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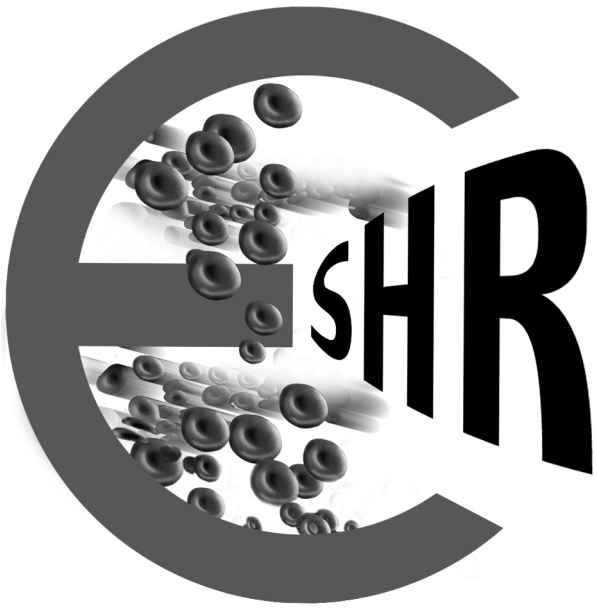

The Official Journal of the
Egyptian Society of Haematology & Research

Vol. 6, No. 2, September 2010

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

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Volume 6 * Number 2 * September 2010

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Journal of the Egyptian Society of Haematology & Research, Vol. 6, No. 2, September: 31-37, 2010

Brain and Acute Leukemia Cytoplasmic (BAALC) Gene Expression in Acute Myeloid Leukemia: A Study of an Egyptian Cohort

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ABSTRACT

Background: Acute myeloid leukemia with normal cytogenetics (CN-AML) constitutes a heterogeneous group with variable prognosis. Among others, high BAALC gene expression is reported as an independent bad prognostic indicator in CN-AML. Egyptian studies on BAALC gene expression in AML are limited.

Objectives: We aimed to study the BAALC gene expression in an Egyptian cohort with AML and to verify its prognostic relevance on CNAML in the studied cohort.

Material and Methods: The cohort comprised 54 AML patients including 38 CNAML, 7 M3, 4 CML in blastic crisis, 2 on top of MDS, 2 (+8) and one AML case with hypodiploidy. Cases were diagnosed according to standard methods and FAB subtype specified. BAALC gene expression was evaluated by RT-PCR.

Results: High BAALC gene expression was detected in 19/38 (50%) of the CNAML cases. No statistically significant association of BAALC gene expression with Hb level, TLC, platelet count or percentage of BM blasts at first presentation, at day 14 or at day 28 post chemotherapy was encountered. High BAALC expression was significantly associated with FAB M0/M1 (p=0.01), lower CR rate (p=0.04), higher frequency of primary resistance to chemotherapy (p=0.02) and shorter OS (p=0.003).

Apart from the CNAML cases, high BAALC gene expression was encountered in 2/2 cases with trisomy 8, 1/1 with hypodiploidy, 2/4 CML in blastic crisis and 2/2 AML on top of MDS; none of the 7 M3 cases showed high BAALC expression.

Conclusion: Our study has further demonstrated, in an Egyptian cohort, that high BAALC gene expression is associated with lower incidence of CR, higher incidence of resistant disease and shorter OS in CNAML patients. This study would strongly recommend including BAALC gene evaluation in the initial molecular characterization of AML cases.

Key Words: *AML – CNAML – BAALC gene.*

INTRODUCTION

Chromosomal abnormalities provide a powerful tool to stratify acute myeloid leukemia (AML) patients into different prognostic risk groups. Patients lacking cytogenetic aberrations, accounting for approximately 45% of newly diagnosed de novo AML cases, were originally contained in an intermediate risk group **[1]**. However, this cytogenetically normal AML (CN-AML) actually constitutes a heterogeneous cohort of patients with favorable, intermediate, or relatively poor clinical outcome **[2]**. This was proved by molecular studies of CN-AML cases that revealed a striking heterogeneity with regards to the presence of acquired gene mutations and changes in gene expression **[3]**. Multiple submicroscopic genetic alterations with prognostic significance have been discovered, including internal tandem duplication of the FLT3 gene **[4]**, partial tandem duplication of the MLL gene **[5]**, mutations in the CEBPA gene **[6]**, NPM1 gene **[7,8,9]**, WT1 gene **[10]**, AML1 gene **[11]** and NRAS gene as well as high expression of ERG gene **[12]**, MN1 gene **[13]** and the BAALC gene **[14,15,16]**. BAALC (for brain and acute leukemia, cytoplasmic) is a relatively recently identified gene on chromosome 8q22.3 which is suspected in the pathogenesis of some AML cases **[14]**. BAALC expression in normal bone marrow is restricted to the compartment of progenitor cells and it shows high expression in a subset of leukemic blasts. It is absent in normal peripheral blood cells. The function of the BAALC protein in hematopoiesis and leu-

kemogenesis contributing to a more aggressive behavior of AML is obscure. Baldus et al., **[17]** suggested that BAALC may be seen as a stage-specific marker that maintains proliferative capacity and inhibits differentiation in a regulated way during hematopoiesis but if aberrantly expressed, this can lead to leukemogenesis. In AML patients with normal cytogenetics, high BAALC expression seems to predict a poor prognosis **[14,15,17]**.

Studies on Egyptian patients are limited. We aimed in this work to study BAALC gene expression in a cohort of Egyptian patients with myeloid malignancies and to verify its prognostic impact on prognosis of CN-AML cases in our cohort.

PATIENTS AND METHODS

The study included 68 cases with AML presented to the NCI, Cairo University, during the period of March 2007 to June 2010.

Patients were evaluated according to standard methods including clinical, radiological and laboratory workup. Diagnosis was confirmed by complete blood picture, bone marrow (BM) examination, cytochemistry as indicated and immunophenotyping. Routine karyotyping and evaluation of t(8;21), t(15;17) and inv (16) were performed. BAALC gene expression was evaluated in all cases.

Of the 68 patients, data was available for 54. Among these 38 were CN-AML; the others included 7 M3 cases, 4 CML cases in blastic crisis, 2 AML cases on top of MDS, 2 AML +8 cases and one AML case with hypodiploidy. Statistical analysis was confined to the 38 CN-AML. Results of the other cases will be presented in a descriptive way.

Patients with normal cytogenetics (38) included 19 males and 19 females; they had an age range of 0.5-80 and a median of 32 years; 7 were children (≤16 years), 20 were adults (16-60) and 5 were elderly AML cases (≥60 years). Of these 26 were followed-up for a period of 11-26 months with a median of 17 months to detect the clinical outcome of treatment emphasizing on achievement of CR, failure to achieve CR at the end of induction therapy, incidence of relapse and OS.

BAALC gene expression was evaluated by reverse transcription-polymerase chain reaction (RT-PCR). RNA extraction was performed by QIAamp® RNA Blood Mini Kit Catalog no. 52304; USA, its concentration (ng/ul.) and purity were measured using The Thermo Scientific NanoDrop™ 1000 Spectrophotometer, Wilmington, DE 19810 USA. The ratio of the readings at 260 nm and 280 nm (A260/A280) provides an estimate of the purity of RNA with respect to contaminants that absorb in UV spectrum, such as protein; pure RNA has an A260/A280 ratio of 1.9-2.1.

The GeneAmp® Gold RNA PCR Reagent Kit (Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404) was used for the reverse transcription (RT) and polymerase chain reaction (PCR) amplification. First-strand cDNA was synthesized from 1µg RNA in a 20µL reaction mix containing 1X RT-PCR Buffer, 25mM $MgCl_2$, 10mM dNTP Blend, 0.5µL RNase Inhibitor 10 unit/20ul, 100 mM DTT, 0.5µL (1.25uM) random hexamers, 0.3µL MultiScribe Reverse Transcriptase (50 units/µL) in RNase-free water. Cycling parameters for the RT included hybridization for 10min at 25°C and reverse transcription for 45min at 42°C.

To check the integrity of the cDNA, the house keeping gene glucose phosphate isomerase (GPI) was amplified as a control gene by single-round PCR with the following primer pair (forward primer, TaqE1F: 5- CCCCCAGTTCCAGAAGCTG 3- and reverse primer: TaqE2R: 5- GCATCACGTCCTCCGTCAC 3-). A 63ng cDNA in 2.5µL was added to a final PCR reaction mixture of 25µL containing 5.0µL of 5 X PCR Buffer, 1.75µL of 10mM dNTPs, 1.9µL of 25mM $MgCl_2$ 0.25µL of 5U/µL AmpliTaq Gold DNA Polymerase and 1.0µL of each of the10 pmole/µL GPI primers. The reaction mixture was subjected to an initial step of denaturation at 95ºC for 10 minutes followed by 40 cycles of denaturation at 95ºC for 15 seconds, annealing at 60ºC for 15 seconds and extension at 72ºC for 1 minute followed by a terminal step of extension at 72ºC for 5.0 minutes. The BAALC cDNA was amplified in the same way using BAALC specific primers:

- Forward primer, 5- GGGAGACAGAATCCACCTG 3-

• Reverse primer: 5- TGGACTCTCTGCTAGT-TGAC 3-.

