-ODN/IL-2; the aberrant clones were present consistently in TPA and/or LPS cultures when successful (Table 1) (Figs. 1,2).

Table (1): Clonal abnormalities detected by conventional cytogenetic analysis in 33/52 chronic lymphocytic leukemia patients.

Case number	Translocation
Case 1	46,XY,add(5)(p13) **[4]**/46,XY**[16]***
Case 4	46,XY,der(15)t(3;15)(q25;q24)**[7]**/46,XY**[13]***
Case 5	46,XX,del(17)(p10)**[7]**/46,XX**[13]**
Case 8	48,XX,+12,+19**[20]**
Case 13	47,XX,+12 **[6]**/46,XX**[14]**
Case 15	46,XY,der(16)t(14;16)(q32;q23)**[11]**/46,XY**[9]***
Case 16	Complex Karyotype**
Case 17	46,XY,del(7)(q21.2q31)**[14]**/46,idem,del(11)(q31)**[2]**/46,XY**[4]**
Case 19	46,XY,add(22)(p12)**[14]**/46,XY**[6]***
Case 21	46,XY,del(6)(q13q25)**[12]**/46,XY,add(18)(p11.2)**[3]**/46,XY**[5]****
Case 22	47,XY,+12**[20]**
Case 23	46,XY,del(6)(q13q25)**[6]**/46,XY**[14]***
Case 24	46,XY,del(11)(q22.3),del(16)(q?)**[3]**/46,idem,del(13)(q14q21)**[1]**/46,XY,del(13)(q14q21)**[4]**/46,XY,del(16)(q?)**[2]**/46,XY**[10]**
Case 25	44,XY,del(1)(p34),-8,-9,-10,del(11)(p11.2),-13,-16,-17,+4mar**[2]**/46,XY**[18]****
Case 27	45,XX,-4,del(11)(q21),der(17)t(4;17)(q13;p11.2)**[20]**
Case 28	46,XX,del(11)(q23)**[3]**/46,XX,del(11)(q13)**[1]**/46,XX**[16]***
Case 29	47,XY,+12**[3]**/46,XY**[17]**
Case 31	46,XX,-17,+mar**[4]**/46,XX **[16]****
Case 33	46,XY,del(13)(q14q21)**[10]**/46,XY,del(13)(q14q21)X2**[1]**/46,XY**[9]**
Case 34	46,XY,-19,+mar**[5]**/46,XY**[15]****
Case 35	47,XY,+12**[12]**/47,idem,del(11)(q22)**[6]**/46,XY**[2]**
Case 36	46,XY,del(11)(q22)**[18]**/45,XY,-11**[1]**/46,XY**[1]**
Case 38	46,XY,del(13)(q14q21)**[4]**/46,XY**[16]**
Case 39	44,XY,del(1)(q42),der(4)t(4;17)(p13;q21),add(8)(p11.2),del(14)(q32),-15,add(17)(p13.1),-17[**15]**/46,XY,del(1)(q42),del(14)(q32)**[2]**/46,XY**[3]**
Case 40	46,XY,t(1;7)(q21;p22),del(2)(p13),del(11)(q22),add(14)(q32)**[2]**/46,XY,del(11)(q22)[**2]**/46,X **[16]**
Case 41	46,XY,der(17)t(9;17)(q22;p13.2)**[3]**/47,XY,+12[**2]**/46,XY**[15]**
Case 42	46,XY,del(13)(q14q21)**[4]**/46,XY**[16]**
Case 44	Complex Karyotype
Case 46	46,XY,del(7)(q22),add(7)(q32).ishdel(7)(q22),t(7;?)(q32;q?)**[3]**/46,X **[17]**
Case 48	46,XY,del(11)(q23),del(13)(q14q21)**[1]**/46,XY,del(11)(q23)**[1]**/46,XY,del(13)(q14q21)**[3]**/46,XY**[15]**
Case 49	46,XY,del(11)(q23)**[5]**/46,XY,del(6)(q13q25),del(11)(q23)**[12]**/46,XY**[3]**
Case 50	47,XX,+mar**[2]**/47,idem,del(9)(q12)**[2]**/46,XX**[16]***
Case 52	46,XY,del(13)(q14q21)**[4]**/46,XY**[16]**

* Cases with a normal panel of interphase FISH analysis. ** Cases with clonal abnormality only for 13q14.3 deletion.

Table (2): Abnormalities detected by interphase FISH analysis in 40/60 of chronic lymphocytic leukemia patients.

Case No.	13q14/13q43	17p13 (p53)	11q22/23 (ATM)	14q32 (IgH)	CEP 12	6q21
Case 3	20%*					
Case 5	19%	90%				
Case 8					50%	
Case 9	40%/50%*/**					
Case 10	20%*					
Case 13					42%	
Case 14	15%*					
Case 16	93%					
Case 17	45% /27%**					
Case 18	60% /7%*/**					
Case 20	30%*					
Case 21	82%/12%**					
Case 22					66%	
Case 24	87%		90%			
Case 25	85%					
Case 26	25%*					
Case 27	90%	30%	80%			
Case 29					23%	
Case 31	20%/33%**					
Case 32	35%*					
Case 33	64%/16%**					
Case 34	85%/5%**					
Case 35			14%		42%	
Case 36			77% /9%***	5%		
Case 38	53% /16%**					
Case 39		33%	14%			

* Cases where 13q14 deletion was not detected cytogenetically.
** Double deletion of the 13q14 locus.
*** Monosomy of chromosome 11.

Interphase FISH analysis was done for 40 cases. A full probe panel of the six commonly involved regions (mentioned above) was applied. One or more abnormalities were detected in 26 cases (65%) (Table 2, Fig. 3). Out of the 33 cases showing clonal abnormalities, 7 (21.2%) had a negative panel of interphase FISH. In cases 23 and 28 (as indicated in Table 1) deletion of chromosome 6 and chromosome 11 were detected respectively. In these 2 cases FISH failed to detect the specific abnormality which was visible at the cytogenetic level. In addition, in 3/7 of these cases whole chromosome painting was done for specific chromosomes to further clarify the results (as indicated in Table 1): For case 4 whole chromosome painting was done for chromosome 3 (Kreatech # pKBI-30003G) (Fig. 4), for case 15 whole chromosome painting was done for chromosome16 (Kreatech # pKBI-30016G) and for case 19 whole chromosome painting was done for chromosome 22 (Kreatech # pKBI-30020R). In addition, 5 cases (15.15%) showed clonal abnormality with positivity only for 13q14 deletion (Fig. 5), of those for case 21 (as indicated in Table 1) whole chromosome painting was done for chromosome 18 (Kreatech # pKBI-30018R) and CEP 18 (Vysis/Abbott # 05J08-028) was also done for further specification of the karyotyping. On the other hand 8/40 cases (20%) showed 13q14 deletion which was invisible by conventional cytogenetic analysis.

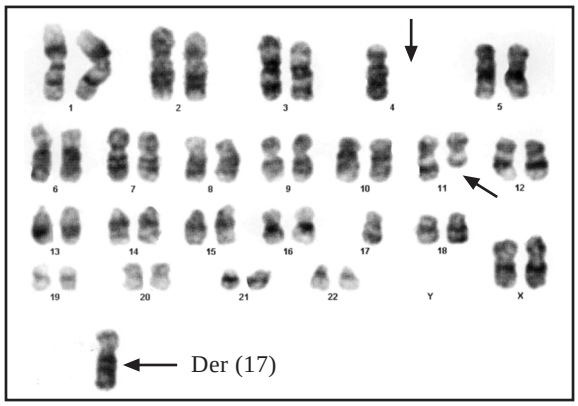


Fig. (1): Case 27: 45,XX,-4,del(11)(q21),der(17)t(4;17)(q13;p11.2)**[20]**.

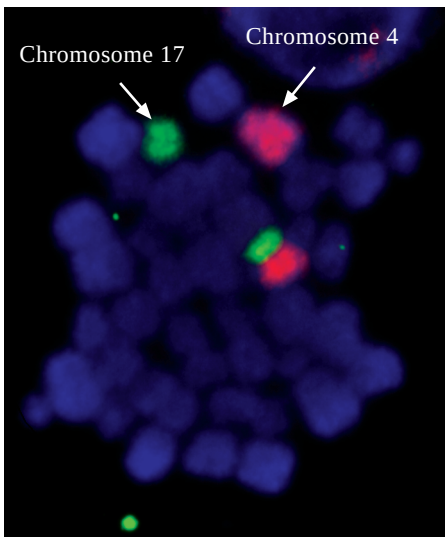


Fig. (2): Case 27: showing der(17)t(4;17)(q13;p11.2) (WCP 4Red, 17Green).

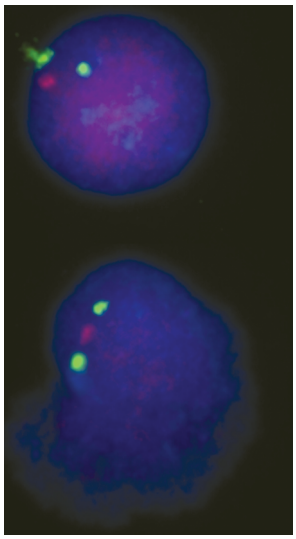


Fig. (3): Case 36: showing ATM 11q22.3 deletion [LSI ATM Red, CEP 11 Green probe].

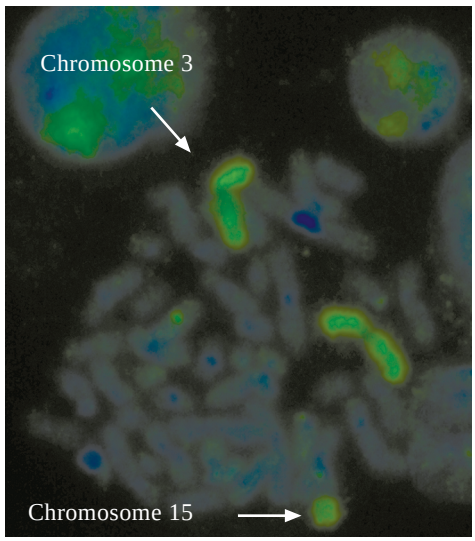


Fig. (4): Case 4: painting of chromosome 3 showing der (15)t(3;15)(q25;q24)(WCP 3 Green).

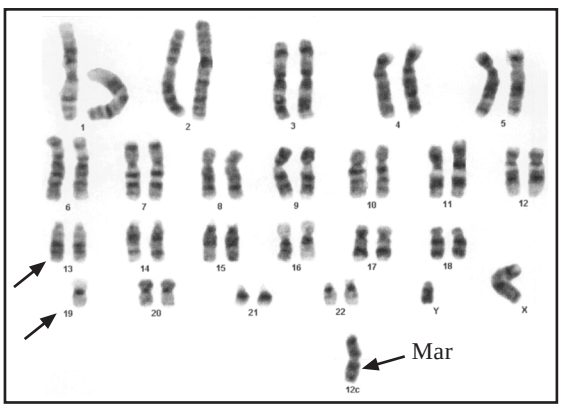


Fig. (5): Case 34: 46,XY,-19,+mar**[5]**/46,XY**[15]**.

DISCUSSION

Cytogenetic abnormalities are considered major prognostic parameters for predicting response to treatment and survival of CLL patients. The heterogeneous biology of the CLL cells, as well as their poor in vitro mitotic index pose a great challenge for obtaining reproducible and reliable abnormal metaphases. Attempts to improve culture conditions to increase the cycling status of the CLL cells for efficient cytogenetic analysis have been investigated. Several B-cell mitogens/stimulatory agents including pokeweed mitogen (PWM), phorbol 12-myristate13-acetate (PMA), TPA and LPS have been applied and used as a standard method in many cytogenetic laboratories. Although each of these mitogens has its own advantages and disadvantages, recently, the use of novel im-

mune-stimulatory agents including CpG ODNs, CD40 ligand, and cytokines such as IL-2, and IL-15 were evaluated **[3,10,11,13,24-26]**. Introduced culture methods with CpG-ODN/IL-2 improved proliferation capacity of CLL cells and yielded detection rates of cytogenetic aberrations comparable with interphase FISH, i.e., 81-83% **[11,12]**. Furthermore, the reproducibility of CpG-ODN/IL-2 stimulation for detecting chromosomally abnormal clones has been established in five laboratories **[19]**. In our study, the overall detection rate of chromosomal aberrations was (86.6%) when stimulated with CpG-ODN/IL-2; this represents a significant improvement over both TPA and LPS cultures which showed a success rate of 48.3% and 40% respectively. The success rate of CpG-ODN/IL-2 is comparable with some studies **[11,13]**, and is higher than others **[14,19]**. The difference was explained by the different methodology in which the cells were not separated; a hypothesis which has been proposed by the authors although it was not ascertained **[11,13]**. In addition, the higher success rate of CpG-ODN/IL-2 as a mitogen in comparison with both TPA and LPS which we report is comparable with some studies **[14,19]** and different from another **[13]**. The latter reported a higher success rate with TPA although they reported a higher proportion of metaphase abnormalities detected using CpG-ODN/IL-2. The variability in the results could be attributed to the slightly different methodologies as each laboratory has its own standardized techniques. In addition we cannot discard the biological heterogeneity of the disease itself and the proliferation capacity of the CLL cells even under standardized conditions. Furthermore, in our study the clonal abnormalities detected were the same among the cultures with CpG-ODN/IL-2 and both TPA and LPS when successful. This finding differed from others who found differences in abnormalities detected and their frequency (an issue which was not found in our study) between different cultures **[13,14,19]**. This could be attributed to the small cohort enrolled in our study compounded by the absolute low success rate with TPA and LPS, a fact which rendered it difficult to fully investigate this point. Interphase FISH identified abnormalities in 65% of cases. In 20% of the cases, FISH detected a 13q14 deletion which was invisible by conventional cytogenetic analysis, an aberration which is often cytogenetically cryptic. Conversely, in 21.2%, FISH failed to detect any abnormality despite abnormal conventional cytogenetics. Hence, the combination of conventional cytogenetics and FISH could increase the detection rate of clonal abnormalities. Of note, the abnormalities observed by conventional cytogenetics and FISH, using classical CLL probe panels, were not always overlapping. In case 23 and 28 (as indicated in Table 1) deletion of chromosome 6 and chromosome 11 were detected respectively. In these 2 cases FISH failed to detect the specific abnormality which was visible at the cytogenetic level. As previously reported this may reflect a selective proliferative response of the CLL clone or inappropriate location of the FISH probe **[14]**. This illustrates the need for a combined routine analysis, including both techniques. Another finding that was observed while doing FISH analysis is that 5 cases showed clonal abnormality with positivity only for 13q14 deletion. Although CLL with 13q deletion as the sole cytogenetic abnormality (del13q-only) usually has good prognosis, more aggressive clinical courses are documented for del13q-only CLL carrying higher percentages of 13q deleted nuclei. Moreover, deletion at 13q of different sizes has been described; its prognostic significance was unknown **[27-30]**. Interestingly in 4/5 cases the percentage of the 13q deleted nuclei was >70%, the cut off % documented by a multi-centric as a bad prognostic parameter. However, the probe we used for detecting 13q14 deletions did not encompass the DLEU2/MIR15A/MIR16-1 and RB1 loci which according to this study influenced the prognosis where cases with larger deletions involving the RB1 locus had poor prognosis even with a low percentage of deleted nuclei rendering it an independent prognostic parameter; this fact might explain our remaining case with a lower percentage of deleted nuclei and cytogenetic clonal abnormalities. This further emphasizes the importance of performing conventional cytogenetics in addition to FISH.