The PCR products were resolved on ethidium bromide-stained 1.5% agarose gel and UV photographed. BAALC gene yields products of 221 and 388bp according to different splicing patterns 1, 8 or 1, 6, 8 respectively, and the GPI control primers result in an amplified fragment of 176bp (Fig. 1).

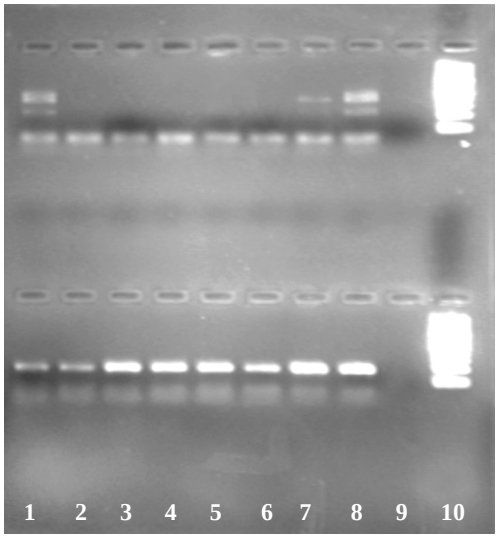

Fig. (1): Gel documentation of BAALC and GPI gene expression.

Lane 10 : 100bp ladder, Lane 9: Empty.
Upper panel : BAALC expression: 221 and 338bp bands are detectable in lanes 1, 7 and 8 and undetectable in lanes 2-6.
Lower panel: GPI gene expression: Lanes 1-8 show a 176 bp band.

Interpretation of BAALC gene expression was done according to Baldus et al. **[17]**. The authors evaluated BAALC gene expression by both real-time PCR and conventional PCR. BAALC gene is expressed in all cases and controls. However, only in cases with high expression levels an apparent band is detected on gel upon electrophoresis of the amplified product. On the other hand, when the expression of the gene is low by real time technique, no band could be detected on the gel. Accordingly, we considered cases with an apparent band as high expressers and those with undetectable bands as low expressers.

Statistical analysis was performed using SPSS version 17 & EpiInfo 6 (WHO) software. Normality of distribution was computed by W Shapiro-Wilk's test. The comparison of quantitative data was performed by independent *t*-test or Man Whitney test for 2 groups and by ANOVA test or Kruskal Wallis test for more than 2 groups. Comparison of qualitative variables were done using X^2 test or Fisher exact test where appropriate. Statistical significance was set at a level of 0.05. Survival curves according to type of gene expression whether high or low were derived using the Kaplan-Meier method and compared using log-rank tests.

RESULTS

BAALC gene expression in CN-AML:

High and low BAALC gene expression was encountered each in 50% of the studied cases. No statistically significant differences were encountered between both groups with regards to age, gender, organomegaly, lymphadenopathy, or occurrence of infection (Table 1). Extramedullary disease was not detected in any of the studied cases.

Table (1): Demographic and clinical features of 38 CN-AML patients in relation to BAALC gene expression.

Parameter	BAALC gene expression			*p* value
	High (N. 19)	Low (N. 19)	Total (N. 38)	
Age: Years	35.3±16.8* (2-61)	30.7±27 (0.5-80)	–	0.79
Gender:				
Male	7 (36.8)**	12 (63.2)	19 (50)	0.1
Female	12 (63.2)	7 (36.8)	19 (50)	
Hepatomegaly	18 (94.7)	14 (73.7)	32 (84.2)	0.18
Splenomegaly	12 (63.2)	15 (78.9)	27 (71.1)	0.28
Lymphadenopathy	3 (15.8)	2 (10.5)	5 (13.2)	1
Infection	10 (52.6)	6 (33.3)	16 (43.2)	0.244

* Mean ± SD (range).
** No (%).

No statistically significant differences were encountered between high and low BAALC expressers with regards to Hb level, TLC, platelet count or percentage of BM blasts at first presentation, at day 14 or at day 28 post chemotherapy (Table 2).

Patients with high BAALC expression tended to have higher blast percentage in peripheral blood at presentation than those with low BAALC expression; the difference is near significance (*p*-value 0.08).

Table (2): Hematological parameters in 38 CN-AML patients in relation to BAALC gene expression.

Parameter	BAALC gene expression		*p* value
	High expressers (N. 19)	Low expressers (N. 19)	
Hb.: (g/dl)	7.6±2.6* (3.6-11.6)	8±1.6 (4.4-11.8)	0.96
TLC: x 10^9/L	52±57 (1.7-374)	57.8±68 (2.7-240)	0.71
Platelets: x 10^9/L	88.9±75.9 (13-280)	67.5±51.9 (18-181)	0.45
PB blast. At presentation: %	61.4±30 (2-90)	44.6±24 (5-90)	0.08
BM blast At presentation: %	60.4±23.6 (24-98)	58.8±18 (31-95)	0.82
On day 14: %	25.4±30.7 (0-75)	8.4±13.7 (0-50)	0.24
On day 28: %	15±23 (0-64)	12±26.5 (0-90)	0.79

* Mean ± SD (range).

FAB classification was available for 36 cases. They included 11M0, 22 M1, 3 M4; no M5, M6 or M7 were encountered in our cohort.

High BAALC expression was encountered in 81.8% of both M0/M1 cases as compared to 55.9% of the M2 and 66.6% of the M4 cases. The difference is statistically significant (*p*=0.01).

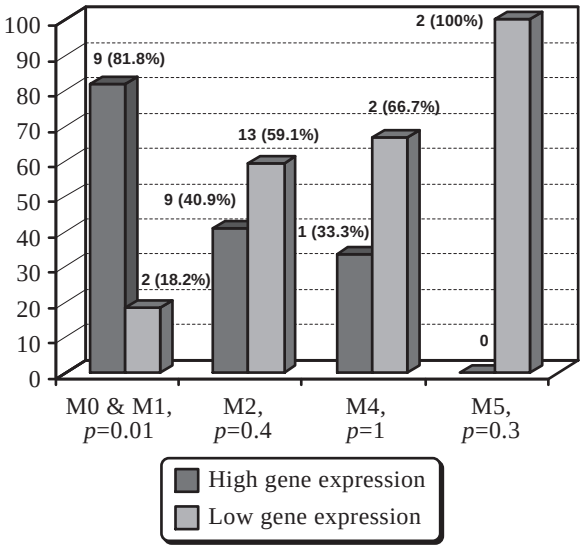

Fig. (2): Distribution of high and low BAALC gene expressers among the different FAB subgroups in 38* AML patients in relation to BAALC gene expression.

No statistically significant association was encountered between high and low BAALC expressers and any of the surface markers expressed by the leukemic cells.

The clinical outcome of treatment in 26 CN-AML patients in relation to BAALC gene expression is presented in Table (3). High BAALC gene expressers had significantly lower CR rate (*p*=0.04) and higher frequency of primary resistance to chemotherapy (*p*-value 0.02).

There was a trend for high expressers to have relapsed after remission (66.7% vs. 27.3%). However the difference did no achieve statistical significance.

Table (3): Clinical outcome of treatment in 26 CN-AML patients in relation to BAALC gene expression.

Outcome	BAALC gene expression			*p* value
	High No. (13)	Low No. (13)	Total No. (26)	
Complete remission	6 (46.2)	11 (84.6)	17 (65.4)	0.04
Primary resistance	9 (69.2)	3 (23.1)	12 (46.2)	0.02
Relapse after remission	4 (66.7	3 (27.3)	7 (41.2)	0.29

The overall survival in 26 AML patients with normal cytogenetics in relation to BAALC gene expression is presented in Table (4) and Fig. (3). Patients with high BAALC gene expression had a median OS of 1.5 with a range of 0-4 months as compared to a median of 10.5 and a range of 1.5-16.5 months in patients with low BAALC gene expression (*p*=0.003).

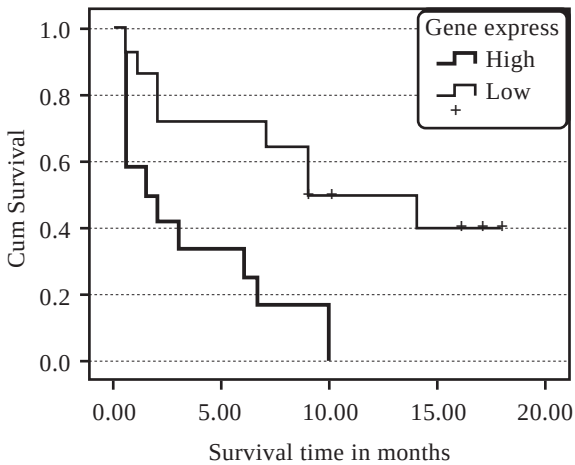

Fig. (3): Relation between overall survival in months and BAALC gene expression in 26 cytogenetically normal AML patients.

Apart from the CN-AML cases, high BAALC gene expression was found in 2/2 AML cases with trisomy 8, 1/1 AML case with hypodiploidy, 2/4 CML cases in blastic crisis and 2/2 secondary AML cases on top of MDS. On the other hand, none of the 7 M3 cases showed high BAALC expression.