In conclusion, we confirm that the detection rate of clonal chromosomal abnormalities is superior after CpG/IL-2 stimulation compared with TPA and LPS. The combination of the three culture techniques does not result in an increase of the detection rate or in the detection of different clonal abnormalities. Therefore, CpG/IL-2 should be preferred for routine conventional cytogenetic analysis of CLL. However,

the lack of detection of differences in clonal abnormalities among cultures using different mitogens as previously reported, could possibly be attributed to our small cohort, the low success rate of TPA and LPS cultures (although comparable with others), or to the biological heterogeneity of the response of CLL cells in our routine settings. Finally, neither conventional cytogenetics nor CLL-specific FISH detected all aberrations, further confirming the complementary nature of both techniques.

REFERENCES

1- Juliusson G, Oscier DG, Fitchett M, et al. Prognostic subgroups in B-cell chronic lymphocytic leukemia defined by specific chromosomal abnormalities. NEJM. 1990; 323: 720-724.

2- Döhner H, Stilgenbauer S, Benner A, et al. Genomic aberrations and survival in chronic lymphocytic leukemia. NEJM. 2000; 343: 1910-1916.

3- Mayr C, Speicher MR, Kofler DM, et al. Chromosomal translocations are associated with poor prognosis in chronic lymphocytic leukemia. Blood, 2006; 107:742-751.

4- Van Den Neste E, Robin V, Francart J, et al. Chromosomal translocations independently predict treatment failure, treatment-free survival and overall survival in B-cell chronic lymphocytic leukemia patients treated with cladribine. Leukemia. 2007; 21: 1715-1722.

5- Byrd JC, Gribben JG, Peterson BL, et al. Select high-risk genetic features predict earlier progression following chemoimmunotherapy with fludarabine and rituximab in chronic lymphocytic leukemia: Justification for risk-adapted therapy. J Clin Oncol. 2006; 24: 437-443.

6- Lin TS, Blum KA, Fischer DB, et al. Flavopiridol, fludarabine, and rituximab in mantle cell lymphoma and indolent B-cell lymphoproliferative disorders. J Clin Oncol. 2010; 28: 418-423.

7- Lozanski G, Heerema NA, Flinn IW, et al. Alemtuzumab is an effective therapy for chronic lymphocytic leukemia with p53 mutations and deletions. Blood, 2004; 103: 3278-3281.

8- Reddy KS. Chronic lymphocytic leukaemia profiled for prognosis using a fluorescence in situ hybridisation panel. Br J Haematol. 2006; 132: 705-722.

9- Stilgenbauer S, Dohner K, Bentz M, et al. Molecular cytogenetic analysis of B-cell chronic lymphocytic leukemia. Ann Hematol. 1998; 76: 101-110.

10- Buhmann R, Kurzeder C, Rehklau J, et al. CD40L stimulation enhances the ability of conventional metaphase cytogenetics to detect chromosome aberrations in B-cell chronic lymphocytic leukaemia cells. Br J Haematol. 2002; 118: 968-975.

11- Dicker F, Schnittger S, Haferlach T, et al. Immunostimulatory oligonucleotide-induced metaphase cytogenetics detect chromosomal aberrations in 80% of CLL patients: A study of 132 CLL cases with correlation to FISH, IgVH status, and CD38 expression. Blood. 2006; 108: 3152-3160.

12- Haferlach C, Dicker F, Schnittger S, et al. Comprehensive genetic characterization of CLL: A study on 506 cases analysed with chromosome banding analysis, interphase FISH, IgV(H) status and immunophenotyping. Leukemia. 2007; 21: 2442-2451.

13- Struski S, Gervais C, Helias C, et al. Stimulation of B-cell lymphoproliferations with CpG-oligonucleotide DSP30 plus IL-2 is more effective than with TPA to detect clonal abnormalities. Leukemia. 2009; 23: 617-619.

14- Put N, Konings P, Rack K, et al. Improved detection of chromosomal abnormalities in chronic lymphocytic leukemia by conventional cytogenetics using CpG oligonucleotide and interleukin-2 stimulation: A Belgian multicentric study. Genes Chromosomes Cancer. 2009; 48: 843-853.

15- Bauer S, Kirschning CJ, Hacker H, et al. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. Proc Natl Acad Sci U.S.A. 2001; 98: 9237-9242.

16- Ilvesaro JM, Merrell MA, Li L, et al. Toll-like receptor 9 mediates CpG oligonucleotide-induced cellular invasion. Mol Cancer Res. 2008; 6: 1534-1543.

17- Manzel L, Macfarlane DE. CpG-oligodeoxynucleotide-resistant variant of WEHI 231 cells. J Leukoc Biol. 1999; 66: 817-821.

18- Liang X, Moseman EA, Farrar MA, et al. Toll-like receptor 9 signaling by CpG-B oligodeoxynucleotides induces an apoptotic pathway in human chronic lymphocytic leukemia B cells. Blood. 2010; 115: 5041-5052.

19- Heerema NA, Byrd JC, Cin PSD, et al. Stimulation of chronic lymphocytic leukemia cells with CpG oligodeoxynucleotide gives consistant karyotypic results among laboratories: A CLL Research Consortium (CRC) Study. Cancer Genetics. 2010; 203: 134-140.

20- Muthusamy N, Breidenbach H, Andritsos L, et al. Enhanced detection of chromosomal abnormalities in chronic lymphocytic leukemia by conventional cytogenetics using CpG oligonucleotide in combination with pokeweed mitogen and phorbol myristate acetate. Cancer Genetics. 2011; 204: 77-83.

21- Muller-Hermelink HK, Montserrat E, Catovsky D, et al. Mature B-cell neoplasms. In: Swerdlow SH, Campo E, Harris NL, et al, editors. WHO classification of tumours of haematopoietic and lymphoid tissues. International agency for research on cancer: Lyon. 2008; p. 179-268.

22- Shaffer LG, Slovak ML, Campbell LJ, editors. ISCN 2009: an international system for human cytogenetic nomenclature. Basel: Karger. 2009.

23- Del Principe MI, Del Poeta G, Buccisano F, et al. Clinical significance of ZAP-70 protein expression in B-cell chronic lymphocytic leukemia. Blood. 2006; 108: 853-861.

24- Decker T, Schneller F, Kronschnabl M, et al. Immunostimulatory CpG-oligonucleotides induce functional high affinity IL-2 receptors on B-CLL cells: costimulation with IL-2 results in a highly immunogenic phenotype. Exp Hematol. 2000; 28: 558-568.

25- Decker T, Schneller F, Sparwasser T, et al. Immunostimulatory CpG-oligonucleotides cause proliferation, cytokine production, and an immunogenic phenotype in chronic lymphocytic leukemia B cells. Blood. 2000; 95: 999-1006.

26- Buhmann R, Nolte A, Westhaus D, et al. CD40-activated Bcell chronic lymphocytic leukemia cells for tumor immunotherapy: Stimulation of allogeneic versus autologous T cells generates different types of effector cells. Blood. 1999; 93: 1992-2002.

27- Birerdinc A, Nohelty E, Marakhonov A, et al. Pro-apoptotic and antiproliferative activity of human KCNRG, a putative tumor suppressor in 13q14 region. Tumour Biol. 2010; 31: 33-45.

28- Klein U, Lia M, Crespo M, et al. The DLEU2/miR-15a/16-1 cluster controls B cell proliferation and its deletion leads to chronic lymphocytic leukemia. Cance, Cell. 2010; 17: 28-40.

29- Mosca L, Fabris S, Lionetti M, et al. Integrative genomics analyses reveal molecularly distinct subgroups of B-cell chronic lymphocytic leukemia patients with 13q14 deletion. Clin Cancer Res. 2010; 16: 5641-5653.

30- Palamarchuk A, Efanov A, Nazaryan N, et al. 13q14 deletions in CLL involve cooperating tumor suppressors. Blood, 2010; 115: 3916-3922.

Journal of the Egyptian Society of Haematology & Research, Vol. 8, No. 2, September: 43-49, 2012

NOTCH1 Versus NOTCH3 in the Pathogenesis of T-Acute Lymphoblastic Leukemia

EMAN R. RADWAN, M.D.[1]; ELISABETTA FERRETTI, Ph.D.[2]; AZZA M. KAMEL, M.D.[3]; PAOLA GRAZIOLI, Ph.D.[1]; AGNESE PO, Ph.D.[2]; CLAUDIO TALORA, Ph.D.[2]; HEBA S. MOUSSA, M.D.[3]; SAULA CHECQUOLO, Ph.D.[2]; ANTONIO F. CAMPESE, M.D.[2]; AMIRA SOLIMAN, M.D.[3]; DINA A YASSIN, M.D.[3]; SAHAR K HUSSEIN, M.D.[1]; MERVAT M EL-ANSARY, M.D.[1] and ISABELLA SCREPANTI, M.D., Ph.D.[2,4]

The Department of Clinical and Chemical Pathology, Faculty of Medicine, Cairo University, Egypt[1], Experimental Medicine & Pathology, University 'La Sapienza', Rome, Italy[2], Clinical Pathology, National Cancer Institute, Cairo University, Egypt[3] and Institute Pasteur-Foundation Cenci Bolognetti, University 'La Sapienza', Rome, Italy[4]

ABSTRACT

Background: It has been established that Notch pathway plays a major role in the pathogenesis of T-ALL. The relative role of Notch1 versus Notch3 is still a controversial issue.

Objectives: The aim of this study was to evaluate the role of NOTCH1 and NOTCH3 in the pathogenesis of TALL. We also wanted to study the correlation of the expression of each of the two genes to various clinical and laboratory parameters.

Patients and Methods: We studied the expression of Notch1 and Notch3 in 46 T-cell acute lymphoblastic leukemia (T-ALL) patients (38 children/8 adults), in comparison with 12 cases of precursor B-ALL and 13 healthy adults, as control groups using Real Time PCR.

Results: The expression of both genes was increased in T-ALL, compared to precursor B-ALL and healthy subjects, and statistically higher for Notch3 (p=0.02) in children compared to adult T-ALL. Expression levels were higher in intermediate and late T-ALL group compared to early T-ALL for both genes (p=0.016 and 0.019) In T-ALL, a correlation was found between Notch1 and Notch3 (r=0.508/ p=0.0001). T-ALL and precursor B-ALL groups showed comparable Notch3/Notch1 relative expression ratios (p=0.312), however, it was significantly higher in comparison to healthy subjects, particularly for T-ALL (p=0.0001). The highest Notch3/Notch1 ratio was observed in T-ALL.

Conclusion: Our results confirm a pivotal role of Notch pathway in the pathogenesis of T-ALL. The higher Notch3/Notch1 ratio suggests that Notch3 dysregulation may play a more central role than Notch1 in T-ALL pathogenesis. This could open the field for Notch3 targeted therapy.

Key Words: *T-cell acute lymphoblastic leukemia – Notch1 – Notch3.*

INTRODUCTION

Acute Lymphoblastic Leukemia (ALL) is a heterogeneous disease with various subtypes that differ significantly in clinical outcome. Hence, there is a need to identify subtype-specific molecular markers, to improve our understanding of pathogenetic events and to facilitate risk assessment. T-cell ALL (T-ALL), which accounts for 10-15% of pediatric and 25% of adult ALL cases, includes several subtypes and these are thought to correlate with genetic aberrations at different stages of thymocyte differentiation **[1]**.

Members of the Notch protein family, have a role in cell-fate choice in several tissues, and control various steps of intrathymic T-cell development. Hence, dysregulated Notch signaling could be involved in the development of T-cell leukemia **[2]**.

Notch proteins are transmembrane receptors and are activated by ligand-mediated proteolysis, involving a series of mutually dependent cleavage events. This process releases the Notch intracellular domain (Notch-IC), which then translocates to the nucleus and forms a large transcriptional activation complex that includes proteins of the Mastermind family thus activating the transcription of target genes (e.g. The Hes family members) **[2]**.

Notch1 and Notch3 have been linked with distinct steps in the T-cell differentiation process that occurs inside the thymus **[3]**.

Regulation of the Notch-dependent T-cell developmental process appears to be affected in T-ALL. Notch3 was shown to be expressed in all the T-ALL patients examined, whereas its expression was dramatically reduced or absent in remission and in other types of ALL **[4]**. Notch1 was discovered as a partner gene in a t(7;9) chromosomal translocation resulting in <1% of T-ALLs **[5]**. Although the t(7;9) is rare, about 50% of human T-ALLs were noted to harbor activating point mutations in Notch1 that lead to aberrant activation of Notch signaling, placing the Notch1 pathway at the center of T-ALL pathogenesis **[6]**. However, other studies reported that Notch1 expression was not pathognomonic for T-ALL, because Notch1, but not Notch3 expression, was generally detected not only in normal peripheral blood T lymphocytes but also in non-T cell leukemias **[4]**. Some studies also reported that Notch1/Fbxw7 mutations are a good prognostic parameter in T-ALL with good overall survival and better outcome **[7,8]**. Alternatively, others reported the exact opposite **[9]**, while others reported that Notch1 mutations do not affect prognosis **[10]**.

Finally, Notch1 mutations have been reported to represent secondary events in both human and mouse T-ALL **[11,12]**.

Thus, the aim of this work was to study the expression of Notch1 and Notch3 and to verify the role of Notch1 versus Notch3 in the pathogenesis of T-ALL and to correlate the level of expression of both genes to other prognostic parameters.

PATIENTS AND METHODS

Patients and cell samples:

The study protocol was approved by the IRB of NCI and Faculty of Medicine, Cairo University. Under informed consent of the patients or their parents bone marrow (BM) or peripheral blood (PB) samples were taken from 46 newly diagnosed T-ALL patients (33 male and 13 female); 38 were children with a median age of 9 years (range: 18 months – <18.0 years), and 8 were adults with a median age of 27 years (range: 18-60 years). The study also included 12 cases of precursor B-ALL and 13 healthy adults as control groups. The choice of healthy adults as one of the control groups was based on ethical restrictions to use healthy children as control. Moreover, expression of the studied genes has not been reported to change with age in normal subjects.

Diagnosis of ALL was based on morphology and on cytochemical and immunophenotypic features. Immunophenotyping was performed on circulating leukemic blasts isolated using whole blood lysis technique, and cell-surface as well as intracytoplasmic antigens were detected by cytofluorimetric assay with a panel of monoclonal antibodies. The criteria for marker positivity and for the subclassification of T lineage ALL (early, intermediate, and late T-ALL) and non-T cell ALL (precursor B), were adopted as previously described (13). Mononuclear cells were isolated from BM and PB samples and stored at –80ºC until RNA extraction.

RNA extraction and cDNA preparation:

RNA was extracted from BM or PB samples of patients with newly diagnosed T-ALL, precursor B-ALL (both with >75% blast cells) and healthy subjects using the QIAamp RNA blood RNeasy Minikit (Qiagen, Hilden, Germany), according to manufacturers' instructions, further processed for RT-PCR, as previously described (4) and stored at –20ºC until use.

Evaluation of the tested genes:

Study of Notch1 and Notch3, genes' expression was carried out using Real-Time PCR (ABI Prism 7900 Sequence Detection System, Applied Biosystems) and TaqMan Universal PCR master mix (Applied Biosystems). Primer sequences are summarized in table (1). Gamma-secretase-resistant T-ALL cell lines with variable levels of expression of the studied genes were used as a reference for the methodology validation namely MOLT3, CCRF CEM, SKW3, JURKAT, HSB2, and LOUCY. The reaction was performed in 25µl mix containing 1X master mix, 1X assay on demand (AOD) mix, 900nM of each primer, 25ng cDNA and probe final concentration of 200nM. The data were analyzed using the relative standard method (relative fold change). For the tested genes, external standard curves were constructed using serial dilutions of known concentration templates (total thymocyte cD-

NA). The measured amount of the template from each gene was divided by the amount of cDNA from the housekeeping gene ß-actin measured in the same sample to normalize for possible variation in the amount and quality of cDNA between different samples **[14,15]**.

Statistical analysis:

SPSS package (version 15) was used for data management. Mean and standard deviation described quantitative data with median and range when appropriate (small number, no normal distribution). Parametric and non-parametric *t*-tests were used for comparing means of 2 independent groups and Kruskal Wallis ANOVA for comparing means for more than 2 independent groups. Parametric and non-parametric correlation analysis was done to elicit association between different genes and prognostic factors. *p*-value is significant at 0.05 level.

RESULTS

Clinical and laboratory characteristics of T-ALL patients' cohort:

Clinical data of T-ALL cohort showed 30% of cases with mediastinal mass, 13.5% with CNS involvement, 62.2% with hepatomegaly, 73% with splenomegaly and 66.7% with lymphadenopathy. Complete blood count showed 66.6% with haemoglobin level <10g/dl and 33.4% with a level ≥10g/dl, 16.2% with total leucocytic count (TLC) <10 x 10^9/L, 21.65% with TLC 10 - <50 x 10^9/L, 13.51% with TLC 50 - <100 x 10^9/L and 48.64% with TLC ≥100 x 10^9/L. BM or PB samples displayed a blast percentage with a range of 53-95% and 30-96% respectively. Immunophenotyping showed 20/46 cases to be early T-ALL (17 children and 3 adults), 20/46 intermediate T-ALL (17 children and 3 adults) and 6/46 late T-ALL (4 children and 2 adults).

Genes' expression of Notch family in T-ALL cases:

Forty six cases of newly diagnosed T-ALL were examined by Real-Time PCR compared to both precursor B-ALL and healthy subjects. Our results showed a significantly increased expression level of both Notch1 and Notch3 in T- ALL cases compared to the other 2 groups (Table 2). A moderate correlation was found between both genes (r=0.508, p=0.0001). Our study showed consistent results even when comparing between pediatric groups for both T-ALL and B-ALL cases (data not shown).

Genes' expression in T-ALL in relation to age:

In this work children showed increased gene expression of Notch1 and Notch3 compared to adults. However, the difference was found to be statistically significant only for Notch3 (Table 3).

Increased genes' expression in relation to T-cell differentiation:

Regarding the maturation stages of T-ALL our study examined the genes level in early, intermediate and late T-ALL. Due to the comparable level of gene's expression in both intermediate and late T-ALL, we considered them as one group as compared to early T-ALL. A statistically significant increase in gene expression in the intermediate and late T-ALL group was found for both genes compared to early T-ALL (Table 4).

Genes' expression in relation to other prognostic parameters in T-ALL:

In our study the level of Notch 1 and Notch3 genes' expression was compared in relation to prognostic parameters including age, hemoglobin level, TLC, mediastinal involvement, CNS involvement, hepatomegaly, splenomegaly, and lymphadenopathy. Significant results were found only for Notch1 expression in relation to hemoglobin level; Notch1 gene expression showed a higher level in the group with hemoglobin level <10g/dl compared to that with a level ≥10g/dl (p-value 0.049). Also a statistically significant moderate negative correlation was found between Notch1 expression and hemoglobin level (Fig. 1).

Notch3/Notch1 ratio among the studied groups:

Evaluation of Notch1 and Notch3 relative expression in the form of a ratio in T-ALL compared to both precursor B-ALL and normal control was done. Our results showed that Notch3/Notch1 ratio among T-ALL cases is comparable to that of precursor B-ALL cases (p-value 0.312), whereas both T-ALL and B-ALL cases showed a statistically significant higher ratio when compared to normal healthy subjects (p-value 0.0001 and 0.013 respectively) (Table 5).

Table (1): Reagents and primers for real time PCR: Applied biosystems.

Gene	Primer	Probe	Assay on demand*
Notch3	Fwd- ggatgagcttgggaaatcagc Rev- tccatttttgagcagggcc (cod. 4304971-80 nM) Rev- cacacctgtgggtagggctg (cod. 4304971-80nM)	ctgcggctgtgaaca (cod.4316033100uM)	
ßactin			Probe dye VIC-MGB 43263 15E-04 06005
Notch1			(cod. HS 00413187-M1 20x mix

* Assay on demand (AOD) sequence not provided.

Table (2): Genes' expression in T-ALL compared to control groups.

Gene	T-ALL (46 cases)	Precursor B-ALL (12 cases)	Healthy subjects (13 cases)	*p*1	*p*2	*p*3
Notch1	18.64±14.8* 0.34–62.53	7.25±9.53 0.15–34.63	1.0±0.6 0.28–2.24	0.019	0.0001	0.20
Notch3	3189.4±5089.02 0.204–21571.99	451.39±554.74 0.16–1877.60	1.0±0.98 0.16–3.86	0.044	0.0001	0.023

Mean±SD and range.
*p*1: *p*-value for T-ALL vs. B-ALL.
*p*2: *p*-value for T-ALL vs. Healthy subjects.
*p*3: *p*-value for B-ALL vs. Healthy subjects.

Table (3): Gene expression in T-ALL in relation to age.

Age group	No.	Notch1	Notch3
Pediatric	38	19.62±14.69*	3749.49±5430.78
Adult	8	13.96±15.39	528.97±895.96
p-value		0.21	0.02

* Mean±SD (Standard Deviation).

Table (4): Genes' expression in T-ALL in relation to immunophenotyping (IPT).

IPT	No.	Notch1	Notch3
Early	20	12.51±11.99* (0.349–33.98)	2077.30± 576.98 (0.20–11962.20)
Intermediate +late	26	22.95±15.25 (0.641–62.53)	3971.99±5867.37 (8.71–21571.99)
p-value		0.016	0.019

* Mean±SD

Table (5): Notch3/Notch1 ratio in T-ALL compared to control groups.

Group	Mean±SD	Range	Median
T-ALL	151.96±181.98	0.05–841.31	84.18
Precursor B-ALL	64.98±73.32	0.32–251.75	56.84
Normal Control	0.95±0.51	0.33–1.90	0.82

SD: Standard deviation.

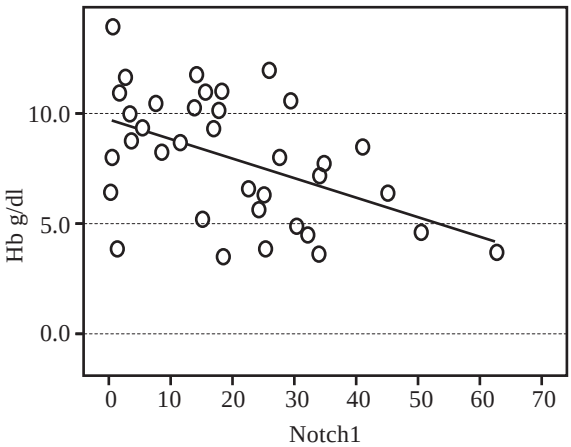


Fig. (1): Correlation between Hemoglobin level and Notch1 in 46 T-ALL cases: *r*-value:–0.476 *p*-value: 0.003.

DISCUSSION

Notch1 mutations are found in over 50% of T-ALL cases **[16,17]** and its role in the pathogenesis of T-ALL has been established. On the other hand, although no mutations were found in Notch3, its oncogenic role was supported in both mouse models and human T-ALL samples **[4]**. In this work we studied both genes to verify the role of Notch1 versus Notch3 in the pathogenesis of T-ALL.

Almost all studies concerning the role of Notch1 gene in T-ALL concentrated on studying the gene mutation rather than directly quantitating the gene expression as we did **[16-18]**. However, we propose that direct measurement of the gene expression would be more relevant as evidenced by the fact that Notch1 mutations were found only in 50% of cases **[16]**, whereas increased gene expression was reported in all T-ALL cases **[19]** as was encountered in the current study. The oncogenic role of Notch3 signaling was supported by mouse models in which enforced expression of Notch3-IC led to aggressive T cell leukemia reminiscent of human T-ALL **[4,20]**. Thus it could be postulated that the gene mutation is not the only factor affecting gene expression and that other mechanisms may influence Notch3 over-expression. Indeed, in the present work and, previously, some of us namely Bellavia et al. **[4]**, evidenced a significantly higher expression of Notch3 in all T-ALL cases examined with no evident mutations, compared to that of the controls. Notably, this expression level was significantly reduced or absent in cases that have undergone remission **[4]**.

In addition our study showed a significantly higher gene expression level for Notch3 in B-ALL cases compared to healthy controls and although there is a controversy regarding the role of activated Notch signaling in the development of B-cell malignancies **[19,22-24]**, the higher expression of this gene raises the possibility that Notch3 may be a contributing factor in the development of B-cell malignancies.

In our study children showed increased gene expression of Notch1 and Notch3 compared to adults; the difference was found to be statistically significant for Notch3. Up to our best knowledge, no other studies addressed this issue. We proposed separating our cohort in relation to age in view of the fact that in general ALL in children differs from adults in prognosis and response to therapy. The relevance of such findings to clinical outcome with children having better prognosis than adults needs further investigations.

With regards to the maturation stages of T-ALL, our results showed a statistically significant increase in gene expression in the intermediate and late T-ALL group, compared to early T-ALL for both genes. This suggests that the level of the genes is higher at both the double positive and the single positive stages in comparison with the double negative stage. Up to our best knowledge, no other studies addressed this issue using the same methodology on human subjects. However, other studies in murine models examining Notch1 mutations reported comparable results **[23,24-28]**.

In our study a correlation was found between both Notch1 and Notch3. A strong correlation between Notch3 and pTa was documented in both murine **[29]** and human T-ALL **[4]**; it was concluded that enforced expression of Notch3, which is ordinarily down-regulated as thymocytes mature, may sustain pre-TCR expression, causing dysregulated hyperplasia **[4]**. On the other hand, Chiaramonte and co-workers reported an increased level of Notch1 gene expression in T-ALL cases and identified pTa as a Notch1 pathway target gene **[19]**. Whether correlating with Notch3 or Notch1 all the previous studies show the strong interaction between the Notch pathway and pre-TCR signaling pathways documenting the role of both in T-cell ontogeny.

Our results showed a statistically significant negative correlation between Notch1 and Hb level. Up to our best knowledge no other studies addressed this issue. Notably Notch pathway is linked to early hematopoiesis during embryonic development **[30]**. Furthermore, it was reported that the Notch/RBPjk signaling pathway induces erythroid apoptosis in different hematopoietic tissues including yolk sac and bone marrow as well as in murine erythroleukemia cells **[31]**.

As one of the aims of our study was to evaluate the role of Notch1 versus that of Notch3 in the pathogenesis of T-ALL, we evaluated their relative expression in the form of a ratio in T-ALL compared to both precursor B-ALL and normal controls. Up to our best knowledge this issue was not addressed before. Our data suggest that dysregulation of the normal pathway of Notch genes occurs in ALL whether precursor B-ALL or T-ALL. The high Notch3/Notch1 ratio raises the possibility that Notch3 dysregulation and involvement in the pathogenesis of ALL may play a more central role than Notch1. Although, the relative ratio between Notch3 and Notch1 seems to be a character of

malignancy being comparable in precursor B-ALL and T-ALL yet the gene expression of both Notch3 and Notch1 is significantly higher in T-ALL cases. This could reflect the pivotal role of Notch pathway in the pathogenesis of T-ALL compared to its controversial role in B-ALL. Altogether, these data may question the hypothesis of some authors who reported that Notch3 could be a target of Notch1 **[32]** and has no real role alone in the pathogenesis of T-ALL, rather supporting the hypothesis of a prominent role of Notch3 in T-ALL development.

In conclusion, our study confirms a pivotal role of the Notch pathway in the pathogenesis of T-ALL. The higher Notch3/Notch1 ratio suggests that Notch3 dysregulation may play a more central role than Notch1, thus placing Notch3 as a major player in T-ALL pathogenesis. This could open the field for the new studies which identified monoclonal antibodies that specifically inhibit or induce activating proteolytic cleavages in Notch3 **[33]**. As was shown, intestinal epithelium cells express only Notch1 and Notch2 receptors, and Notch signaling plays an important role in homeostasis of intestinal progenitors **[34]**, thus one of the main drawbacks of using g-secretase inhibitors as a Notch signaling pathway inhibitor was the severe gut toxicity induced **[35]**. Consequently, it can be postulated that using monoclonal antibodies targeting only Notch3 could spare the gut these severe side effects. Thus proving Notch3 as a center stage player in the pathogenesis of T-ALL could revolutionize the ongoing targeted therapy into a new direction of targeting only a specific receptor instead of the whole pathway.

REFERENCES

1- Staudt LM. It is ALL in the diagnosis. Cancer Cell. 2002; 1: 109-110.

2- Screpanti I, Bellavia D, Campese AF, et al. Notch, a unifying target in T-cell acute lymphoblastic leukemia? Trends in Mol Med. 2003; 9: 30-35.

3- Rothenberg EV. Notchless T cell maturation? Nat. Immunol. 2001; 2: 189-190.

4- Bellavia D, Campese AF, Checquolo S, et al. Combined expression of pTalpha and Notch3 in T cell leukemia identifies the requirement of preTCR for leukemogenesis. Proc Natl Acad Sci USA. 2002; 99: 3788-3793.

5- Ellisen LW, Bird J, West DC, et al. TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. Cell. 1991; 66: 649-661.

6- Aster JC. Deregulated Notch signaling in acute T-cell lymphoblastic leukemia/lymphoma: New insights, Questions, and Opportunities. Int. J. of Hematol. 2005; 82: 295-301.

7- Malyukova A, Dohda T, von der Lehr N, et al. The tumor suppressor gene hCDC4 is frequently mutated in human T-cell acute lymphoblastic leukemia with functional consequences for Notch signaling. Cancer Res. 2007; 67: 5611-5616.

8- Asnafi V, Buzyn A, Le Noir S, et al. NOTCH1/FBXW7 mutation identifies a large subgroup with favorable outcome in adult T-cell acute lymphoblastic leukemia (T-ALL): A Group for Research on Adult Acute Lymphoblastic Leukemia (GRAALL) study. Blood. 2009; 113: 3918-3924.

9- Mansour MR, Sulis ML, Duke V, et al. Prognostic implications of NOTCH1 and FBXW7 mutations in adults with T-cell acute lymphoblastic leukemia treated on the MRC UKALLXII/ECOG E2993 protocol. J Clin Oncol. 2009; 27: 4352-4356.

10- van Grotel M, Meijerink JP, Beverloo H.B, et al. The outcome of molecular-cytogenetic subgroups in pediatric T-cell acute lymphoblastic leukemia: a retrospective study of patients treated according to DCOG or COALL protocols. Haematologica. 2006; 91: 1212-1221.

11- Mansour MR, Duke V, Foroni L, et al. Notch-1 mutations are secondary events in some patients with T-cell acute lymphoblastic leukemia. Clin Cancer Res. 2007; 13: 6964-6969.

12- O'Neil J, Calvo J, McKenna K, Krishnamoorthy V, Aster JC, Bassing CH et al. Activating Notch1 mutations in mouse models of T-ALL. Blood. 2006; 107: 781-785.

13- Kamel AM, Ghaleb FM, Assem MM, et al. Phenotypic analysis of T-cell acute lymphoblastic leukemia in Egypt. Leuk Res. 1990; 14: 601-609.

14- Nailis H, Coenye T, Van Nieuwerburgh F, et al. Development and evaluation of different normalization strategies for gene expression studies in Candida albicans biofilms by real-time PCR. BMC Mol Biol. 2006; 7: 25.

15- Nolan T, Hands RE, Bustin SA. Quantification of mRNA using real-time RT-PCR. Nat Protoc. 2006; 1: 1559-1582.

16. Weng AP, Ferrando AA, Lee W,et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science. 2004; 306: 269-271.

17- Malecki MJ, Sanchez-Irizarry C, Mitchell JL, et al. Leukemia-associated mutations within the NOTCH1 heterodimerization domain fall into at least two distinct mechanistic classes. Mol Cell Biol. 2006; 26: 4642-4651.