DISCUSSION

In this work we have evaluated the BAALC gene expression in 68 AML cases. Patients were judged as high or low expressers according to the detect ability of the amplified band on gel electrophoresis. Most of the previous studies evaluated BAALC expression by real-time PCR considering the median level obtained in each cohort as the discriminator between high and low expression. Accordingly the cutoffs are different according to the studied cohort and comparison between studies is difficult. This issue was objectively addressed in the study of Baldus et al., **[17]** and Tanner et al. **[18]**. The authors evaluated the expression level by both real-time and conventional PCR. In high expressers, as judged by real-time, the amplicon gave a detectable band on gel electrophoresis while no band could be visualized in low expressers. Thus conventional electrophoresis could be considered as simple relatively objective method for categorization of cases into high and low BAALC gene expression which was adopted in the current study.

In the present work, no statistically significant differences were encountered between high and low BAALC gene expresser CN-AML patients with regards to age, gender, organomegaly, lymphadenopathy, or infection. This is in agreement with Baldus et al., **[15,17]**. One previous study reported male predominance **[19]** and another reported female predominance **[20]**. Both studies used the median level obtained by real-time PCR as the discriminating level which makes comparison difficult.

In the current study, no statistically significant differences were encountered between the high and low BAALC gene expresser CN-AML patients with regards to Hb level, TLC, platelet count or number of blasts in bone marrow at presentation. This is in agreement with previous studies **[15,17,21]**. However, we encountered a higher PB blast% at presentation in the high expresser group with a near significant difference (p=0.08). This is supported by the findings of Baldus et al. **[15]** who reported that high BAALC expressers had a 56% PB blasts at presentation vs. 31%, in low BAALC expressers, (p-value 0.004). Failure of achieving statistical significance in our cohort may be attributed to our small sample size. The association of high BAALC expression with higher PB blasts may be explained by the effect of BAALC gene on hematopoietic progenitors; it inhibits differentiation of the progenitors and favors their proliferation **[18]**. In contrast, Baldus et al. **[17]** reported the association of low BAALC expression with a significantly higher white blood count. The discrepancy is difficult to explain and it may be just a coincidence rather than a real association.

In the current study, high BAALC gene expression was found to be associated with the primitive FAB subgroup (M0 and M1). Similar findings were reported by Bienz et al., **[14]** and Tanner et al. **[18]**. The higher frequency of high BAALC gene expression among M0 (Minimally differentiated AML) and M1 (Acute myeloid leukemia without maturation) FAB subgroups can be attributed to the effect of BAALC gene on differentiation and proliferation, inhibiting differentiation and promoting proliferation **[17]**.

In the current study, no significant differences were reported between high BAALC- and low BAALC-expresser CN-AML patients with respect to the marker expression by the leukemic blasts including CD34, as well as different myeloid, monocytic and aberrant lymphoid markers. In contrast significant association between CD34 and high BAALC expression was previously reported **[14]**. The finding of the latter authors is in harmony with the assumed role of BAALC gene in inhibiting differentiation and favoring proliferation of hematopoietic progenitors **[17]**. We have to study a larger cohort to verify this potential association in our series.

In the current study, the response to treatment was impacted by the level of BAALC gene expression. The BM% blasts tended to be higher in CN-AML patients with high BAALC gene expression both on the 14th and 28th days after the start of chemotherapy though statistical significance was not achieved. Blasts on day 14 post chemotherapy is an early indicator of the prognosis; data from UK MRC AML10 study as well as from German Cooperative

Group showed that if patients failed to adequately clear their blast count, they will do badly even if CR is subsequently achieved **[22]**.

In the current study, failure of remission induction either due to death in induction or failure to reduce the blast count to less than 5% of nucleated marrow cells, at the end of 2nd induction cycle, was significantly higher in high than in low BAALC gene expressers. This is in agreement with previous studies that reported a higher rate of primary resistance and refractoriness to chemotherapy among high BAALC gene expressers **[15,21,23]**. Also, the current study showed a statistically significant association between low BAALC-expression and achievement of CR. Though this association was denied in earlier studies **[14,17]**; yet it was documented in more recent ones **[15,21]**.

Despite being statistically insignificant; that might be due to the small sample size, the present work showed a high tendency for relapse in the high BAALC gene expressers. 66.7% of high BAALC gene expression patients had relapses vs. 27.3% of low BAALC gene expression patients. This is in harmony with previous studies **[15,17,21]**.

In the present study, OS time was longer among the low BAALC gene expression patients, mean 10.8 (Range 7.1, 14.5) with a median of 9 months compared to a mean of 3.5 (Range 1.4, 5.6) with a median of 1.5 months in the high BAALC gene expression group (p=0.003). This is in agreement with several reports **[14,15,17,21]**. Baldus et al. **[17]** reported that high BAALC expression remained a significant adverse prognostic factor for FLT3ITD/WT and FLT3WT/WT patients, compared to those with low BAALC expression (OS, p=.002; hazard ratio [HR]=2.7; DFS, p=.04, HR=2.2). The emphasized that high BAALC expression is an independent adverse prognostic factor for EFS regardless of the FLT3 status (p=.008, HR=2.2). The adverse effects observed in high BAALC expresser CNAML patients was attributed, at least partly, to the highly up-regulated multidrug resistance genes MDR1 and ABCB1 **[24]**.

In the current study, apart from the CN-AML cases, high BAALC gene expression was detected in 2/2 AML cases with trisomy 8, 1/1 AML case with hypodiploidy, 2/4 CML cases in blastic crisis and 2/2 secondary AML cases on top of MDS but none of the 7 M3 cases. This is because BAALC gene expression in hematopoietic cells is restricted to the compartment of progenitor cells, whereas no expression could be detected in mature bone marrow or circulating normal white blood cells **[25]**. High BAALC gene expression levels were first identified in a study of AML patients with trisomy 8 as a sole abnormality **[25]**. Subsequent studies also suggested a trend towards higher BAALC in patients with trisomy 8 as compared to CN-AML patients **[21]**. This is reasonable because BAALC gene is located on chromosome 8q22.3 **[18]**. Trisomy 8 is known to have a negative prognostic relevance in AML and it might be hypothesized that over expression of BAALC might be the molecular basis of such outcome even in AML patients lacking this cytogenetic abnormalities **[25]**. High BAALC gene expression was previously reported in CML in blastic crisis while none of the chronic phase CML cases showed high BAALC gene expression **[17,18]**.

In conclusion, our study has proved, in an Egyptian cohort of CN-AML patients, that high-level expression of, BAALC gene, can predispose to an adverse outcome including lower incidence of CR, higher incidence of resistant disease, shorter OS and a trend to a higher incidence of relapse. Currently, in Egypt, evaluation of BAALC gene is not included in the routine workup of AML patients. This study would strongly recommend its inclusion in the initial molecular characterization of newly diagnosed cases.

REFERENCES

1- Slovak ML, Kopecky KJ, Cassileth PA, et al. Karyotypic analysis predicts outcome of preremission and postremission therapy in adult acute myeloid leukemia: A SouthwestOncology Group/Eastern Cooperative Oncology Group study. Blood. 2000; 96: 4075-4083.

2- Byrd JC, Mrózek K, Dodge RK, et al. Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with de novo acute myeloid leukemia: Results from Cancer and Leukemia Group B (CALGB 8461). Blood. 2002; 100 (13): 4325-36.

3- Schlenk RF, Döhner K, Krauter J, et al. German-Austrian Acute Myeloid Leukemia Study Group. Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. N Engl J Med. 2008; 358 (18): 1909-18.

4- Stirewalt DL, Radich JP. The role of FLT3 in hematopoietic malignancies. Nat Rev Cancer. 2003; 3: 650-665.

5- Whitman SP, Ruppert AS, Marcucci G, et al. Long term disease free survival with cytogenetically normal MLL partial tandem duplication: A cancer and leukemia group B study. Blood. 2007; 109: 5164-5167.

6- Fröhling S, Schlenk RF, Stolze I, et al. CEBPA mutations in younger adults with acute myeloid leukemia and normal cytogenetics: Prognostic analysis of cooperating mutations. J Clin Oncol. 2004; 22: 624-633.

7- Thiede C, Koch S, Creutzig E, et al. Prevalence and prognostic impact of NPM1 mutations in 1485 adult patients with acute myeloid leukemia. Blood. 2006; 107: 4011-4020.

8- Schlenk RF, Döhner K, Kneba M, et al. German-Austrian AML Study Group (AMLSG). Gene mutations and response to treatment with all-trans retinoic acid in elderly patients with acute myeloid leukemia. Results from the AMLSG Trial AML HD98B. Haematologica. 2009; 94: 54-60.

9- Schiffer CA. Molecular characterization of AML: A significant advance or just another prognostic factor? Best Practice & Research Clinical Haematology. 2008; 21 (4): 621–628

10- Virappane P, Gale R, Hills R, et al. Mutations of the Wilms 1 tumor gene is a poor prognostic factor associated with chemoresistance in normal karyotype acute myeloid leukemia: The United Kingdom Medical Research Council Adult Leukaemia Working Party. J Clin Oncol. 2008; 26 (33): 5429-5435.

11- Dicker F, Haferlach C, Kern W, Haferlach T, Schnittger S. Trisomy 13 is strongly associated with AML1/RUNX1 mutations and increased FLT3 expression in acute myeloid leukemia. Blood. 2007; 110: 1308-1316.

12- Marcucci G, Baldus CD, Ruppert AS, et al. Overexpression of the ETS-relatedgene ERG predicts a worse outcome in acute myeloid leukemiaGroup B study. J Clin Oncol. 2005; 23: 9234-9242.