18- Sulis ML, Williams O, Palomero T, et al. NOTCH1 extracellular juxtamembrane expansion mutations in T-ALL. Blood. 2008; 112: 733-740.

19- Chiaramonte R, Basile A, Tassi E, et al. A wide role for NOTCH1 signaling in acute leukemia. Cancer Lett. 2005; 219: 113-120.

20- Zweidler-McKay PA, Pear WS. Notch and T cell malignancy. Semin Cancer Biol. 2004; 14: 329-340.

21- Hubmann R, Schwarzmeier JD, Shehata M, et al. Notch2 is involved in the overexpression of CD23 in B-cell chronic lymphocytic leukemia. Blood. 2002; 99: 3742-3747.

22- Jundt F, Pröbsting KS, Anagnostopoulos I, et al. Jagged1-induced Notch signaling drives proliferation of multiple myeloma cells. Blood. 2004; 103: 3511-3515.

23- Chiaramonte R, Calzavara E, Balordi F, et al. Differential regulation of Notch signal transduction in leukaemia and lymphoma cells in culture. J Cell Biochem. 2003; 88: 569-577.

24- Aster JC, Xu L, Karnell FG, et al. Essential roles for ankyrin repeat and transactivation domains in induction of T-cell leukemia by Notch1. Mol Cell Biol. 2000; 20: 7505-7515.

25- Pear WS, Aster JC, Scott ML, et al. Exclusive development of T cell neoplasms in mice transplanted with bone marrow expressing activated Notch alleles. J Exp Med. 1996; 183: 2283-2291.

26- Allman D, Karnell FG, Punt JA, et al. Separation of Notch1 promoted lineage commitment and expansion/transformation in developing T cells. J Exp Med. 2001; 194: 99-106.

27- Izon DJ, Punt JA, Xu L, et al. Notch1 regulates maturation of CD4+ and CD8+ thymocytes by modulating TCR signal strength. Immunity. 2001; 14: 253-264.

28- Ciofani M, Schmitt TM, Ciofani A, et al. Obligatory role for cooperative signaling by pre-TCR and Notch during thymocyte differentiation. J Immunol. 2004; 172: 5230-5239.

29- Bellavia D, Campese AF, Alesse E, et al. Constitutive activation of NF-kappaB and T-cell leukemia / lymphoma in Notch3 transgenic mice. EMBO J. 2000; 19: 3337-3

30- Kumano K, Chiba S, Kunisato A,et al. Notch1 but not Notch2 is essential for generating hematopoietic stem cells from endothelial cells. Immunity. 2003; 18: 699-711.

31- Robert-Moreno A, Espinosa L, Sanchez MJ, et al. The notch pathway positively regulates programmed cell death during erythroid differentiation. Leukemia. 2007; 21: 1496-1503.

32- Palomero T, Lim WK, Odom DT, et al. NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. Proc Natl Acad Sci USA. 2006; 103: 18261-18266.

33- Li K, Li Y, Wu W, et al. Modulation of Notch Signaling by Antibodies Specific for the Extracellular negative Regulatory region of NOTCH3. J Biol Chem. 2008; 283: 8046-8054.

34- Riccio O, van Gijn ME, Bezdek AC, et al. Loss of intestinal crypt progenitor cells owing to inactivation of both Notch1 and Notch2 is accompanied by derepression of CDK inhibitors p27Kip1 and p57Kip2. EMBO Rep. 2008; 9: 377-383.

35- Real PJ, Tosello V, Palomero T, et al. Gamma-secretase inhibitors reverse glucocorticoid resistance in T cell acute lymphoblastic leukemia. Nat Med. 2009; 15: 50-58.

Journal of the Egyptian Society of Haematology & Research, Vol. 8, No. 2, September: 51-56, 2012

Subclinical Cardiac Dysfunctions in ß-Thalassemia Patients

MUHAMAD R. ABD EL-HAMEED, M.D.; OSAMA A. IBRAHIEM, M.D. and
SHIMAA A. MAHMOUD, M.Sc.

The Departments of Internal Medicine, Clinical Hematology Unit, Faculty of Medicine, Assiut University Hospital, Assiut, Egypt

ABSTRACT

Background: Prediction of potential cardiac injury from iron overload in Thalassemia patients is necessary to assess the efficacy of the treatment regimes, particularly the chelation therapy and to propose any modification.

Aim: Early detection of preclinical markers of left and right ventricular dysfunction in patients with suspected myocardial iron overload especially in the absence of clinical signs of cardiac failure by echocardiography.

Patients and Methods: Thirty six Thalassemia patients were included in the study with another 36 matched healthy control persons. M-mode, two-dimensional echo, and echodoppler were performed to both.

Results: Pulmonary artery systolic pressure (PASP), Left atrial diameter (LAD), Right ventricular diameter (RVD), Left ventricular end-diastolic diameter (LVEDD), Left ventricular end-systolic diameter (LVESD) and Ejection fraction (EF) were significantly higher in patients than controls. Also, RVD, LVEDD and LVESD were lower in thalassemia major than in thalassemia intermedia patients. Moreover, hemoglobin level correlated negatively with EF and fraction shortening (FS) and also, Serum ferritin correlated negatively with LVESD and LVEDD.

Conclusion: Echocardiography remains a valuable tool in the cardiovascular function assessment. It is particularly suited to assess both systolic and diastolic functions and pulmonary pressure so it should be included in the follow-up scheme of thalassemic patients.

Key Words: *ß-Thalassemia – Systolic functions – Diastolic functions – Pulmonary pressure – Echocardiography.*

INTRODUCTION

The complications that occur with ß-thalassemia major or intermedia are related to over stimulation of the bone marrow, ineffective erythropoiesis, and iron overload from regular blood transfusions. Iron is deposited in visceral organs (mainly the heart, liver, and endocrine glands), and most patient deaths are caused by cardiac complications **[1,2,3]**.

In patients with various types of ß-Thalassemia, mortality and morbidity vary according to the severity of the disease and the quality of care provided. Severe cases of ß-Thalassemia major are fatal if not treated. Heart failure due to severe anemia or iron overload is a common cause of death in affected persons **[2,4-6]**. Iron overload interferes in the cardiomyocytes' capacity to catalyze the formation of deleterious oxygen free radicals. The quantification of myocardial iron content is not generally easy and only T2* Cardiovascular magnetic resonance (CMR) has allowed a reliable estimation in a large number of ß-Thalassemia major patients **[7]**.

Before the availability of iron chelation therapy, the majority of transfused ß-Thalassemia major patients died, usually in the second and third decade of life, from cardiac failure that was due to iron overload **[4]**. In Thalassemia intermedia the increased gastro-intestinal absorption of iron, which is much higher than that in normal individuals is most likely due to a paradoxical suppression of Hepcidin **[8]**.

The iron induced cardiac toxicity is often complicated by arrhythmias such as extra atrial and ventricular beats, paroxysmal atrial tachycardia, flutter or fibrillation. The high output state may also be related to the incidence of arrhythmias to a lesser extent. Life threatening ventricular tachycardia is rare and often associated with reduced LV function. Short runs of non specific ventricular tachycardia are quite common and are more common with elevated cardiac iron **[2,5]**.

In patients with high output state, the heart's systolic function index and ejection fraction

(EF) is expected to be higher than in normal subjects. Thus, for TM patients, it has been recommended that a normal LVEF should be above 60% **[9]** and the degree of cardiac output (CO) increase should be taken into account when assessing EF in each individual patient **[3,10]**.

PATIENTS AND METHODS

This study was conducted on 36 patients with thalassemia major and intermedia who were attending the Clinical Hematology Unit of Assiut University Hospital during the year 2010. The standard evaluation consisted of a thorough medical history and physical examination, electrocardiography, chest radiographs, and echocardiography. Thirty-six age- and sex-matched control subjects were randomly selected; they were non-smokers and had no evidence of anemia or liver, respiratory, or cardiovascular disease. Informed consents were taken from all subjects.

Complete M-mode, 2-dimensional, and Doppler (pulsed-wave, continuous wave, and color) echocardiography was performed at rest. Cardiac dimensions were measured according to the recommendations of the American Society of Echocardiography (ASE).

Statistical analysis:

Statistical analysis was performed using the SPSS 9.0 statistical software Package. Continuous variables were expressed as mean±SD. Categorical variables were expressed as numbers and percentages. *p*-value $p>0.05$ was considered statistically insignificant, $p<0.05$ was considered statistically significant, $p<0.001$ was considered statistically highly significant. Paired-samples and independent-samples student t tests were used to compare variables between patients and controls or between patient groups. Bivariate Pearson correlation was used to investigate potential relations between variables.

RESULTS

The clinical characteristics and demographic data of the patients and controls are represented in Table (1), while laboratory data as regards peripheral blood counts and some hepatic biochemical results are presented in Table (2). Electrocardiographic (ECG) findings of the studied patients are: Twenty patients (55.6%) had ECG abnormalities as left ventricular hypertrophy was detected in 15 patients (41.7% of total), left axis deviation (LAD) and LVH in two patients (5.6%), right axis deviation (RAD) with right bundle branch block (RBBB) in another 2 patients (5.6%) and P-pulmonale and RAD in one patient (2.8%), while 16 patients (44.4%) had normal ECG findings.

Table (1): Characteristics of 36 thalassemia patients and 36 controls.

Parameters	Patients	Controls	*p*-value
Gender:			
Male	25 (69.4%)	19 (52.8%)	
Female	11 (30.6%)	17 (47.2%)	
Age (years)	22±11	25±7	
Pattern:			
Thalssemia major	17 (47.2%)		
Thalassemia intermedia	19 (52.8%)		
Positive family history	14 (38.9%)		
Splenectomy	14 (38.9%)		
Positive hepatitis C virus	5 (13.9%)		
Frequent blood transfusion	27 (75%)		
Therapy:			
Deferoprone	13 (36.1%)		
Deferoxamine	8 (22.2%)		
No treatment	15 (41.7%)		
Serum ferritin	985.4±853.7	89.8±30	<0.001***

*=$p<0.05$, ***=$p<0.001$

Table (2): Peripheral hemogram and liver functions in thalassemic patients and controls.

Parameter	Patients (n=36)	Control (n=36)	Parameter	Patients (n=36)	Control (n=36)
Total Leucocytic Count (x10^9/L)	9.2±4.7***	6.3±2	Total Proteins (g/L)	73.3±6 *	76.6±5
Hemoglobin (g/dl)	7.7±1.7***	13±1	Albumin (g/L)	40±4.5*	42±2.6
Platelets Count (x10^9/L)	468±268***	271±102	Total bilirubin (umol/L)	47.6±32.7***	11±4
Reticulocytic Count %	8.0±6***	1±.6	Indirect bilirubin (umol/l)	33±29***	8±5
			Direct bilirubin (umol/l)	14.5± 10.4***	2.9±1.3
			AST (u/l)	29.1±29.6***	12±3.4
			ALT (u/l)	31.3±34.4***	12.6±3
			ALP (u/l)	91.3±68.0*	76±25

AST: Aspartase Transaminase. * : $p<0.05$.
ALT: Alanine Transaminase. *** : $p<0.001$
ALP: Alkaline Phosphatase.

The detected morphological changes by echocardiography in thalassemic patients was mitral valve incompetence in 9 patients (25%) 4 of them (11%) had mild form and 5 patients (14%) had trace incompetence, while tricusped valve incompetence was found in 14 patients (38.9%) 2 of them (5.6%) had moderate, 4 (11.1%) had mild and 8 patients (22.2%) had trace incompetence. Mitral valve prolapse was found in 2 patients (5.6%) pericardial effusion in one (2.8%) and hypokinesia was observed also in one patient (2.8%). Patients with pulmonary hypertension were 11 (30.6%); one patient had diastolic dysfunction (2.8%).

Comparison between thalassemic patients and their controls as regards conventional echocardiographic parameters are presented in Table (3), while Table (4) and Fig. (1) show echocardiographic parameters in relation to both serum ferritin and hemoglobin levels of the studied thalassemic patients.

Table (3): Echocardiographic parameters in 36 thalassemic patients and 36 controls

Parameters	Patients	Controls	*p*-value
PASP (mmHg)	28.7±10.4	16±2	<0.001***
RVD (cm)	1.6±0.5	1.24±0.4	<0.05*
LA D (cm)	3.1±0.6	2.8±0.2	<0.05*
LVEDD (cm)	5±0.7	4±0.4	<0.001***
LVESD (cm)	3±0.6	2.7±0.3	<0.001***
LVEF (%)	64.8±7	60.8±2.5	<0.05*
LVFS (%)	35.5±5.3	33.5±5	
PWD (cm)	0.9±0.16	0.8±.13	
IVSD (cm)	0.9±0.18	0.8±.13	

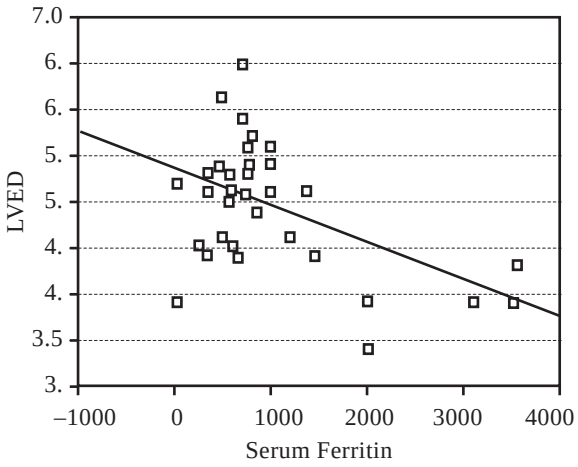
RVD : Right Ventricular Diameter.
LAD : Left Atrial Diameter.
LVEDD : Left Ventricular End Diastolic Diameter.
LVESD : Left Ventricular End Systolic Diameter.
LVEF : Left Ventricular Ejection Fraction.
LVFS. : Left Ventricular Fractional Shortening.
PWD : Diastolic Posterior Wall Thickness.
IVSD : Diastolic Interventricular Septal Thickness.
$p<0.05$: Significant*.
$p<0.001$: Highly Significant***.

Table (4): Echocardiographic parameters in correlation with serum ferritin and hemoglobin levels in 36 thalassemia patients.

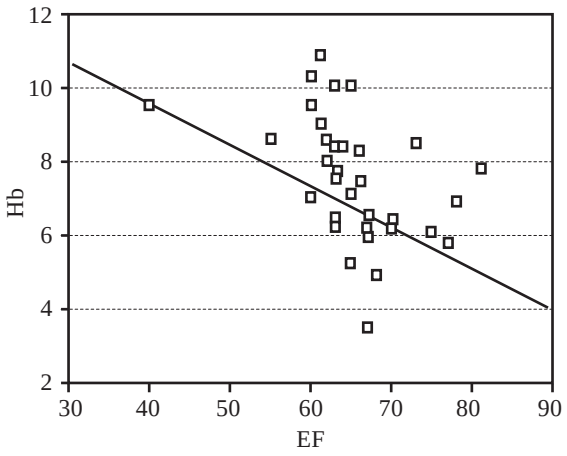
Parameters	Serum Ferritin		Hemoglobin level	
	r	*p*-value	*r*	*p*-value
PASP (mmHg)	0.091	NS	0.050	NS
RVD (cm)	–0.162	NS	0.050	NS
LA (cm)	–0.153	NS	0.080	NS
LVEDD (cm)	–0.494	<0.01*	–0.021	NS
LVESD (cm)	–0.368	<0.05*	0.188	NS
EF (%)	0.153	NS	–0.461	<0.01*
FS (%)	0.044	NS	–0.443	<0.01*
LVPWD (cm)	–0.065	NS	0.049	NS
IVSD (cm)	–0.142	NS	0.124	NS

RVD : Right ventricular diameter.
LAD : Left atrial diameter.
LVEDD : Left ventricular end diastolic diameter.
LVESD : Left ventricular end systolic diameter.
LVEF : Left ventricular ejection fraction.
LVFS : Left ventricular fractional shortening.
PWD : Diastolic posterior wall thickness.
IVSD : Diastolic interventricular septal thickness.
$p<0.05$: Significant*.
$p<0.001$: Highly significant***.
LAD : Left axis deviation.
LVH : Left ventricular hypertrophy.
RAD : Right axis deviation.
RBBB : Right bundle branch block.
PP : *p*-pulmonale

Fig. (1)



Negative correlation between serum ferritin and left ventricular end diastolic diameter (LVEDD) r=–0.494, p<0.01*.