13- Heuser M, Beutel G, Krauter J, et al. High Meningioma 1 (MN1) expression as a predictor of poor outcome in acute myeloid leukemia with normal cytogenetics. Blood. 2006; 108: 3898-3905.

14- Bienz M, Ludwig M, Leibundgut EO, et al. Risk assessment in patients with acute myeloid leukemia and a normal karyotype. Clin Cancer Res. 2005; 11: 1416-1424.

15- Baldus CD, Thiede C, Soucek S, Bloomfield CD, Thiel E, Ehninger G. BAALC expression and FLT3 internal tandem duplication mutations in acute myeloid leukemia patients with normal cytogenetics: Prognostic implications. J Clin Oncol. 2006; 24: 790-797.

16- Mrózek K, Marcucci G, Paschka P, Whitman SP, Bloomfield CD. Clinical relevance of mutations and gene-expression changes in adult acute myeloid leukemia with normal cytogenetics: Are we ready for a prognostically prioritized molecular classification? Blood. 2007; 109 (2): 431-448.

17- Baldus CD, Tanner SM, Ruppert AS, et al. BAALC expression predicts clinical outcome of de novo acute myeloid leukemia patients with normal cytogenetics: a Cancer and Leukemia Group B Study. Blood. 2003; 102 (5): 1613-1618.

18- Tanner SM, Austin JL, Leone G, et al. BAALC, the human member of a novel mammalian neuroectoderm gene lineage, is implicated in hematopoiesis and acute leukemia. Proc Natl Acad Sci USA. 2001; 98 (24): 13901-13906.

19- Metzeler KH, Dufour A, Benthaus T, et al. ERG expression is an independent prognostic factor and allows refined risk stratification in cytogenetically normal acute myeloid leukemia: A comprehensive analysis of ERG, MN1, and BAALC transcript levels using oligonucleotide microarrays. J Clin Oncol. 2009; 27 (30): 5031-5038.

20- El-Hoseiny S, Gawdat R, Abdelfattah R, Elhadidy K. High BAALC gene expression is associated with poor outcome in ccute myeloid leukemia patients with normal cytogenetics. Journal of the ESHR. 2010.

21- Santamaría C, Chillón MC, García-Sanz R, et al. BAALC is an important predictor of refractoriness to chemotherapy and poor survival in intermediate-risk acute myeloid leukemia (AML). Ann Hematol. 2010; 89 (5): 453-458.

22- Kern W, Haferlach T, Schoch C, et al. Early blast clearance by remission induction therapy is a major independent prognostic factor for both achievement of complete remission and long-term outcome in acute myeloid leukemia: Data from the German AML Cooperative Group (AMLCG) 1992 Trial. Blood. 2003; 101 (1): 64-70.

23- Ferrara F, Palmieri S, Leoni F. Clinically useful prognostic factors in acute myeloid leukemia. Crit Rev Oncol Hematol. 2008; 66 (3): 181-193.

24- Langer C, Radmacher MD, Ruppert AS, et al. Cancer and Leukemia Group B (CALGB). High BAALC expression associates with other molecular prognostic markers, poor outcome, and a distinct gene-expression signature in cytogenetically normal patients younger than 60 years with acute myeloid leukemia: A Cancer and Leukemia Group B (CALGB) study. Blood. 2008; 111 (11): 5371-9.

25- Baldus CD, Tanner SM, Guimond M, et al. Overexpression of BAALC Suppresses Normal Hematopoiesis. Blood. 2002; 100 (11): 164b (abstract # 4147).

Journal of the Egyptian Society of Haematology & Research, Vol. 6, No. 2, September: 39-45, 2010

Transforming Growth Factor-ß1 C-509T Polymorphism and its Association with Prevalence and Severity of Asthma in Egyptian Children

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ABSTRACT

Background: Bronchial asthma is a complex genetic disorder regulated by the release of cytokines and inflammatory mediators. Transforming growth factor beta (TGF-ß1) cytokine plays a pivotal role in the inflammatory response of the airways. Differential production of this cytokine is associated with allelic variations in the transcriptional regulatory region of *TGF-ß1* gene.

Aims: The objective of the present study was to investigate the C-to-T single-nucleotide polymorphism (C–509T) in the *TGF-ß1* gene promoter for its association with bronchial asthma in children.

Material and Methods: DNA isolated from 84 asthmatic children and 55 control children was screened for this polymorphism using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method.

Results: Genotype frequencies of the C-509T polymorphisms showed no statistically significant differences between cases and controls regarding both CC and TT genotypes, however, the heterozygous CT genotype showed a significant increase in controls more than cases with a *p*-value 0.04. The interaction between these polymorphisms revealed statistically significant association between the high producer T-allele of *TGF-ß1* (in TT & CT genotypes) and asthma severity with a *p*-value 0.03.

Conclusion: Our results showed no significant association between the C-509T polymorphisms and the prevalence of asthma. However, our findings provide evidence that *TGF-ß1* plays an important role in determining disease severity in asthmatic children.

Key Words: *Bronchial asthma – Transforming growth factor-ß1.*

INTRODUCTION

Asthma is a chronic inflammatory disease of the airways characterized by recurrent episodes of airway obstruction and wheezing **[1]**. Airway inflammation and remodeling are critical pathophysiologic events in asthma. Genes involved in these processes are candidate genes for evaluation in association studies **[2]**. Transforming growth factor-ß1 (TGF-ß1) is a multifunctional cytokine that plays a critical role in cell growth and differentiation, immune modulation, airway development, and inflammation. TGF-ß1 is ubiquitously expressed in the lung and is involved in both normal cellular processes and numerous disease states **[3]**. Several lines of evidence suggest that TGF-ß1 contributes to the development of asthma, airway responsiveness, and airway remodeling. This cytokine has been also observed in the asthmatic airways and their bronchoalveolar lavage **[4]**. Differential production of this secreted cytokine is under genetic control and accumulating evidence indicates that functional polymorphisms in the *TGF-ß1* gene that affect its expression may modulate asthma occurrence. These allelic variations are attributed to the presence of single nucleotide polymorphisms (SNPs) in the *TGF-ß1* gene **[5]**. One such functional variant, a C-to-T base substitution at position –509 (i.e., C-509T) in the *TGF-ß1* gene promoter, increases *TGF-ß1* gene transcription **[6]** and plasma TGF-ß1 concentrations **[7]**. Although some epidemiologic studies found significant associations between C-509T and asthma occurrence in adults, data on childhood asthma are limited and inconsistent **[8]**.

The aim of this work was to investigate the C-to-T single-nucleotide polymorphism (C-509T) in the *TGF-ß1* gene promoter for its association with the incidence and severity of bronchial asthma in Egyptian children.

PATIENTS AND METHODS

Study population:

Eighty four children with bronchial asthma were enrolled in this study. They included 51 males (60.7%) and 33 females (39.3%), their ages ranged from 0.5 to 12 years old (mean= 4.6±2.9, median=4). All patients were under follow-up in the Chest Clinic of New Cairo University Children's Hospital (group I). Bronchial asthma was diagnosed according to GINA guidelines 2009 **[9]**. They were assessed for history of chest wheezes, dyspnea, cough and respiratory distress. Other symptoms of atopy as eczema, urticaria, conjunctivitis or rhinitis were also determined. Fifty five age and sex matched healthy children were enrolled in the study as control group (group II). They included 33 males (60%) and 22 females (40%), their ages ranged from 0.5 to 5 years old (mean=2.5± 0.9, median=2.6). Informed consents were obtained from the parents of participating children before enrollment and the study was approved by the ethical committee of the Faculty of Medicine, Cairo University.

Sample collection and laboratory investigations:

Blood samples were drawn from each child in groups I and II by sterile venipunctures for performing; a complete blood count including manual differential, genotyping following DNA extraction and determination of serum IgE (IMX, Abott technology).

Stool and urine analysis were performed to exclude eosinophilia caused by parasitic infestations.

Genotyping:

Genomic DNA was purified from collected peripheral blood samples using DNA extraction kit (Qiagen). The *TGF-ß1* genotypes were determined by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) technique as described by Lario, et al. **[10]**.

The primers' sequences for C509T gene locus are:

5' GGGACACCATCTACAGTG 3' (forward) and 5' GGGGACACCATCTACAGTG 3' (reverse). The extracted DNA was amplified in a reaction mixture of 50µl containing 25µl Taq PCR Master Mix (2.5 units Taq DNA polymerase, 1X Qiagen PCR buffer, 200µM of each dNTP (Qiagen), 2µl of each primer (0.4µM final concentration) and distilled water. PCR reactions were initially denaturated at 94ºC for 5min. Amplification was then carried out for 35 cycles, each cycle consisting of denaturation at 94ºC for 30s, annealing at 60ºC for 20s, extention at 72ºC for 30s and finally a 5min extension at 72ºC. This produced a 455bp fragment.

The PCR products were digested by the restriction enzyme AoC I (Fermentas). Seventeen µl of the PCR product, 2µl of the restriction enzyme buffer and 1µl of the enzyme were mixed and incubated for 16 hours at 37ºC. Digested DNA fragments were separated by electrophoresis on 2% agarose gel and visualized by ethidium bromide staining. A visible band at 429bp was significant for the wild CC genotype, a band at 455bp was significant for the homozygous TT genotype, while the presence of two bands at both 429bp and 455bp signified the heterozygous CT genotype.