Negative correlation between Hemoglobin (Hb) and ejection fraction (EF) r=–0.461, p=<0.01*.

DISCUSSION

Cardiac complications are still the most common cause of death in patients with thalassemia major. Iron overload causes severe and permanent cardiac damage even more than untreated anemia. Cardiac complications due to iron overload are recurrent pericarditis, heart block, ectopic ventricular beats, ventricular tachycardia, ventricular fibrillation, cardiomegaly, left ventricular (LV) dysfunction and finally heart failure resistant to any therapeutic measures **[2,3]**.

In the current study, pericarditis, was present in 2.8% of patients a figure which is less than that reported by Aessopos and his colleagues **[11]** as they stated that pericarditis was present in 8.1%; the difference may be explained by the larger sample of patients in their study. The pathogenesis of pericarditis was unclear; a likely cause was increased susceptibility to viral infection due to anemia, iron overload, and splenectomy in some patients.

As regards valvular lesions, the current study showed that valvular incompetence was recorded as trace, mild or moderate degrees. These results are in agreement with Aessopos and his colleagues **[11]**. The hyperkinetic state due to the high output, iron overloads, cardiac chamber dilatation and primarily elastic tissue abnormalities have been suggested as the responsible pathogenic mechanisms **[12]**. Although the hemodynamic consequences of these valvular abnormalities are not usually significant, they may have an additive effect when associated with other pathogenic mechanisms in the development of heart disease.

Pulmonary hypertension and right ventricular dysfunction are important components of cardiac dysfunction in *ß*-Thalassaemia. Right ventricular diameter (RVD) and pulmonary artery systolic pressure (PASP) were significantly higher in patients compared to controls with positive correlation between PASP and RVD. These results are concordant to the results obtained by both Aessopos and his colleagues **[11]** and Atichartakarn et al. **[13]**; they suggested that pulmonary hypertension is secondary to increased pulmonary vascular resistance due to a chronic low-grade hypercoagulable condition associated with thalassemia major. Pulmonary hypertension may be reversible with correction of anemia, iron chelation therapy, aspirin use and anticoagulation with warfarin. Blood transfusion to prevent pulmonary hypertension in thalassemia intermedia is currently a subject of much debate.

Also, in the current study, left ventricular end-diastolic diameter (LVEDD) and end-systolic diameter (LVESD) were significantly higher in patients with *ß*-thalassemia in relation to controls with negative correlation between LVEDD and patient's hemoglobin level. These results are coinciding with those of Aessopos et al. **[14]** and Ebru et al. **[15]**. Also, Left ventric-

ular ejection fraction (LVEF) showed statistically significant higher levels in ß-thalassemia patients than in controls with negative correlation with patient's hemoglobin levels and left ventricular fractional shortening (LVFS) which are similar to the findings of Stakos et al. **[15]**. In patients with high output state due to anemia, the heart's systolic function index and ejection fraction (EF) are expected to be higher than in normal subjects. Thus, for ß-thalassemia patients, even well transfused, it has been recommended that a normal LVEF should be above 60% **[9]** and the degree of cardiac output increase should be taken into account when assessing EF in each individual patient **[10]**. In those ß-thalassemia patients who are poorly transfused the increased cardiac output will be greater. On the other hand, Hahalis et al. **[17]** reported that their patients exhibited insignificant differences in LVEF in relation to controls, while Ebru et al. **[15]** reported that ejection fraction in their patients group was less than in control group that may be due to higher mean hemoglobin level (12±1.3 g/dl) in their ß-thalassemia patients which is not associated with increased cardiac output compared to our patients group who had lower mean hemoglobin level.

Thalassemia intermedia is a clinical definition applied to patients whose clinical phenotype is milder than that of thalassemia major. Criteria used to define thalassemia intermedia include age at presentation, hemoglobin or fetal hemoglobin levels and transfusion independence. Nevertheless, because of several factors that interact in the disease expression on molecular basis, the ß-genotype alone is not predictive of the phenotype in all cases. Although benign, the clinical course of thalassemia intermedia is characterized by several complications that can be prevented by an accurate follow-up **[18]**. In the current study, there is positive correlation between serum ferritin and PASP, EF and FS with negative correlation between serum ferritin level and left atrial diameter (LAD), right ventricular diameter (RVD), LVESD, diastolic interventricular septal thickness (IVSD) and LVEDD which are similar to that reported by Aessopos et al. **[14]**. These findings may be explained by that ß-thalassemia major patients are universally kept on an intensive transfusion regimen to maintain their hemoglobin level close to normal allowing an adequate tissue oxygen delivery. Patients with thalassemia intermedia, in contrast, remained without transfusions due to their less severe molecular defect, a fact that leads to a lower overall hemoglobin level. The resulting chronic hemolysis and ineffective erythropoiesis lead to chronic tissue hypoxia.

In conclusion: Prediction of potential cardiac injury from iron overload in thalassemia major patients is considered necessary in order to assess the efficacy of the treatment regimes, particularly the chelation therapy and to propose any modification. Echocardiography remains an indispensable tool in the cardiovascular assessment of patients, it provides many insights into cardiovascular function, and its use allows improved management of patients.

REFERENCES

1- Cunningham MJ, Macklin EA, Neufeld EJ, Cohen AR. Complications of beta-thalassemia major in North America. Blood. 2004; 104: 34-39.

2- Taher A, Ismaeel H, D Cappellini M. Chronic transfusion, iron overload and cardiac dysfunction: A multidimensional perspective. Br J Cardiol. 2008; 15: 40-45.

3- Walker MJ, Nair S. Detection of the cardiovascular complications of thalassemia by echocardiography, Ann N Y Acad Sci. 2010; 1202: 165-172.

4- Piga A, Gaglioti C, Fogliacco E, Tricta F. Comparative effects of deferiprone and deferoxamine on survival and cardiac disease in patients with thalassemia major: A retrospective analysis. Haematologica. 2003; 88: 489-496.

5- Lekawanvijit S, Chattipakorn N. Iron overload thalassemic cardiomyopathy: Iron status assessment and mechanisms of mechanical and electrical disturbance due to iron toxicity. Can J Cardiol. 2009; 25: 213-218.

6- Dimitrios T, Kremastinos D, Athanasios A, Hahalis G. ß-Thalassemia Cardiomyopathy History, Present Considerations, and Future Perspectives. Circ Heart Fail. 2010; 3: 451-458.

7- Anderson J, Holden S, Davis B. Cardiovascular T2* magnetic resonance for the early diagnosis of myocardial iron overload. Eur Heart J. 2001; 22: 2171-2179.

8- Nemeth E, Ganz T. Hepcidin and iron-loading anemias. Haematologica. 2006; 91: 727-732.

9- Pepe A, Positano V, Santarelli MF. Multislice multiecho T2* cardiovascular magnetic resonance for detection of the heterogeneous distribution of myocardial iron overload. J Magn Reson Imaging. 2006; 23: 662-668.

10- Aessopos A, Deftereos S, Tsironi M. Predictive echo-Doppler indices of left ventricular impairment in B-

thalassemic patients. Ann Hematol. 2007; 86: 429-434.

11- Aessopos A, Farmakis D, Karagiorga M. Cardiac involvement in thalassemia intermedia: A multicenter study. Blood. 2001; 97: 3411-3416.

12- Aessopos A, Deftereos S, Farmakis D. Cardiovascular adaptation to chronic anemia in the elderly: An echocardiographic study. Clin Invest Med. 2004; 27: 265-273.

13- Atichartakarn V, Likittanasombat K, Chuncharunee S. Pulmonary arterial hypertension in previously splenectomized patients with beta-thalassemic disorders. Int J Hematol. 2003; 78: 139-45.

14- Aessopos A, Farmakis D, Deftereos S. Thalassemia heart disease: A comparative evaluation of thalassemia major and thalassemia intermedia. Chest. 2005; 127: 1523-1530.

15- Ebru A, Dursun A, Tuncay H, Fatma G. The efficacy of tissue Doppler imaging in predicting myocardial iron load in patients with beta-thalassemia major: Correlation with T2* cardiovascular magnetic resonance, Int J Cardiovasc Imaging. 2010; 26: 413-421.

16- Stakos D, Margaritis D, Tziakas I, Kotsianidis G, Boudoula S. Cardiovascular involvement in patients with ß-thalassemia major without cardiac iron overload. International Journal of Cardiology. 2009; 134: 207-211.

17- Hahalis G, Alexopoulos D, Kremastinos D. Heart failure in beta thalassemia syndromes: A decade of progress. Am J Med. 2005; 118: 957-6.

18- Camaschella C, Cappellini M. Thalassemia Intermedia, molecular basis of disease. Haematologica. 1995; 80: 58-68.

Journal of the Egyptian Society of Haematology & Research, Vol. 8, No. 2, September: 57-61, 2012

A Randomized Comparison of Deferasirox Versus Deferoxamine for the Treatment of Transfusional Iron Overload in Patients with ß-Thalassemia Major in Upper Egypt

OSAMA A. IBRAHIEM, M.D. and AHMAD F. THABET, M.D.

The Departments of Internal Medicine, Clinical Hematology Unit, Faculty of Medicine, Assiut University, Assiut, Egypt

ABSTRACT

Background: Many patients with transfusional iron overload are at risk of progressive organ dysfunction and early death. Poor compliance with iron chelators is believed to be a major contributing factor.

Objective: The aim of this study is to evaluate the efficacy of deferasirox in comparison with deferoxamine for the treatment of transfusional iron overload in patients with ß-thalassemia major in Upper Egypt.

Patients and Methods: A prospective study was designed to evaluate once-daily deferasirox (Exjade) for 48 weeks in 44 patients diagnosed as ß-thalassaemia major ≥2 years old with iron overload that previously either had received no chelating agent or chelated with deferoxamine. Most patients began treatment with deferasirox 10mg/kg /day and may be increased to 30mg/kg/day. Serum ferritin level was assessed before and after beginning of deferasirox (Exjade) treatment at 3 months interval for 48 weeks.

Results: Adverse events, most commonly associated with deferasirox, were mild including transient nausea, vomiting, diarrhea, abdominal pain and skin rash. The mean serum ferritin level had significantly decreased in all ß-thalassaemia major patients with iron overload treated with deferasirox compared to those on deferoxamine.

Conclusion: Administration of Exjade therapy as an oral drug is considered to be preferable and more effective than the parentral iron chelating therapy in the Upper Egypt due to the poor patient compliance and poor practical regimen of parenteral infusions.

Key Words: *ß-thalassemia major – Iron overload – Deferasirox – Deferoxamine.*

INTRODUCTION

Chronic iron overload is a serious complication of the repeated blood transfusions that are necessary for the treatment of patients with blood disorders such as thalassemia, sickle cell disease (SCD), myelodysplastic syndromes (MDS) and various other rare anemias, including aplastic anemia (AA). Without chelation therapy, humans are unable to eliminate the iron released from the breakdown of transfused red blood cells and the excess iron is deposited as hemosiderin and ferritin in the liver, spleen, endocrine organs and myocardium, leading to organ failure, particularly of the liver, heart and endocrine glands **[1,2]**. Diverse manifestations of iron overload are commonly seen in regularly transfused children and adolescents with B-thalassemia. These may include growth impairment and delayed sexual maturation due to impaired pituitary function, diabetes mellitus due to damage to pancreatic islet cells, and cardiac complications later in life **[3]**.

Morbidity and mortality in regularly-transfused thalassemia patients are due primarily to the effects of iron overload rather than to the underlying disease, with over half of all deaths attributable to cardiac complications **[4]**. Following extensive clinical research in the management of iron overload, patients with thalassemia major receiving effective chelation therapy were found to have significant improvements in survival **[4]**. Iron chelators mobilize tissue iron by forming soluble, stable complexes that are then excreted in the feces and/or urine **[5]**.

Iron overload can be effectively managed by adequate chelation therapy as documented by experience with deferoxamine (Desferal®, DFO), which has been in clinical use for more than 40 years and is the current reference standard chelating agent **[6]**. The poor oral bioavailability and short plasma half-life of DFO necessitates parenteral administration and prolonged

infusions. The standard regimen to remove excess iron accumulated through regular transfusion is a subcutaneous (SC) infusion over 8-12 hours, on 3 to 7 days each week. This inconvenient schedule has a negative impact on compliance and eventually on long-term outcome, with some deaths being directly attributable to poor compliance with therapy **[7,8]**. Poor compliance to deferoxamine therapy is even more pronounced among adolescents **[9]**.

There is, therefore, a clear requirement for an effective, well-tolerated iron chelator with a less demanding mode of administration to ensure patient compliance to life-long chelation therapy in transfusion-dependent anemia.

Deferasirox (Exjade®, ICL670), an N- substituted bis-hydroxyphenyl-triazol was selected from more than 700 compounds as part of a rational drug development program **[10]**. Deferasirox represents a new class of tridentate iron chelators with a high specificity for iron **[11]**. In prior studies evaluating the efficacy and safety of deferasirox, dosing was based on baseline liver iron concentration (LIC) as assessed by either liver biopsy, superconducting quantum interference device (SQUID) or magnetic resonance imaging (MRI) **[12]**. Biopsies are uncomfortable for the patient, particularly the elderly, and can lead to complications such as bleeding and infection, especially in MDS or AA patients with hemostatic impairment **[13]**. The consistency of results obtained from studies measuring the accuracy of LIC by SQUID is generally poor, with the underestimation of SQUID-determined LIC compared with biopsy-determined LIC being a critical factor **[8]**. Measurement of LIC by MRI is not used routinely as it requires special software and expertise and is often unavailable or relatively expensive in many regions worldwide. Hence, serum ferritin concentration remains a convenient, less expensive and widely used way of assessing body iron and, when followed serially, is a suitable alternative marker of trends in body iron burden as significant correlations between changes in LIC and serum ferritin have been identified in various types of anemia **[14]**.

PATIENTS AND METHODS

Eligibility and enrolment procedures:

The patients were divided into two groups, group (I) included 15 patients (11 males and 4 females) (≥2 years old) with ß-thalassaemia major and transfusional iron overload who required ≥8 blood transfusions /year and had a serum ferritin level of ≥1000ng/mL with no iron chelating therapy before, while group (II) included 29 ß-thalassaemia major patients (19 males and 10 female) (≥2 years old) had a serum ferritin level of ≥1000ng/mL and treated with prior mono or combination therapy with deferoxamine and/or deferiprone but had experienced unacceptable toxicity to deferoxamine, had poor response despite proper compliance with deferoxamine, had documented non-compliance of taking <50% of prescribed deferoxamine doses in the previous year or if deferoxamine treatment was contraindicated.

All patients commenced deferasirox at a dose of 10mg/kg/day, the lowest dose of the therapeutic range reported in previous studies **[2]**. Deferasirox was administered once daily, 30 minutes before breakfast, and doses were dispersed in a glass of non-carbonated bottled water and ingested immediately.

Blood transfusions were regularly administered during the study period according to the patients' requirements with the aim of maintaining haemoglobin level ≥8g/dL.

Safety assessments: Laboratory assessments were performed at monthly intervals and included complete blood counts, serum gamma-glutaryl-transferase, total protein, urea and creatinin. Also, Iron parameters including total iron and serum ferritin were assessed at 3 months interval for 48 weeks during the study and the change was determined using the baseline and final ferritin levels.