Statistical analysis:

Data were collected and tabulated. Statistical Package for Social Science (SPSS) program version 17.0 was used for data analysis. Mean and standard deviation (SD) were estimates of quantitative data while frequency and percentage were estimates of qualitative data. Differences in clinical and biochemical characteristics were tested by Student's *t*-test or Mann Whitney U test, when appropriate, for continuous variables and by chi-square test for categorical data. A two-sided *p*-value <0.05 was considered statistically significant. Odds ratio were used to measure the strength of associations.

RESULTS

In our study, children with asthma were more likely to be boys than girls (60.7% versus 39.3%). Respiratory symptoms and atopic manifestations were recorded in the asthmatic children with variable presentations which are described in Table 1(a). While, seasonal variation, pattern of asthma and precipitating factors of asthmatic episodes in the studied cases are shown in Table 1(b).

According to the severity of the symptoms on the basis of GINA guidelines 2008 **[11]**, we categorized our cases into 3 groups; those with mild persistent symptoms constituted 31% of cases, those with intermittent symptoms constituted 35.7% of cases, and those with moderate persistent symptoms constituted 33.3% of cases while none of them had severe persistent symptoms.

The peripheral blood eosinophilic count and serum IgE levels showed a highly significant increase in the cases as compared to controls (p=0.000 for both) as shown in Table (2).

Total serum IgE of patients showed a significant positive correlation with the peripheral blood eosinophils (r=0.3 and p=0.0009) as shown in Fig. (1).

Results of the PCR-RFLP are shown in Fig. (2) demonstrating the different patterns of polymorphisms of the *TGF-ß1* gene. Genotype frequencies of the C-509T polymorphisms showed no statistically significant differences between cases and controls except for the heterozygous CT genotype with a p-value 0.04 (Table 3).

The presence of the mutant T-allele (in TT & CT genotypes) showed a borderline significant increase in males than in females among the asthmatic children included in our study (p=0.05). We also recorded a significant association between the severity of asthma and the presence of T-allele in TT & CT genotypes (p=0.03). There were no other statistically significant associations of the different genotypes with either the eosinophilic count or the serum IgE levels (Table 4).

Table (1a): Distribution of symptoms among 84 bronchial asthma cases.

(a) Symptoms	Number	Percentage
Upper respiratory:		
Sneezing	82	97.6
Rhinorrhea	77	91.6
Snuffling	22	26.2
Itchy nose	22	26.2
Chest symptoms:		
Wheezes	84	100
Dyspnea	84	100
Cough	84	100
Tightness	35	41.6
Respiratory distress	79	94
Atopic manifestations:		
Eczema	7	8.3
Urticaria	29	34.5
Conjunctivitis	7	8.3
Rhinitis	4	4.7

Table (1b): Characteristics of wheezing episodes in 84 asthmatic children.

(b) Characteristics of wheezing episodes	Number	Percentage
Season:		
Winter	66	78.5
Perennial	15	17.8
Spring	2	2.4
Pattern:		
Paroxysmal	84	100
Precipitating factors:		
Infection	77	91.6
Passive smoking	71	84.5
Exercises	57	67.8
Dust	7	8.3
Diet	22	26.2
Severity:		
Intermittent	30	35.7
Mild persistent	26	31
Moderate persistent	28	33.3

Table (2): Eosinophils and serum IgE levels in asthmatic children as compared to controls.

Parameter	Cases (N=84)	Controls (N=55)	p-value
Eosinophilic count	751.9±513.8 $^{+}$ 11-2019 $^{++}$	311.8±120.7 $^{+}$ 79-893 $^{++}$	0.000*
Ig E levels	136.6±111.1 $^{+}$ 5-500 $^{++}$	38.4±44.1 $^{+}$ 6-320 $^{++}$	0.000*
IgE >100 N (%)	38 (45.2%)	3 (5.5%)	0.000*

+: Mean ± SD, ++: Range, *: p-value >0.01: Highly significant difference.

Table (3): *TGFß1* C-509T genotypic frequencies in 84 bronchial asthma cases as compared to controls.

C-509T genotype	Cases N (%)	Controls N (%)	*p*-value	Odds ratio	95% Confidence Interval
Wild type CC	23 (27.4)	8 (14.5)	0.08	2.2	0.9-5.4
Base exchange TT	16 (19)	8 (14.5)	0.5	0.7	0.3-1.
Heterozygous CT	45 (53.6)	39 (70.9)	0.04*	2.1	1.03-4.4

Table (4): Association between the severity of asthma and the presence of TGF-ß1 genotype among 84 children with bronchial asthma.

Genotype \ Severity of asthma	Intermittent N (%)	Mild persistent N (%)	Moderate persistent N (%)	*p*-value
Presence of T allele (TT or CT)	23 (76.7)	14 (53.8)	24 (85.7)	0.03*
Absence of T allele (CC)	7 (23.3)	12 (46.2)	4 (14.3)	

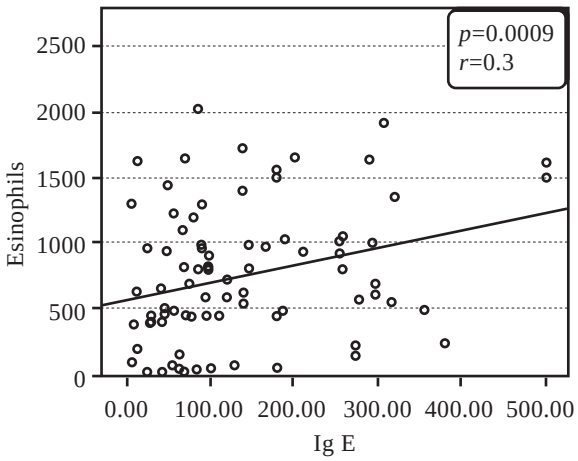

Fig. (1): Pearson correlation between IgE levels and eosinophilic counts among 84 children with bronchial asthma (*r*=0.3 & *p*=0.0009).

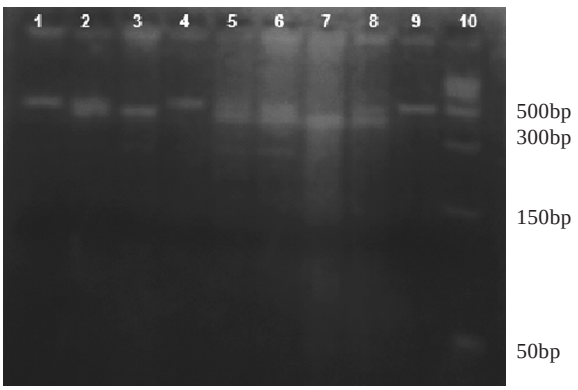

Fig. (2): PCR-RFLP of the *TGF-ß1* gene polymorphism.

- Lanes 1, 4, and 9 show homozygous TT genotype with single band at 455bp.
- Lanes 2, 5, 6 and 8 show heterozygous cases with two bands at 429 and 455bp respectively.
- Lanes 3 and 7 show homozygous wild CC genotype with single band at 429bp.
- Lane 10: Molecular weight marker (Promega), with the following base pairs: 50,150,300,500,750 and 1000 respectively.

DISCUSSION

Asthma is known as a complex genetic disease with a broad range of phenotypes distinguished by hyper responsiveness and airway inflammation to various extrinsic and intrinsic stimuli **[12]**. Quite clearly, more emphasis is now laid on the role of immunological mechanisms involved in the process of inflammation. Cytokines are cell signaling proteins playing a central role in immunological and inflammatory mechanisms by relaying the necessary instruction to their target cell via specific receptor(s) in an autocrine, paracrine and endocrine fashion **[5]**.

TGF-ß1 is a multifunctional cytokine that is increased in the airways of individuals with asthma compared with those without asthma and is further increased in patients with status asthmaticus **[13,14]**. The increased TGF-ß1 is localized principally in the extracellular connective tissue of the subepithelial space of the airways in association with the binding proteoglycan decorin **[15]**; however, the precise cellular source of increased TGF-ß1 in the airways of individuals with asthma is unknown **[6]**. TGF-ß1 is secreted as a latent complex that must be cleaved via proteases, acid, or reactive oxygen species to become active **[16]**. Although multiple mechanisms are involved, transcriptional mechanisms have a very important role in the control of TGF-ß1 activity, and are regulated by inflammatory molecules found in the airways of individuals with asthma **[17]**.

The expression of TGF-ß1 is influenced by polymorphisms in the *TGF-ß1* gene, and some of these polymorphisms may be associated with

asthma and other diseases **[18,19,20]**. In particular, there is a C-to-T promoter polymorphism at base pair position –509 that alters a Yin Yang 1 (YY1) transcription factor consensus binding site (–CCATCTC/TG–) and is associated with higher circulating concentrations of TGF-ß1 in plasma. Grainger and colleagues **[7]** reported that the C-509T SNP accounted for 8.2% of the additive genetic variance of plasma TGF-ß1 concentrations. It has been hypothesized that the T allele enhances the YY1 binding site on the *TGF-ß1* promoter and is responsible for increased *TGF-ß1* transcription. Silverman and colleagues **[6]** demonstrated that the –509T SNP increases the TGF-ß1 promoter affinity for the transcription factor Yin Yang 1 by 30% resulting in a 30% increase in basal promoter activity. Taken together, findings from these studies indicate that the –509C SNP is likely to be a genetic determinant of levels of TGF-ß1 and support involvement of this SNP in asthma pathogenesis **[2]**.

In the present study, we utilized the PCR-RFLP technique to determine the C–509T gene polymorphism in the *TGF-ß1* gene promoter and to assess its association with the incidence and severity of bronchial asthma in Egyptian children compared to a control group of similar age and sex distribution and with no history for asthmatic episodes.

Regarding the age groups on which the study was performed, it was limited to childhood as this age harbors most of asthma reporting cases and high prevalence **[21]**; moreover, asthma exacerbations are among the leading causes of morbidity in children and have resulted in increased healthcare expenditures in the pediatric population over the last decade **[22]**.

Asthmatic children, included in our study, were more likely to be boys than girls (60.7% versus 39.3%). This increased risk of recurrent wheezing in males coincides with other studies that stated that asthma is more common in males compared to females **[2]**. Gissler et al., (1999) **[23]** reported that this could be related to the narrower airways, increased airway tone and possibly higher IgE levels in boys.

On studying the genotypic analysis of both groups, we found that genotype frequencies of the C-509T polymorphisms were as follows; the homozygous CC wild genotype was more prevalent in cases than controls (27.4% Vs 14.5%) while the TT mutant homozygous genotype was slightly increased in cases than controls (19% Vs 14.5%) but without statistically significant differences for both genotypes, however, there was a significant increase in the heterozygous CT genotype within controls when compared to cases (70.9% Vs 53.6%, *p*-value 0.04). When comparing the presence or absence of T allele between cases and controls, we found that the T allele being the high producer of TGF-ß1 was detected more among controls (85.5%) than cases (72.6%); however, this difference is not statistically significant (*p*-value 0.08, odds ratio 2.2). This is in accordance with some authors who failed to find significant associations between C-509T and asthma occurrence in children **[24,25]** or adults **[26,27]**; however, these findings are against many studies that found that the TT genotype is associated with an increased risk of asthma **[2,6,8,28,29]**. Also, Kumor, et al. **[5]** observed that individuals carrying T allele are at greater risk of developing asthma. However, in a study by Sharma et al., the TT and CT genotypes of a coding polymorphism in *TGF-ß1* were inversely associated with asthma exacerbations **[4]**.

The plausible reason for this controversy could be either the diverse ethnic background of the studied populations or the diverse etiologies that may contribute to the development of asthma, which might suggest that the physiological basis of this polymorphism might have a broader role in the susceptibility to asthma. Furthermore, determination of the levels of TGF-ß1 in serum or bronchoalveolar lavage fluid might prove helpful in better understanding of the role of this cytokine in asthma.

Another explanation would be attributable to the fact that there are at least two possible mechanisms by which TGF-ß1 may impact the development and severity of asthma. Some studies suggest that increased TGF-ß1 has a beneficial role in asthma by suppressing airway inflammation and hyper-responsiveness through the inhibition of T lymphocytes, dendritic cells, eosinophils, and mast cells. In this way, TGF-ß1 may be part of a negative-feedback loop, turning off inflammation that augments its production **[30]**. Under this paradigm, genetic variants that are associated with increased TGF-ß1 activity, such as the T allele of C–509T, would

be expected to be associated with decreased asthma prevalence or decreased asthma severity. Other studies suggest that TGF-ß1 has harmful effects in the airways of individuals with asthma. TGF-ß1 is profibrotic, and its sustained elevation may stimulate airway remodeling. Under this paradigm, genetic variants that are associated with increased TGF-ß1 activity, such as the T allele of C–509T, would be expected to be associated with higher asthma prevalence or increased asthma severity **[6]**. We have detected a statistically significant association between the presence of the high producing T allele and asthma severity in our asthmatic children (*p*-value 0.03). Pulleyn and coworkers **[31]**, also reported an increased frequency of homozygous 509T allele in severely asthmatic patient group compared to the mild and control groups included in their study. The same results were obtained in a study by Salam and coworkers **[2]** who mentioned that the TT genotype had a 5 fold increased risk of early persistent asthma. These findings are also supported by the study of Silverman and coworkers **[6]**.

The presence of the mutant T-allele (in TT & CT genotypes) showed a borderline significant increase in males than in females among the asthmatic children included in our study (p=0.05). But this may be due to unequel gender distribution among the studied population.

As regards the absolute eosinophilic count and the total IgE levels, there was a highly significant increase in these two atopic markers within the cases compared to the control group (p=0.000 for both). This is in accordance with several authors who stated that increased IgE levels and absolute eosinophilia are hallmarks for the diagnosis of bronchial asthma. A particular challenge is to distinguish children with transient wheezing from those whose wheezing persists and later develop asthma. Airway tissue inflammation leading to airway remodeling occurs at an early age and is fundamental for the development of asthma. Thus characteristic features of inflammation such as eosinophilia and increased IgE levels can be used to distinguish asthma-related wheezing from wheezing caused by viral infection **[32]**.

We found no association between 509T allele and IgE levels or eosinophil count. Our results are in agreement with other studies that found inconsistent associations between C-509T polymorphism and atopic markers like serum IgE, eosinophil count and positive skin test to allergens **[6,8,25]**. Therefore, it is unlikely that the relationship of *TGF-ß1* C-509T with asthma is primarily due to effects of this polymorphism on atopy.

In conclusion our study failed to find an association between the *TGF-ß1* C-509T polymorphisms and the prevalence of asthma taking into consideration the relatively limited number of the studied groups. However, our findings provide further evidence that TGF-ß1 plays an important role in determining disease severity in asthmatic children.

REFERENCES

1- Cookson WO, Moffatt MF. Genetics of asthma and allergic disease. Mol Genet. 2000; 9: 2359-64.

2- Salam MT, Gauderman WJ, McConnell R, et al. Transforming Growth Factor-ß1 C-509T Polymorphism, Oxidant Stress, and Early-Onset Childhood Asthma. Am J Respir Crit Care Med. 2007; 176: 1192-1199.

3- Duvernelle C, Freund V, Frossard N. Transforming growth factor-beta and its role in asthma. Pulm Pharmacol Ther. 2003; 16: 181-196.

4- Sharma S, Raby BA, Hunninghake GM, et al. Variants in TGFB1, Dust Mite Exposure, and Disease Severity in Children with Asthma. Am J Respir Crit Care Med. 2009; 179: 356-362.

5- Kumar A, Gupta V, Changotra H, et al. Tumor necrosis factor-α and transforming growth factor-ß1 polymorphisms in bronchial asthma. Indian Journal of Medical sciences. 2008; 62: 323-330.

6- Silverman ES, Palmer LJ, Subramaniam V, et al. Transforming growth factor-beta1 promoter polymorphism C-509T is associated with asthma. Am J Respir Crit Care Med. 2004; 169: 214-219.

7- Grainger DJ, Heathcote K, Chiano M, Snieder H, Kemp PR, Metcalfe JC, Carter ND, Spector TD. Genetic control of the circulating concentration of transforming growth factor type ß1. Hum Mol Genet 1999; 8: 93-97.

8- Li H, Romieu I, Wu H, Sienra-Monge J-J, Ramírez-Aguilar M, del Río-Navarro BE, del Lara-Sanchez IC, Kistner EO, Gjessing HK, London SJ. Genetic polymorphisms in transforming growth factor beta-1 (TGF-ß1) and childhood asthma and atopy. Hum Genet. 2007; 121: 529-538.

9- Global Strategy for Asthma Management and Prevention, Global Initiative for Asthma (GINA) 2009, available from :hhh://www.ginasthma.org/.

10- Lario S, Inigo P, Compistol J, et al. Restriction enzyme based method for TGFB1 genotyping: Non-isotopic detection of polymorphism in codons 10 and 25 and

the 5' flanking region. Clinical Chemistry. 1999; 45: 1290-1292.

11-Global Strategy for Asthma Management and Prevention, Global Initiative for Asthma (GINA) 2008, available from :hhh://www.ginasthma.org/.

12- Barnes PJ. Cytokines as mediators of chronic asthma. J Respir Crit Care Med. 1994; 150: S42-9.

13- Redington AE, Madden J, Frew AJ, Djukanovic R, Roche WR, Holgate ST, Howarth PH. Transforming growth factor-ß1 in asthma: Measurement in bronchoalveolar lavage fluid. Am J Respir Crit Care Med. 1997; 156: 642-647.

14- Tillie-Leblond I, Pugin J, Marquette CH, Lamblin C, Saulnier F, Brichet A, Wallaert B, Tonnel AB, Gosset P. Balance between proinflammatory cytokines and their inhibitors in bronchial lavage from patients with status asthmaticus. Am J Respir Crit Care Med. 1999; 159: 487-494.

15- Redington AE, Roche WR, Holgate ST, Howarth PH. Co-localization of immunoreactive transforming growth factor-ß1 and decorin in bronchial biopsies from asthmatic and normal subjects. J Pathol 1998; 186: 410-415.

16- Blobe GC, Schiemann WP, Lodish HF. Role of transforming growth factor ß in human disease. N Engl J Med. 2000; 342: 1350-1358.

17- Bellocq A, Azoulay E, Marullo S, Flahault A, Fouqueray B, Philippe C, Cadranel J, Baud L. Reactive oxygen and nitrogen intermediates increase transforming growth factor-ß1 release from human epithelial alveolar cells through two different mechanisms. Am J Respir Cell Mol Biol. 1999; 21: 128-136.