Patients were excluded from the study if they had a mean alanine aminotransferase and/or serum creatinin above the upper limit of normal, significant proteinuria, uncontrolled hypertension, chronic hepatitis B or active hepatitis C receiving specific treatment and/or a history of nephrotic syndrome or any medical condition that may affect absorption, distribution, metabolism or excretion of deferasirox. Patients were also excluded if they had a history of noncompliance either with treatment or the protocol (e.g. patients who were considered potentially unreliable and/or not cooperative).

Statistical methods:

Data were analyzed and expressed as mean values±standard deviations (SD). SPSS version 16 program was used for data processing. One Way ANOVA was used in comparison of numerical data. A value of $p<0.05$ was considered to be statistically significant.

RESULTS

This study included 44 ß-thalassemia major patients with iron overload, 30 males and 14 females; their ages ranged from 2 to 15 with a mean of 6.9±4.1 and a median of 6 years. Adverse events, most commonly associated with deferasirox, were mild including transient nausea, vomiting, diarrhoea, abdominal pain and skin rash. The gastrointestinal adverse events that patients experienced with deferasirox were generally transient in nature and lasted about 1 week maximum. They are all transfusion dependant with rate of transfusion ranging from 6 to 25 times per year (13.5±4.4). Six patients from the 44 patients were splenectomized (13.6%), 10 patients were Hepatitis C antibodies positive (22.8%) one patient was Hepatitis B positive (2.27%) and two patients were positive for both C and B viruses (4.54%).

Prior to deferasirox therapy, the patients were divided into two groups, group (I) did not receive any form of iron chelation before while group (II) were on iron chelatores deferoxamine and/or deferiprone; their demographic data are summarized in Table (1). Follow-up serum ferritin level was measured every three months after Exjade therapy, in which there was a significant decrease of the mean serum ferritin level after (3,6,9,12) months of initiation of therapy when compared to that before Exjade therapy in both groups (Table 2, Figs. 1,2).

Table (1): Demographic data of 44 ß-thalassemia major patients prior to Exjade therapy.

Variable	Group (I) n=15	Group (II) n=29
Age in years	2-4 (3±.6547)	2-4 (3±.6547)
Gender:		
Male	11(25%)	11(25%)
Female	4 (9%)	4 (9%)
Packed RBCs Transfusion per year	6-15 (10.7±3.2)	6 -15 (10.7±3.2)
Splenectomy	0	0
Base line serum ferritin ng/ml	1646.67±528.97	1646.67±528.97

Group (I) : Did not receive any form of iron chelation before.
Group (II): Were on iron chelatores deferoxamine and/or deferiprone.

Table (2): Base line serum ferritin level (ng/mL) before and at 3,6,9 and 12 months after Exjade therapy in 44 ß-thalassemia major patients.

Variable	Serum Ferittin				
	Base line	3 months	6 months	9 months	12 months
Group I: (No: 15)	1646.66±528.97*	1603.33±537.68	1486.67±479.38	1360±418.84	1176±457
p-value**		0.01	0.001	0.001	0.001
Group II: (No: 29)	2131.03±374.26	2044.83±365.1	1858.62±338.60	1675.86±336.63	1541.38±328.98
p-value**		0.001	0.001	0.001	0.001

* Mean ± standard deviation.
***p*-value: Serum ferritin levels after vs. before Exjade therapy.
Group (I) : Did not receive any form of iron chelation before
Group (II): Were on iron chelatores deferoxamine and/or deferiprone.

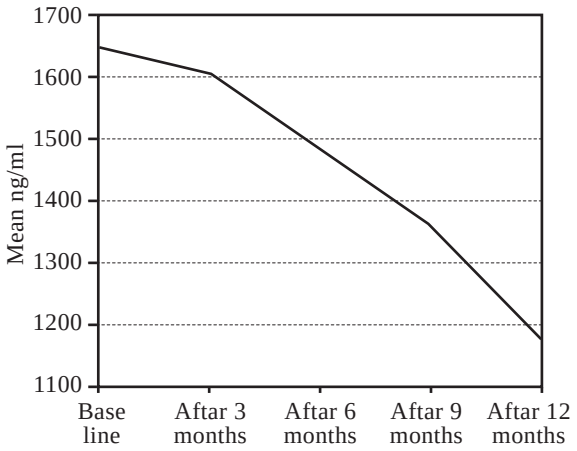


Fig. (1): Serum ferritin level before and after. Exjade therapy in Group (I) patients.
(Did not receive any form of iron chelation before)

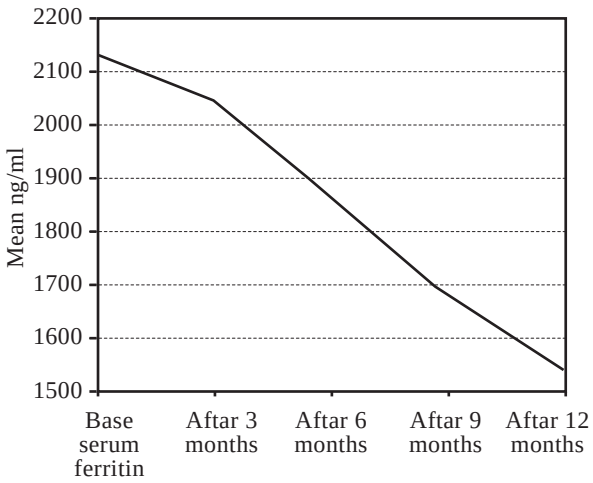


Fig. (2): Serum ferritin level before and after. Exjade therapy in Group (II) patients.
(Were on iron chelation deferoxamine and/or deferiprone)

DISCUSSION

Chronic iron overload due to blood transfusions leads to significant morbidity and early mortality unless adequate chelation therapy is administered. Deferoxamine is the reference chelation therapy that has a well-established safety and efficacy profile. Patients who are treated adequately with deferoxamine from early on in life do not develop typical complications of iron overload, including cardiac, endocrine, and hepatic failure **[15]**. However, because deferoxamine must be administered by prolonged subcutaneous or intravenous infusion, patient acceptance of and compliance with therapy are often poor. So, despite the availability of an effective chelating agent, the compliance issues with deferoxamine mean that many patients still develop clinically significant iron overload, with the related impact on morbidity and mortality.

In prior studies evaluating the efficacy and safety of deferasirox, dosing was based on baseline liver iron concentration (LIC) as assessed by liver biopsy **[12]**. Biopsies are uncomfortable for the patient, and can lead to complications such as bleeding and infection **[13]**. The measurement of LIC by MRI is not used routinely as it requires special software and expertise and is often unavailable or relatively expensive. Hence, serum ferritin concentration remains a convenient, less expensive and widely used way of assessing body iron and, when followed serially, is a suitable alternative marker of trends in body iron burden as significant correlations between changes in LIC and serum ferritin have been identified **[14]**. These findings support the use of regular serum ferritin assessments for the monitoring of deferasirox therapy **[16]**. In the current study, we used serial serum ferritin levels to assess body iron level in thalassemic patients.

Our results are in agreement with Cappellini et al. **[14]** who stated that the compliance with the administration of parenteral deferoxamine chelation therapy has proved challenging to all groups of patients with transfusional iron overload.

Deferasirox was developed in response to the need for an oral iron-chelating agent. In particular, it was desirable to have an agent that could be administered conveniently to patients of all ages, and across a range of iron burdens. Previous clinical studies indicated the potential of deferasirox to meet this need **[17]**. This current study was performed to compare this agent to deferoxamine. Because complications of chronic iron overload have been best studied in thalassemia, this population of patients was used for the demonstration of efficacy for deferasirox.

A significant decrease in serum ferritin levels was observed in our study after the usage of deferasirox; these results are consistent with the studies of Nisbet et al. **[18]** and Cappellini et al. **[14]** in their previously published short-term study examining the ability of deferasirox to remove iron from the body.

The same finding was observed in Porter **[1]** study who reported that the effective adminis-

tration of iron chelation therapy has been limited by the route of its administration. Although deferoxamine is effective in removing iron from the body, yet due to very poor oral bioavailability and a short half-life it must be administered by subcutaneous or intravenous infusion; the compliance with this regimen is often poor. Also Treadwell et al. **[19]** had reported that the availability of a once-daily, oral alternative deferasirox would potentially facilitate improved compliance, and thereby reduce morbidity and mortality from iron overload.

Also our results are in agreement with Elliott et al. **[20]** and Stumpf **[21]** as they reported that in routine clinical practice, compliance with a once-daily, oral regimen offers a promising alternative for patients unwilling or unable to comply with parentral deferoxamine therapy.

Conclusion:

In conclusion, these data provide evidence that patients with ß-thalassaemia major with iron overload may be effectively managed using deferasirox regimen and considered to be preferable and effective than the parenteral iron chelating therapy due to the poor patient compliance and poor practical regimen of parenteral infusions especially at our locality.

REFERENCES

1- Porter JB. Practical management of iron overload. Br J Haematol. 2001; 115 (2): 239-52.

2- Piga A, Galanello R., Luca Forni G., Cappellini MD., Origa R., Zappu A, Donato G. Randomized phase II trial of deferasirox (Exjade®, ICL670), a once-daily, orally-administered iron chelator, in comparison to deferoxamine in thalassemia patients with transfusional iron overload. Haematologica. 2006; 91(7): 873-80.

3- Britton RS, Leicester KL, Bacon BR.: Iron toxicity and chelation therapy. Int J Hematol. 2002; 76: 219-28.

4- Borgna-Pignatti C, Rugolotto S, De Stefano P, Zhao H, Cappellini MD, Del Vecchio GC. Survival and complications in patients with thalassemia major treated with transfusion and deferoxamine. Haematologica. 2004; 89 (10): 1187-93.

5- Olivieri NF, Brittenham GM. Iron-chelating therapy and the treatment of thalassemia. Blood. 1997; 89: 739-61.

6- Davis BA, Porter JB. Results of long term iron chelation treatment with deferoxamine. Adv Exp Med Biol. 2002; 509: 91-125.

7- Modell B, Khan M, Darlison M. Survival in ß-thalassaemia major in the UK. Data from the UK Thalassaemia Register. Lancet. 2000; 355: 2051-2.

8- Piga A, Fischer R, St Pierre T, Longo F, Fung E, Engelhardt R. Comparison of LIC obtained from biopsy, BLS and R2-MRI in iron overloaded patients with beta-thalassemia, treated with deferasirox (Exjade®, ICL670). Blood. 2005; 106 (11): Abst 2689.

9- Gabutti V and Piga A: Results of long-term iron-chelating therapy. Acta Haematol. 1996; 95: 26-36.

10- Heinz U, Hegetschweiler K, Acklin P, Faller B, Lattmann R, Schnebli HP.: 4-[3,5- Bis (2-hydroxyphenyl)-1,2,4-triazol-1-yl]- benzoic acid: A novel, efficient and selective iron (iii) complexing agent. Angew Chem Int Ed Engl. 1999; 38: 2568-70.

11- Nick H, Acklin P, Lattmann R, Buehlmayer P, Hauffe S, Schupp J. Development of tridentate iron chelators: From desferrithiocin to ICL670. Curr Med Chem. 2003; 10: 1065-76.

12- Porter J, Galanello R, Saglio G, Neufeld EJ,Vichinsky E, Cappellini MD. Relative response of patients with myelodysplastic syndromes and other transfusion-dependent anaemias to deferasirox (ICL670): A 1-yr prospective study. Eur J Haematol. 2008; 80 (2): 168-76.

13- Valent P, Krieger O, Stauder R, Wimazal F, Nösslinger T, Sperr WR. Iron overload in myelodysplastic syndromes (MDS) -diagnosis, management, and response criteria: A proposal of the Austrian MDS platform. Eur J Clin Invest. 2008; 38 (3): 143-9.

14- Cappellini MD, Cohen A, Piga A, Bejaoui M, Perrotta S, Agaoglu L. A phase 3 study of deferasirox (ICL670), a once-daily oral iron che lator, in patients with ß-thalassemia. Blood. 2006; 107 (9): 3455-62.

15- Aessopos A, Farmakis D, and Hatziliami A.: Cardiac status in well-treated patients with thalassemia major. Eur J Haematol. 2004; 73: 359-366.

16- John P, Renzo G, Giuseppe, Ellis J, Elliott V. Relative response of patients with myelodysplastic syndromes and other transfusion-dependent anaemias to deferasirox (ICL670): A 1-yr prospective study. European Journal of Haematology. 2007. 902: 4441-5.

17- Cohen AR, Galanello R, Piga A.: Safety and effectiveness of long-term therapy with the oral iron chelator deferiprone. Blood. 2003; 102:1583-87.

18- Nisbet-Brown E, Olivieri NF, Giardina PJ, et al. Effectiveness and safety of ICL670 in iron-loaded patients with thalassemia: A randomised, double-blind,placebo-controlled, dose-escalation trial. Lancet. 2003; 361: 1597-1602.

19- Treadwell MJ, Law AW, Sung J, Hackney-Stephens, E, Quirolo K, Murray E, Glendenning GA,Vichinsky E. Barriers to adherence of deferoxamine usage in sickle cell disease. Pediatric Blood and Cancer. 2005; 44, 500-7.

20- Elliott V, Onyinye O, Peter L. A randomised comparison of deferasirox versus deferoxamine for the treatment of transfusional iron overload in sickle cell disease. British Journal of Haematology. 2006; 136, 501-8.

21- Stumpf JL. Defrasirox. Am J Health Syst Pharm. 2007; 15; 64 (6): 606-16.

Journal of the Egyptian Society of Haematology & Research, Vol. 8, No. 2, September: 63-71, 2012

MDM2, P53 and P21 Gene Polymorphisms in Acute Myeloid Leukemia

BASMA M. ELGAMAL, M.D.

The Department of Clinical Pathology, National Cancer Institute, Cairo University

ABSTRACT

Background: P53 is a tumor suppressor gene that initiates apoptosis in response to severe DNA damage. MDM2 is a major negative regulator of P53. P21 is a cell cycle checkpoint gene functioning as a downstream effector of p53. A single nucleotide polymorphism (SNP) in the promoter of MDM2 gene, SNP309 can increase the expression level of MDM2, thereby causing an impairment of p53 tumor suppressor activity. A G-C exchange at p53 codon 72 polymorphism alters the primary structure of the p53 protein. Both polymorphisms have been implicated in cancer. As regards p21, a nonsynonymous polymorphism of Ser31Arg was shown to be associated with increased risk of cancer.

Aim of the Study: The aim of this work was to study the frequency of p53, p21, and MDM2 polymorphisms among AML patients and in apparently normal healthy controls to describe the prevalence of such mutations and to detect whether or not they have an implication on the development of AML.

Results: A significant association was found between p21 polymorphism and acute myeloid leukemia, while borderline significance was found with MDM2 and no association with p53 polymorphisms. When the MDM2, p53 and p21 polymorphisms were combined, no multiplicative joint effect concerning the risk of development of AML could be found, except for MDM2 and p21, where a borderline significance existed between the frequencies of the mutant versus the wild types, denoting a possible increased risk for the development of AML with the presence of those two mutations together.

Conclusion: In conclusion, it is suggested that the p21 Ser31Arg polymorphism may be a genetic susceptibility factor in the pathogenesis of AML. Future studies are recommended to investigate the biological role of P21 gene polymorphism in AML and to exploit its role in prognosis and therapy.

Key Words: *Acute myeloid leukemia – Gene polymorphism – MDM2 – P53 – P21.*

INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous disease with numerous genetic abnormalities. In fact, genetic abnormalities are present in more than 90% of AML, and a majority of these genetic abnormalities are recurrent. Most acute myeloid leukemias appear to be the consequence of a combination between gene mutations and gene rearrangements that confer a proliferative and/or survival advantage to hematopoietic differentiation and subsequent apoptosis of cells, resulting in the accumulation of primitive cells and the development of leukemia **[1]**.