18- Awad MR, El-Gamel A, Hasleton P, Turner DM, Sinnott PJ, Hutchinson IV. Genotypic variation in the transforming growth factor-ß1 gene: Association with transforming growth factor-ß1 production, fibrotic lung disease, and graft fibrosis after lung transplantation. Transplantation. 1998; 66: 1014-1020.

19- Watanabe Y, Kinoshita A, Yamada T, Ohta T, Kishino T, Matsumoto N, Ishikawa M, Niikawa N, Yoshiura K. A catalog of 106 single-nucleotide polymorphisms (SNPs) and 11 other types of variations in genes for transforming growth factor-ß1 (TGF-ß1) and its signaling pathway. J Hum Genet. 2002; 47: 478-483.

20- Xaubet A, Marin-Arguedas A, Lario S, Ancochea J, Morell F, Ruiz-Manzano J, Rodriguez-Becerra E, Rodriguez-Arias JM, Inigo P, Sanz S, et al. Transforming growth factor-ß1 gene polymorphisms are associated with disease progression in idiopathic pulmonary fibrosis. Am J Respir Crit Care Med. 2003; 168: 431-435.

21- Sublett J. The environment risk factors for atopy and allergy. Current Allergy and Asthma 2005; 5: 445-450.

22- Masoli M, Fabian D, Holt S, Beasley R. The global burden of asthma: executive summary of the GINA Dissemination Committee Report. Allergy. 2004; 59: 469-478.

23- Gissler M, Jarvelin M, Louhiala P, Hemminiki E. Boys have more health problems in childhood than girls; follow-up of the 1987 birth cohort. Acta Pediatr. 1999; 88: 310-314.

24- Hobbs K, Negri J, Klinnert M, Rosenwasser LJ, Borish L. Interleukin-10 and transforming growth factor-beta promoter polymorphisms in allergies and asthma. Am J Respir Crit Care Med. 1998; 158: 1958-1962.

25- Boulet LP, Lemiere C, Archambault F, Carrier G, Descary MC, Deschesnes F. Smoking and asthma: Clinical and radiologic features, lung function, and airway inflammation. Chest. 2006; 129: 661-668.

26- Buckova D, Izakovicova Holla L, Benes P, Znojil V, Vacha J. TGF-beta1 gene polymorphisms. Allergy. 2001; 56: 1236-1237.

27- Hakonarson H, Bjornsdottir US, Ostermann E, Arnason T, Adalsteinsdottir AE, Halapi E, Shkolny D, Kristjansson K, Gudnadottir SA, Frigge ML, et al. Allelic frequencies and patterns of single-nucleotide polymorphisms in candidate genes for asthma and atopy in Iceland. Am J Respir Crit Care Med. 2001; 164: 2036-2044.

28- Mak JC, Leung HC, Ho SP, Law BK, Ho AS, Lam WK, Ip MS, Chan-Yeung MM. Analysis of TGF-beta(1) gene polymorphisms in Hong Kong Chinese patients with asthma. J Allergy Clin Immunol. 2006; 117: 92-96.

29- Huiling L, Romieu I, Hao W, Monge JS, Aguilar M, et al. Genetic polymorphisms in transforming growth factor beta-1 (TGFB1) and childhood asthma and atopy. Hum Genet. 2007; 121: 529-538.

30- Nakao A. Is TGF-ß1 the key to suppression of human asthma? Trends Immunol. 2001; 22: 115-118.

31- Pulleyn LJ, Newton R, Adcock IM, Barnes PJ. TGFß1 allele association with asthma severity. Hum Genet. 2001; 109: 623-627.

32- Lemansue RF. Inflammation in childhood asthma and other wheezing disorders. Pediat Ann. 2002; 69; 368-373.

Journal of the Egyptian Society of Haematology & Research, Vol. 6, No. 2, September: 47-54, 2010

Polymorphisms of GSTT1 and GSTM1 Genes in Diffuse Large B Cell Lymphoma Egyptian Patients

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ABSTRACT

Background: Evidence, although not so extensive, did show that genetic variations in glutathione S-transferase (GST) might be associated with risk of developing lymphoma; overall and/or subtype. Genetic data on the contribution of GST genes in the prognosis of lymphomas is scarce even in occupationally exposed populations.

Aim: The study aimed to investigate the influence of polymorphisms in GSTM1 and GSTT1 genes on both the risk and prognosis of diffuse large B-cell lymphoma (DLBCL).

Subjects and Methods: The study included newly diagnosed 83 DLBCL cases and 89 age and gender matched control subjects. DLBCL patients underwent IPI scoring, CHOP treatment and follow-up for treatment outcome, relapse/progression and death. For all subjects, GSTM1 and GSTT1 genotyping was performed by a multiplex PCR protocol.

Results: We found 2.35 fold increase in the risk of DLBCL associated with GSTT1-null genotype (OR=2.35, 95% CI: 1.02-5.40, p=0.04). Three times increased risk in individuals with the GSTM1/T1-double null genotype (OR3.06, 95% CI: 1.04-8.95, p=0.03) compared with both GSTM1 and GSTT1 genes undeleted (wild genotype) was observed. Patients; overall and those with favorable IPI (<3), showing one GST-null genotype and those showing GSTM1/T1-double null genotype significantly had better progression free survival (PFS) and overall survival (OS) when compared with those showing both GST wild genes. Multivariate analysis showed that the presence of at least one GST-null genotype was associated with a 60% reduced risk of relapse/progression and 71% reduced risk of death.

Conclusion: Our results have shown a role for the GSTT1-null genotype and the GSTM1/T1-double null genotype as risk factors for DLBCL. The presence of at least one GST-null genotype tended to have a positive prognostic value for DLBCL patients independent from both the IPI score and the treatment outcome. Large studies on rituximab plus CHOP treated patients will be needed to either support or modify these findings.

Key Words: *GSTT1 – GSTM1 – DLBCL – Egyptian.*

INTRODUCTION

B-cell lymphomas comprise the majority of cases of non-Hodgkin lymphomas (NHL) and, of these, diffuse large B-cell lymphoma (DLBCL) is the major subtype. DLBCL is the most common of the aggressive NHL **[1]**.

For the vast majority of patients, the etiology of diffuse large B-cell lymphoma is unknown. Factors thought to potentially confer increased risk include immunosuppression (including AIDS, and iatrogenic etiologies in the setting of transplantation or autoimmune diseases), ultraviolet radiation, pesticides, hair dyes, and diet **[2]**. Implicated pesticides include phenoxyacetic acid herbicides, organophosphate insecticides, triazine herbicides and fungicides **[3]**.

Established adverse prognostic factors for Non-Hodgkin lymphoma (NHL), as delineated in the International Prognostic Factor Index (IPI), include older age at diagnosis, higher tumor stage, poor performance score, extranodal involvement, and above-normal lactate dehydrogenase **[4]**.

Glutathione S-transferases (GSTM1, GSTT1, GSTP1) are involved in the detoxification of a wide range of carcinogens, including benzene, organochlorine compounds, organophosphate pesticides, tobacco smoke, chemotherapeutic agents, and reactive oxygen species **[5]**.

Polymorphisms in genes that code various types of GSTs manifest as decreased or lack of enzyme activity **[6]**, prompting the hypothesis that allelic variants may be associated with an

impaired detoxification capacity as well as drug metabolism. Subsequently, they may play a role in increased susceptibility to cancer and may also influence tumor-response to anticancer drugs.

Evidence, although not so extensive, did show that genetic variations in GST might be associated with risk of developing NHL overall and/or subtype **[7,8,9]**. Genetic data on the contribution of GST genes in the prognosis of lymphomas is scarce even in occupationally exposed populations.

Genetic polymorphism that confers susceptibility to or protection from certain cancer type may be quite different in different ethnic populations and the existence of racial and ethnic disparities in healthcare access and outcomes is well-documented.

To ascertain whether there is any association between GST gene polymorphisms and DLBCL risk in Egyptian population, we determined the frequencies of GSTT1 and GSTM1 gene polymorphisms in Egyptian population inhabiting a farming locality (Menofiya governorate) and demonstrating a known insecticide exposure and compared them with the frequencies found in age and gender matched control subjects inhabiting the same locality. Moreover, we studied the implications of these gene polymorphisms on DLBCL patients' prognosis and survival.

SUBJECTS AND METHODS

Subjects:

The study included 83 DLBCL patients (median age 60 years, range 18-84 years; 26 females and 57 males). Forty five bone marrow samples (withdrawn during routine staging of patients) and 38 Peripheral blood samples were obtained at the time of initial diagnosis. All were treated with standard chemotherapy regimen (CHOP) Doxorubicin 50mg/m^2 on day 1, Cyclophosphamide 750mg/m^2 on day 1, Vincristine 1.5mg/m^2 on day 1, Prednisone 100mg on day 1-5. Cycle was repeated every 3 weeks. Patients who achieved complete remission by the 4th cycle continued up to six cycles (for stage I and II disease) or eight cycles (for advanced disease). Patients were evaluated every 2 cycles and after completion of all planed cycles of CHOP to score response according to international working group criteria **[10]**. Patients who did not enter into complete remission or those who relapsed were shifted to second line chemotherapy; DHAP (dexamethasone, cytarabine and cisplatin) or ICE (ifosfamide, carboplatin and etoposide). None of the patients underwent bone marrow transplantation after relapse. Peripheral blood samples from 89 age and gender matched control subjects (median age 57 years, range 18-85 years; 26 females and 64 males), were also obtained. Informed consent was obtained from all subjects included in the study. The procedures followed were in accordance with the ethical standards of the responsible institutional committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 1983.