P53 is a tumor suppressor gene that initiates apoptosis in response to severe DNA damage. P21 (CDKN1A, Waf1) is a cell cycle checkpoint gene functioning as downstream effectors of p53 and acts as an inhibitor of cyclin-dependent kinase. In response to DNA damage, cell cycle arrest at the G1 to S phase is caused by p21 through p53 up-regulation **[2]**.

MDM2 is a crucial negative regulator of p53 through several mechanisms. MDM2 directly binds to p53, resulting in the inhibition of p53 transactivation activity **[3]**. MDM2 also acts as an ubiquitin protein ligase and controls p53 by targeting it for proteasomal degradation. Therefore, overexpression of MDM2 leads to the increased degradation of p53 and down-regulates its tumor suppressor function **[4]**.

The importance of MDM2 over expression, p53 and p21 inactivation in acute Myeloid Leukemia (AML) remains controversial, al-

though a few studies have suggested that p53 and MDM2 up-regulation affect the risk of AML. It is suggested that the MDM2 and TP53 variants interact to modulate responses to genotoxic therapy and are determinants of risk for t-AML. Recently, Xiong et al. **[5]** suggested that the MDM2 SNP309 homozygous GG genotype may be a genetic susceptibility factor in AML.

The p53 pathway is an important response to oncogenic stress, and p53 regulates its own intracellular levels through an autoregulatory feedback pathway with MDM2 **[6]**. MDM2 binds to p53 and inactivates it through ubiquitination. MDM2 is a proto-oncogene and loss of p53 function is caused by MDM2 overexpression, mutations, and other mechanisms, resulting in malignant transformation or carcinogenesis **[7]**. In the p53 pathway, p53, p21, and MDM2 play a crucial role together. Polymorphisms in p53-MDM2 **[8]** and p53-p21 **[9]** have been reported to be associated with other cancers, such as lung, esophageal, colorectal, breast, and gastric cancer. Based on this evidence, we investigated whether these gene polymorphisms and their gene-gene interaction may be important in AML. In this study for each polymorphism, a significant association with AML was observed only for the p21 polymorphism, although additional polymorphisms in the other genes have been linked to susceptibility for other cancers.

In this work we analyzed the frequency of p53, p21, and MDM2 polymorphisms among AML patients referring to the National Cancer Institute (NCI) and in apparently normal healthy controls aiming to describe the prevalence of such mutations.

MATERIAL AND METHODS

This study included 77 newly diagnosed AML patients who presented to the Medical and Pediatric Oncology Departments, NCI, Cairo University in the period between April 2010 and October 2011. Diagnosis was performed according to clinical, morphological, cytochemical and immunophentypic examination. The criteria for inclusion in this group were:

1- Egyptian origin residing in Egypt as judged by their names, language and places of birth.

2- Availability of biological material.

The recruited patients comprised 43 males and 34 females between the ages of 4 and 83 years with a median of 35 years.

A general population control group composed of 72 individuals comprising 53 males and 19 females was randomly selected from blood donors. The criteria for inclusion in the control group were:

1- Anonymous, healthy, and unrelated individuals.

2- Egyptian origin residing in Egypt as judged by their language and place of birth. Informed consent was obtained from all participants involved in the study or their parents.

The study was performed according to Helsinki declaration and theNCI IRB approved the study.

Cases were subjected to the following routine investigation:

1- Thorough history taking.

2- Full clinical examination, particularly for hepatomegaly, splenomegaly and lymphadenopathy.

3- Complete blood picture.

4- Bone marrow aspiration and morphological examination using Romanowsky stain, supplemented with cytochemical stains such as Myeloperoxidase (MPO), Sudan Black B Stain (SBB), Esterases and Acid Phosphatase when indicated.

5- Immunophenotyping by Flow cytometry: To confirm the diagnosis of AML with a wide panel of myeloid markers (MPO, CD13, CD33, CD117, CD14 and CD15), lymphoid markers (CD10, CD19, CD22, CD79a, CD20, Cyto μ, Kappa and Lambda for B lymphoid series, and CD3, CD2, CD4, CD8, CD7 and CD5 for T lymphoid series) and the stem cell marker CD34 as well as CD56 and HLADR on routine basis.

Genotyping:

DNA was isolated from peripheral blood and its concentration was measured as described by Gupta et al. **[9]**.

Genotyping for all studied loci was performed by PCR-RFLP method. Primers sequences, restriction enzymes and fragments obtained are presented in Table (1).

MDM2 T309G Genotyping:

The MDM2 T309G polymorphism was determined by using the method described by Hirata et al. **[10]**. Each PCR assay was performed using 100ng of genomic DNA, 0.2µM of each primer (Table 1), 1U of Hot Start Taq DNA polymerase (Quiagen), 200µM dNTP, 1.5mM MgC l2, 10mM Tris-HCl (pH 8.4), and 50mM KCl. After initial denaturation for 10 minutes at 95°C, the PCR was performed for 35 cycles of 45 seconds at 95°C, 45 seconds at 59°C, and 1 minute at 72°C. The last elongation step was extended to 7 minutes. The amplified fragments targeted the site of polymorphism: The 158-bp fragment for MDM2 T309G contained the T → G bp substitution at nucleotide 309 that creates a MspA1I restriction site. The digestion products were visualized with ethidium bromide after electrophoresis on 3.5% agarose gel at 100 volts for 30min. The MDM2 309TT wild type homozygous was identified by the presence of only a 158bp fragment. 309TG heterozygous was identified by 158, 112, and 46 bp fragments, and 309GG homozygous variant was identified by 112 and 46bp fragments (Fig. 1).

P53 Codon arg72pro Polymorphism:

PCR was performed in 25µL containing 100ng of genomic DNA, 0.5µmol/L of primers (Table 1), 200µmol/L dNTPs, 10mmol/L Tris-HCl (pH 8.3), 2.5mmol/L MgCl2, 50mmol/L KCl, and 1U of Hot Start Taq DNA polymerase (Quiagen). After initial denaturation for 10 minutes at 95°C, the PCR was performed for 35 cycles of 45 seconds at 95°C, 45 seconds at 58°C, and 1 minute at 72°C. The last elongation step was extended to 7 minutes. The Arg → Pro substitution abolishes a restriction site on digestion with BstUI restriction enzyme (10 U). The resulting restricted fragments were evaluated on a 3.5% agarose gel at 100 volt for 30 min **[7]**. showing 113bp and 86bp bands for the wild type and 199bp, 113bp and 86bp bands for heterozygous variant and the homozygous variant remains undigested showing 199bp (Fig. 2).

P21 codon 31 Ser/arg Polymorphism:

P21 codon 31 Ser/arg Polymorphism was characterized by the PCR-RFLP **[7]**. DNA fragment of 225bp was amplified in 25 µL containing 100ng of genomic DNA, 0.5µmol/L of primers (Table 1), 200µmol/L dNTPs, 10mmol/L Tris-HCl (pH 8.3), 2.5mmol/L MgCl2, 50mmol/L KCl, and 1 U of Hot Start Taq DNA polymerase (Quiagen). After denaturation for 10 minutes at 95°C, the PCR was performed for 35 cycles of 1 minute at 95°C, 1 minute at 58°C, and 2 minutes at 72°C. The last elongation step was extended to 7 minutes. The presence of polymorphic variant arg results in abolishing the restriction site of Blp I enzyme. The PCR product (5 to 10µL) was digested with Blp I (10U, 37°C), and subjected to electrophoresis on a 2.5% agarose gel at 100 volt for 30 min. The wild type (ser/ser) resulted in two smaller fragments (122 and 103bp) (Fig. 3) while the heterozygous variant resulted in 225,122,103bp and the homozygous variant prevents digestion resulting in only 225bp band.

Table (1): Primer sequence and PCR, PCR/RFLP fragment size.

Gene	Primer	Primer sequences	Enzyme	Fragment sizes
MDM2 SNP309	Forward	5'- CGCGGGAGTTCA GGG TAAG-3'	MspA1I (10 U)	158 bp (TT) wild 158,112,46 bp (TG) heterozygous
	Reverse	5-CTGAGTCAACCTG CC C A CTG-3'		112,46 bp (GG) homozygous
P53 codon 72	Forward	5'-TTGCCGTCCCAA GCAAT GGATGA-3'	BstUI (10U)	113,86 bp (GG) wild 199,113,86 bp (GC) heterozygous
	Reverse	5'-TCTGGGAAGGG ACAGA AGATGAC-3'		199 bp (CC) homozygous
P21 codon 31	Forward	5'ACCAGGGCCTTCC TTGT ATC-3'	BlpI (10U)	122,103 bp (ser/ser) wild 225,122,103 bp (ser/arg) heterozygous
	Reverse	5'-GTCACCCTCCAG TGGTG TCT-3'		225 bp (arg/arg) homozygous

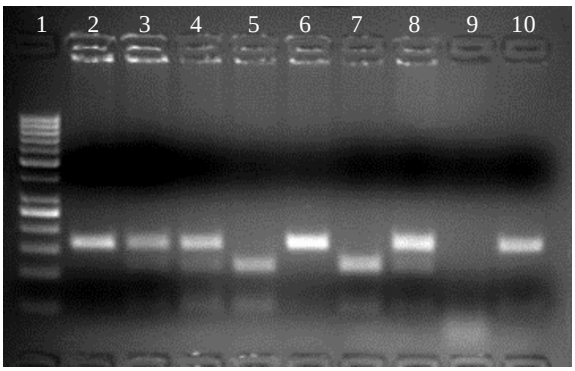


Fig. (1): MDM2 T309G polymorphism after MspA1I digestion.

Lane 1: 50 bp ladder

Lanes 2, 6, 10: Wild type (TT) showing one band at 158 bp

Lanes 3, 4, 8: Heterozygous variant (TG) showing bands at 158,112,46 bp

Lanes 5, 7: Homozygous variant (GG) showing bands at 112,46bp

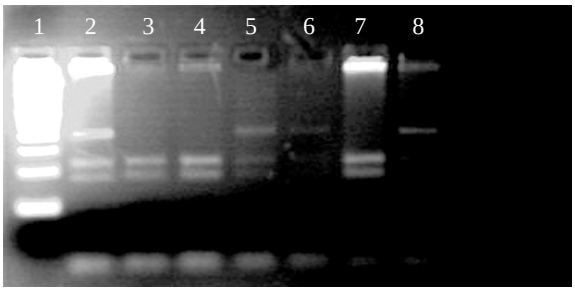


Fig. (2): P 53 arg72Pro polymorpsim after BstUI digestion.

Lane 1: 50 bp ladder

Lanes 2, 5, 8: Heterozygous (Arg/Pro) showing bands at 199,113,86 bp

Lanes 3, 4, 6, 7: Wild type (Arg/Arg) showing bands at 113, 86bp

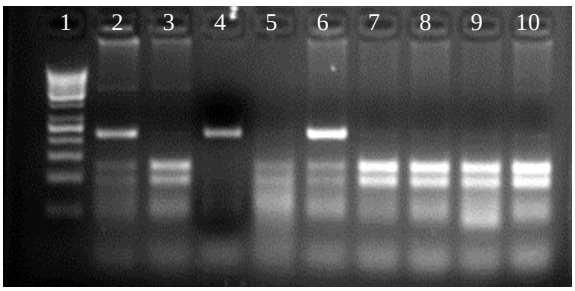


Fig. (3): P 21 Ser31arg gene polymorphism after digestion by BlpI.

Lane 1: 50 bp ladder

Lanes 2, 6: Heterozygous (Ser/Arg) showing bands at 225, 122,103bp

Lanes 3, 5, 7-10: Wild type (Ser/Ser) showing bands at 122,103bp

Lane 4: Homozygous variant (Arg/Arg) showing one band at 225.

Statistical methods:

Data was analyzed using SPSSwin statistical package version 17 (SPSS Inc., Chicago, IL). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test (Fisher's exact test) was used to examine the relation between qualitative variables. For quantitative data, comparison between two groups was done using Mann-Whitney test (non-parametric t-test). Comparison between 3 groups was done using Kruskal-Wallis test (non-parametric ANOVA). Logistic regression was used for calculation of odds ratio (OR) with 95% confidence interval (CI) for risk estimation. A p-value <0.05 was considered significant.

RESULTS

This study was conducted on 77 cases of newly diagnosed AML who presented to the Adult and Pediatric oncology departments of the National Cancer Institute (NCI), Cairo University in the period from April 2010 to October 2011 as well as 72 age and sex comparable healthy individuals as a control group. The relative frequency of MDM2, P53 and P21 genes regarding the wild, heterozygous and homozygous types in the two studied groups was investigated.

The age of cases ranged from 4 to 83 years with a median of 34 years and a mean of 38±17.3 years. The patient group included 43 males (55.8%) and 34 females (44.2%).

The presenting total leucocytic count in the study cases ranged from 2 to 180 with a mean of 34.71±31.22 and a median of $29x10^9$/L. The platelet count ranged from 2 to 271 with a mean of 45±41 and a median of $33x10^{12}$/L. Hemoglobin ranged from 4 to 13, with a mean of 7.7±3.2, and a median of 7.3gm/dl. Blasts in peripheral blood ranged from 0 to 90% with a mean of 38.3%±28 and a median of 32%. The mean percentage of blasts in marrow was 69.2% ±18.5, the median was 72% and the range was 20-95%. Bone marrow megakaryocytes and platelets were reduced in 98.7% of AML cases.

Cytochemistry:

Myeloperoxidase was positive in 94.7% of cases while NASDA/NASDAF was positive/

partially inhibited in 18.2% of cases and positive /totally inhibited in 2.6% of cases.

Immunophenotyping:

The myeloid lineage markers including MPO were detected in 96.1% of cases, CD33 in 93.5% of cases and CD13 in 98.7% of cases. Also CD 117 was positive in 57% of cases and HLA-DR was positive in 71.4% of cases. Myeloid with monocytic markers including CD14 and CD64 were positive in 11.7% and 6.5% of cases respectively. Aberrant expression of lymphoid markers was detected in 19.5% of cases with CD7 showing the highest expression in (7.8%) of cases.

FAB classification:

The most commonly encountered FAB subtype was M2 (32.5%), followed by M1 (29.9%), M4 (18.2%), M3 (14.3%), M5 (2.6%) and finally both M0 and M7 (1.3%).

MDM2:

Table (2) demonstrates the frequency of MDM2 polymorphisms and compares mutant versus wild types in cases versus controls. Fig. (1) demonstrates the different PCR MDM2 fragments.

P53:

Table (3) demonstrates the frequency of p53 polymorphisms and compares mutant versus wild types in cases versus controls. (Fig. 2) demonstrates the different PCR p53 fragments.

P21:

Table (4) demonstrates the frequency of p53 polymorphisms and compares mutant versus wild types in cases versus controls. (Fig. 3) demonstrates the different PCR p53 fragments.

Combinations:

Combination between MDM2-P21 showed that the frequency of the mutant versus wild types was 78.1% versus 21.9% in AML group and 63.1% versus 36.9% in the control group with a *p*-value=0.061 which is a borderline significance (Table 5).

Also combination between P53-MDM2 showed that the frequency of the mutant versus wild types was 76.6% versus 23.4% in AML group and 72.3% versus 27.7% in the control group with a *p*-value=0.580 denoting no significant difference between the control group and the AML group.

Finally combination between P53-21 showed that mutant versus wild types frequencies were 67.8% versus 32.2% in AML group and 53.6% versus 46.4% in the control group with a *p*-value=0.103, also denoting no significant difference between the control group and the AML group.

From the previous results, when the MDM2, P53 and P21 polymorphisms were combined, no multiplicative joint effect existed concerning the risk of developing AML except for MDM2 and P21, where a borderline significance exists denoting a possible increased risk for AML.

Correlation of the clinical, hematological and immunophenotypic parameters with the three gene polymorphisms expression in AML cases showed that its expression was not related to any of these parameters.

Table (2): Comparison between the frequencies of MDM2 types in AML versus control groups.

	MDM2 (GG)	MDM2 (TG)	MDM2 (TT)	*p*-value	MDM2 (TG+GG)	*p*-value
AML	14 (20.6%)	33 (48.5%)	21 (30.9%)	0.083	47 (69.1%)	0.07
Control	6 (9.2%)	29 (44.6%)	30 (46.2%)		35 (53.8%)	
Total	20 (15%)	62 (46.6%)	51 (38.3%)		82 (61.7%)	

* TG+GG vs. TT

Table (3): Comparison between the frequencies of P53 types in AML versus control groups.

Group	p53 GG	p53 GC	p53 CC	*p*-value	p53 (GC+CC)	*p*-value
AML	36 (52.9%)	26 (38.2%)	6 (8.8%)	0.792	32 (47.1%)	0.820
Control	38 (55.1%)	27 (39.1%)	4 (5.8%)		31 (44.9%)	
Total	74 (54.0%)	53 (38.7%)	10 (7.3%)		63 (46.0%)	

* GC+CC vs. GG

Table (4): Comparison between the frequencies of P21 types in AML versus control groups.

Group	p21 arg/arg	p21 ser/arg	p21 ser/ser	*p*-value	P21 (ser/arg+arg/arg)	*p*-value
AML	3 (4.6%)	19 (29.2%)	43 (66.2%)	0.017	22 (38.8%)	0.008
Control	1 (1.4%)	9 (12.9%)	60 (85.7%)		10 (14.3%)	
Total	4 (3.0%)	28 (20.7%)	103 (76.3%)		32 (23.7%)	

* ser/arg+arg/arg vs. ser/ser

Table (5): Comparison between combined MDM2-P21 mutants versus wild types in AML compared to control.

Group	MDM2/P21 (mutant combination)	MDM2/P21 (both wild)	Total	*p*-value
AML	50 (78.1%)	14 (21.9%)	64 (100%)	0.061
Control	41 (63.1%)	24 (36.9%)	65 (100%)	
Total	91 (70.5%)	38 (29.5%)	129 (100%)	

DISCUSSION

In the present study, the presence of MDM2, p53 and p21 gene polymorphism in 77 newly diagnosed AML cases and 72 age and sex comparable healthy controls was analyzed.

To the best of the author's knowledge, no previous studies addressed the correlation of the 3 previous parameters in AML cases. Previous studies only investigated either one or two of them in relation with AML.

Numerous studies have investigated the association between the MDM2 SNP309 polymorphism and the risk of cancer but the results have been inconsistent. In the present study, common polymorphisms in MDM2 were examined to investigate whether or not they would affect the pathogenesis of AML in the Egyptian patients at NCI. A borderline significance was observed between AML cases and controls, with a *p*-value of 0.083. This result is inconsistent with a case-control study on the role of both polymorphisms p53 and MDM2 among 231 patients with AML and 128 normal controls from a northern Chinese population. They observed a nearly 3.52-fold increase in AML risk associated with the MDM2 GG genotype compared with the MDM2 TT genotype (p=0.001) **[5]**. This difference in results may be due to the larger number of patients in the Chinese study and/or to ethnic variation.

A meta-analysis of 21 case-control studies conducted on 14,770 cases with different tumor types and 14,524 controls from 25 published case-control studies to estimate the effect of SNP309 on tumor risk, as well as to quantify the potential between-study heterogeneity showed that ORs of a variety of cancers associated with the MDM2 GG and TG genotypes were 1.17 (95% CI=1.04-1.33) p=0.0002 and 1.15 (95% CI=1.03-1.28) p=0.0005. The analyses suggested that MDM2 SNP309 serves as a low-penetrance susceptibility tumor marker **[11]**. The present finding is partially consistent with the more recent report by Ellis et al. **[12]** who tested associations between patients with t-AML (n=171) and 2 common functional p53-pathway variants, the MDM2 SNP309 and the TP53 codon 72 polymorphism and showed that MDM2 SNP309G allele was associated with a modest increased risk in de novo AML but not in therapy-related AML. So far there exists one published leukemia study claiming that the MDM2 SNP309 G allele reduced the risk of the disease in a Singaporean Chinese population, which is in discordance with the present results **[13]**. The discrepancy between their studies and this one could perhaps be due to ethnic and/or geographic variations of the frequency of these alleles in different healthy populations **[14]**. It is noteworthy that the basal frequency of the G allele in Singaporean Chinese population is much higher than that in our studied healthy population. Hence, it appears that ethnicity and/or geography bias may influence the effect of the MDM2 G allele on AL risk, perhaps in combination with genetic background, carcinogen exposure in different populations, or just simply sample sizes.

Since the discovery by Bond et al., that SNP309 of the MDM2 gene can accelerate the onset of leukemia at a young age in the patients, there have been quite few studies assessing the impact of the G allele on timing of leukemia onset **[14]**. The evidence from studies of leukemia is controversial, showing an association with early onset in Caucasian and Black populations but not in Hispanic and Singporean populations. Hence, it appears that the SNP309 has common susceptibility across populations with different ethnicity-specific effects. Their work in northern Chinese population showed that the SNP309 had no effect on the timing of acute leukemia onset, which further consolidated the idea that the SNP309 has different ethnic effects. Other studies **[15,16]** investigating genetic polymorphisms have demonstrated similar ethnicity-specific effects, which might suggest interactions between gene polymorphisms and unidentified factors associated with ethnic status.

Another study who genotyped children (n=575) with de novo acute myeloid leukemia (AML) treated on three Children's Oncology Group protocols for the presence of SNP309 and healthy blood donors as control population, showed that MDM2 polymorphism increased the susceptibility to childhood AML where the variant G/G genotype was associated with an increased susceptibility to AML (OR 1.5; p=0.049) **[19]**. The difference in results may be due to different age groups as the patients in the present study have a mean of 38 years and a median of 34 years. This finding of increased risk of malignancy in association with the homozygous variant genotype is in agreement with a number of prior studies. Studies of solid tumors including renal cell carcinoma, colorectal cancer, pancreatic cancer, gastric carcinoma, lung cancer, soft tissue sarcoma and neuroblastoma have reported an increased risk of malignancy in persons with a homozygous variant genotype, with odds ratios typically between 1.5 and 2.0 **[14,17]**.

Numerous molecular epidemiological studies have attempted to demonstrate a correlation between inheritance of p53 codon 72 polymorphism and increased susceptibility to various cancers. The role of this polymorphism remains controversial, with some studies reporting increased risk associated with the Pro 72 allele; which results in the substitution of proline for arginine, for certain cancer types and others failing to reach such conclusions. Even meta-analyses of the various studies have not fully resolved this issue **[18,19]**. In the present study, it was found that there is no additional effect from the p53 Arg72Pro polymorphism in the pathogenesis of AML. This is consistent with a case-control study consisting of 231 AML patients and 128 normal individuals from a northern Chinese population (p=0.25) **[5]**. Also these results are lending weight to the conclusion of Nakano et al., that the p53 Arg72Pro was not associated with risk or clinical parameters of AML **[20]**. Similarly, there are also inconsistent associations between the codon 72 polymorphism with age of tumor onset **[21]**. Similarly another study of twenty-five patients with ALL and 65 patients with AML, both recently diagnosed mutation of the P53 gene was found in one patient of the 25 with ALL and in five patients of the 65 with AML **[22]**. Another study about therapy-related acute myeloid leukemia susceptibility, showed that the polymorphism alone cannot influence the risk of t-AML, an interactive effect was detected such that MDM2 TT TP53 Arg/Arg double homozygotes, and individuals carrying both a MDM2 G allele and a TP53 Pro allele, were at increased risk of t-AML (p-value for interaction is 0.009) **[12]**.

In the present study, the p21 codon 31 polymorphism was significantly associated with risk for AML. This result is consistent with the other studies that investigated this polymorphism in various types of cancers. A study on 104 cancer cervix patients and 160 control in Chinese southern women showed that the frequencies of p21 Arg/Arg, Ser/Arg, and Ser/Ser genotypes among patients were significantly different from those among controls (p<0.05) **[23]**. In another study on 53 bladder cancer patients, 119 healthy controls in Taiwanese patients, there was a significant difference in p21 codon 31 polymorphism between the control and the cancer patients (p<0.01). The arginine form was prominent in the cancer patients (per copy of the A allele, odds ratio=2.03, 95% confidence interval =1.23-3.37) **[24]**.

Also another study from northeastern Iran on 126 eosophageal squamous cell carcinoma cases and 100 controls showed that the frequencies of the wild type and variant genotypes for each of these SNPs were the same and equal to

78.57% for the wild type genotype and 21.43% for the variant genotype, respectively, among cases and 82% and 18%, respectively, among controls and this difference was not statistically significant (p=0.52) **[25]**.

When the MDM2, P53 and P21 polymorphisms were combined, the author did not find any multiplicative joint effect concerning the risk of developing AML except for MDM2 and p21 where a borderline significance existed between the frequency of the mutant versus the wild types and which was 78.1% versus 21.9% in the AML group and 63.1% versus 36.9% in the control group with a p-value=0.061, denoting a possible increased risk for AML. This is consistent with Xiong et al. **[5]**, where no multiplicative effect was found when they combine MDM2 GG and p53 Pro/Pro genotypes in the risk of AML in a case control study of 231 AML patients and 128 normal controls from a northern Chinese population.

The present results are inconsistent with another study about therapy-related acute myeloid leukemia susceptibility, which showed that the polymorphism alone cannot influence the risk of t-AML, an interactive effect was detected such that MDM2 TT TP53 Arg/Arg double homozygotes, and individuals carrying both a MDM2 G allele and a TP53 Pro allele, were at increased risk of t-AML (p-value for interaction is 0.009) **[11]**. The difference in results may be attributed to the fact that this study observed this interactive effect only in a certain type of patients e.g. In patients previously treated with chemotherapy but not in patients treated with radiotherapy, and in patients with loss of chromosomes 5 and/or 7, acquired abnormalities associated with prior exposure to alkylator chemotherapy.

In conclusion, this is the first report to the best of the author's knowledge to show the relation between AML and the 3 gene mutations in the P53 pathway where there is a borderline significant association between a functional polymorphism in MDM2 and AML, insignificant association between polymorphism in p53 and AML and a significant association between polymorphism in p21 and AML. It is thus suggested that the p21 codon 31 polymorphism might be a genetic susceptibility factor in the pathogenesis of AML; another study on higher numbers is needed to validate these findings.

REFERENCES

1- Look AT. Oncogenic transcription factors in the human acute leukemias. Science. 1997; 278 (5340): 1059-1064.

2- Levine AJ. P53, the cellular gatekeeper for growth and division. Cell. 1997; 88: 323-31.

3- Haupt Y, Maya R, Kazaz A, Oren M. Mdm2 promotes the rapid degradation of p53. Nature. 1997; 387: 296-9.

4- Bond GL, Hu W, Levine A. A single nucleotide polymorphism in the MDM2 gene from a molecular and cellular explanation to clinical effect. Cancer Res. 2005; 65: 5481-4.

5- Xiong X, Wang M, Wang L, et al. Risk of MDM2 SNP309 alone or in combination with the p53 codon 72 polymorphism in acute myeloid leukemia. Leuk Res. 2009; 33 (11): 1454-8.

6- Ara S, Lee PS, HansenMF, Saya H. Codon 72 polymorphism of theTP53 gene. Nucleic Acids Res. 1990; 18: 4961.

7- Onel K, Cordon-Cardo C. MDM2 and prognosis. Mol Cancer Res. 2004; 2: 1-8.

8- Freedman DA, Levine AJ. Regulation of the p53 protein by the MDM2 oncoprotein-thirty-eighth G.H.A. Clowes Memorial Award Lecture. Cancer Res. 1999; 59: 1-7.

9- Gupta RC, Earley K, Sharma S. Use of human peripheral blood lymphocytes to measure DNA binding capacity of chemical carsinogens. Proc Natl Acad Sci USA. 1988; 85: 3513-7.

10- Hirata H, Hinoda Y, Kikuno N, et al. MDM2 SNP 309 polymorphism as risk factor for susceptibility and poor prognosis in renal cell carcinoma. Clin Cancer Res. 2007; 13: 4123-4129.

11- Hu W, Feng Z, Teresky AK, Levine AJ. P53 regulates maternal reproduction through LIF. Nature. 2007, 450 (7170): 721-4.

12- Ellis NA, Huo D, Yildiz O, et al. MDM2 SNP309 and TP53 Arg72Pro interact to alter therapy-related acute myeloid leukemia susceptibility. Blood. 2010; 1; 112 (3): 458-9.

13- Phang B, Linn Y, Li H, Sabapathy K. MDM2 SNP309 G allele decreases risk but does not affect onset age or survival of Chinese leukaemia patients. European Journal of Cancer. 2008; 449 (5): 760-6.

14- Bond GL, Hirshfield KM, Kirchhoff T, et al. MDM2 SNP 309 accelerates tumor formation in a gender-specific and hormone dependent manner. Cancer Res. 2006; 66: 5104-5110.

15- Ishibe N, Wiencke JK, Zuo ZF, et al. Susceptibility to lung cancer in light smokers associated with CYP1A1 polymorphisms in Mexican- and African-Americans. Cancer Epidemiol Biomarkers Prev. 1997; 6: 1075-1080.

16- WrenschKelsey KT, Liu M, McMillan A, et al. CYP1A1 variants and smoking-related lung cancer

in San Francisco Bay area Latinos and African Americans. Int J Cancer. 2005; 113: 141-147.

17- Cattelani S, Defferrari, Marsilio S, et al. Impact of a single nucleotide polymorphism in the MDM2 gene on neuroblastoma development and aggressiveness: Results of a pilot study on 239 patients. Clin Cancer Res. 2008; 14: 3248-3253.

18- Weston A, Godbold JH. Polymorphisms of H-ras-1 and p53 in breast cancer and lung cancer: A meta-analysis. Environ Health Perspect. 1997; 105 (4): 919-926.

19- Matakidou A, Eisen T, Houlston RS. TP53 polymorphisms and lung cancer risk: A systematic review and meta-analysis. Mutagenesis. 2003; 18 (4): 377-385.

20- Nakano Y, T Naoe, H Kiyoi, et al. Poor clinical significance of p53 gene polymorphism in acute myeloid leukemia. Leuk Res. 2000; 24 (4): 349-352.

21- Sotamaa K, Liyanarachchi S, Mecklin JP, et al. P53 codon 72 and MDM2 SNP309 polymorphisms and age of colorectal cancer onset in Lynch syndrome. Clin Cancer Res. 2005; 11: 6840-4.

22- Wojcik I, Szybka M, Golanska E, et al. Abnormalities of the P53, MDM2, BCL2 and BAX genes in acute leukemias. Neoplasma. 2005; 52 (4): 318-24.

23- Jiang P, Liu J, Li W, et al. Role of p53 and p21 polymorphisms in the risk of cervical cancer among Chinese women. Acta Biochim Biophys Sin. 2010; 42 (9): 671-676.

24- Chen WC, Wu HC, Hsu CD, et al. P21 gene codon 31 polymorphism is associated with bladder cancer. Urol Oncol. 2002; 7 (2): 63-6.

25- Taghavi N, Biramijamal F, Abbaszadegan MR, et al. P21 (Waf1/Cip1) Gene Polymorphisms and Possible Interaction with Cigarette Smoking in Esophageal Squamous Cell Carcinoma in Northeastern Iran: A Preliminary Study. National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran. Arch Iran Med. 2010; 13 (3): 235-42.