GSTT1 and GSTM1 genotyping:

DNA was extracted from bone marrow / peripheral blood leukocytes using DNA purification kit (QIAamp®DNA Blood Mini Kit, Qiagen, 28159 Avenue Stanford, Valencia, USA). DNA was quantified spectrophotometrically at 260nm and 280nm and stored at –20°C. The DNA quality was checked using *ß*-globin gene amplification (indicated by a 273-bp fragment) as a control gene using sense 5'-CAA CTT CAT CCA CGT TCA CC 3' and antisense 5'-GAA GAG CCA AGG ACA GGT AC 3'. Genotyping of the GSTM1 and GSTT1 loci was carried out by a multiplex PCR reaction in a Perkin Elmer thermal cycler (Gene amp, PCR system 2400 PERKIN ELMER, version 2.11, USA). The genotypes were analyzed according to the protocol of Arand et al. **[11]**. Genomic DNA was amplified by using 2 sets of primers: GSTM1 (F): 5'-GAA CTC CCT GAA AAG CTA AAG C-3'; GSTM1 (R): 5'GTT GGG CTC AAA TAT ACG GTG G-3'; GSTT1 (F): 5'-TTC CTT ACT GGT CCT CAG ATC TC-3'; GSTT1(R): 5'-TCA CCG GAT CAT GGC CAG CA-3'. Reaction mixture was 50µL containing 1µg DNA, 200µM dNTPs, 2.5U Taq polymerase (DreamTaq™ DNA Polymerase, Fermentas Inc. USA, 798 Cromwell Park Drive, Glen Burnie, MD 21061), 0.5µM of GSTM1 and 0.3µM of GSTT1 primers. A total of 40 PCR cycles with denaturation at 94°C for 1 minute, annealing at 59°C for 1 minute, and extension at 72°C for 1 minute were conducted. An initial DNA denaturation at 95°C and a final extension at 72°C were carried out for 5 minutes each. The PCR product was then subjected to

electrophoresis on a 2% agarose gel. The presence of bands of 480 and 215bp was indicative of the GSTT1 and GSTM1 genotypes respectively, whereas the absence indicated the null genotype for that gene (Fig. 1). This method cannot differentiate between the wild type and heterozygous state. It determines only homozygous deletion of GSTM1 and/or GSTT1 genes.

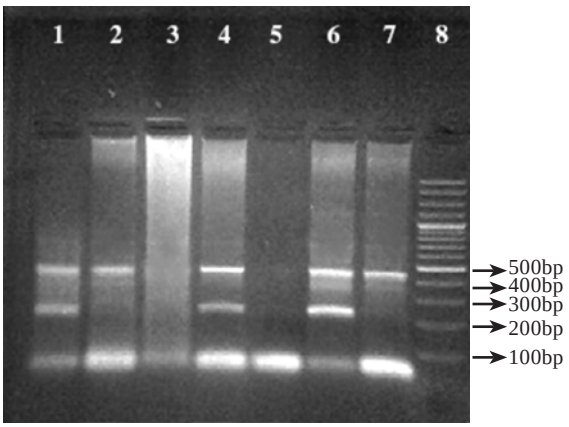

Fig. (1): Agarose gel electrophoresis of PCR products after amplification. Lane 8 shows the DNA marker, lane 5 shows non-template control, lanes 1,4 and 6 show both GSTM1 and GSTT1 undeleted genotype, lanes 2 and 7 show GSTM1-null genotype, lane 3 shows GST double-null genotype.

Statistical analysis: The frequency of the various enzyme genotypes in NHL cases and controls were compared using Fisher's exact tests. Odds ratios (OR) express the relative risk of NHL with a specific genotype. Survival curves were estimated using the Kaplan-Meier product limit method. Differences in the survival curves were evaluated with the log-rank test. Survival analyses were done for both overall survival (OS) and progression-free survival (PFS). In OS analysis, deaths were events and being alive was censoring. In PFS analysis, relapses/progressions were events and otherwise were censorings. Cox regression was used for multivariate models that analyzed differences between groups, including patient characteristics with strongest prognostic significance in the univariate analysis (IPI, treatment outcome and GSTM1/T1 genotyping).

RESULTS

The study included 83 DLBCL patients and 89 age and gender matched control subjects. Patient characteristics are detailed in Table (1).

Table (1): DLBCL patient characteristics.

	Total cases N = 83	
Age (year):		
X ± SD	56.95±14.17	
Median	60	
Range	84-18	
LDH (U/L):		
X ± SD	846.86±245.26	
Median	800	
Range	430-1500	
Performance score:	No	%
0	9	10.8
1	26	31.3
2	48	57.8
Stage:		
1	5	6.0
2	28	33.7
3	40	48.2
4	10	12.0
International prognostic index:		
Low	5	6.0
Low intermediate	30	36.1
High intermediate	28	33.7
High	20	24.1
Treatment response:		
CR	71	85.5
PR	12	14.5

X ± SD = Mean ± standard deviation.
LDH = Lactate dehydrogenase.
CR = Complete remission.
PR = Partial remission.

The frequency of GST deletions in 83 Egyptian patients with DLBCL was as follows: GSTM1-null: 62.7% (52/83); GSTT1-null: 22.9% (19/83); GSTM1/T1-double null: 18.1% (15/83). The frequency of GST deletions in 89 Egyptian age and gender matched control subjects was as follows: GSTM1-null: 58.4% (52/89); GSTT1-null: 11.2% (10/89); GSTM1/T1-double null: 6.7% (6/89) (Tables 2,3).

Consequently there was 2.35 fold increase in the risk of DLBCL associated with GSTT1 null genotype (OR=2.35, 95% CI: 1.02-5.40, p=0.04). No significant differences were found in the frequency of GSTM1, genotype in DLBCL cases and controls.

When individuals with both GSTM1 and GSTT1 genes undeleted were considered as a reference, analysis demonstrated comparatively 3 times increased risk for DLBCL in individuals with the GSTM1/T1-double null genotype (OR3.06, 95% CI: 1.04-8.95, p=0.03) (Table 3).

Table (2): Comparison of GSTM1 and GSTT1 genotype frequencies in DLBCL patients and controls (univariate analysis).

Parameter	Studied groups		*p*-value	OR (95% CI)
	Patients N = 83	Controls N = 89		
GSTM1:				
Present	31 (37.3%)	37 (41.6%)	0.57	1.0 (Reference)
Null	52 (62.7%)	52 (58.4%)		1.19 (0.65-2.20)
GSTT1:				
Present	64 (77.1%)	79 (88.8%)	0.04	1.0 (Reference)
Null	19 (22.9%)	10 (11.2%)		2.35 (1.02-5.40)

GST = Glutathione S-transferase gene.
p-value = Probability of error.
$p \leq 0.05$ (Significant).
OR = Odds ratio.
CI = Confidence interval.

Table (3): Combined effects of GSTM1 and GSTT1 null genotypes (multivariate analysis).

		Studied groups		*p*-value	OR (95% CI)
		Patients N = 83	Controls N = 89		
GSTM1:	*GSTT1:*				
Present	Present	27 (32.5%)	33 (37.1%)		1.0 (Reference)
Null	Present	37 (44.6%)	46 (51.7%)	0.96	0.98 (0.50-1.92)
Present	Null	4 (4.8%)	4 (4.5%)	0.78	1.22 (0.28-5.35)
Null	Null	15 (18.1%)	6 (6.7%)	0.03	3.06 (1.04-8.95)

GST = Glutathione S-transferase gene.
p-value = Probability of error.
$p \leq 0.05$ (Significant).
OR = Odds ratio.
CI = Confidence interval.

We found no association between GST genotypes and IPI score. We also found no effect of GST genotypes on the probability to achieve complete remission.

Both Patients showing one GST-null genotype (GSTM1-null genotype or GSTT1-null genotype) and those showing GSTM1/T1-double null genotype significantly had better PFS (p=0.02) and OS (p=0.005) when compared with those showing both or wild GST genes undeleted or wild (Fig. 3A).

Both the IPI score and the treatment outcome proved to be a strong prognostic parameters in our patient group regarding both PFS and OS (p<0.001) (Fig. 2). The probability of PFS and OS in patients with a favorable IPI score (<3) significantly differed according to the GST genotype; patients with one GST deletion and those with GSTM1/T1-double null genotype showed significantly better PFS and OS (p<0.001) (Fig. 3B), while in patients with IPI score ≥3 the OS significantly differed (p=0.001) and the PFS did not differ (p=0.49) according to the GST genotype (Fig. 3C).

Multivariate analysis using Cox regression showed that the presence of at least one GST-null genotype was associated with a 60% reduced risk of relapse/progression (HR=0.40, 95% CI: 0.23-0.69, p=0.001), and 71% reduced risk of death (HR=0.29, 95% CI: 0.16-0.51, p<0.001) (Table 4) i.e. the presence of at least one GST-null genotype tended to have a positive prognostic value independent from both the IPI score and the treatment outcome.

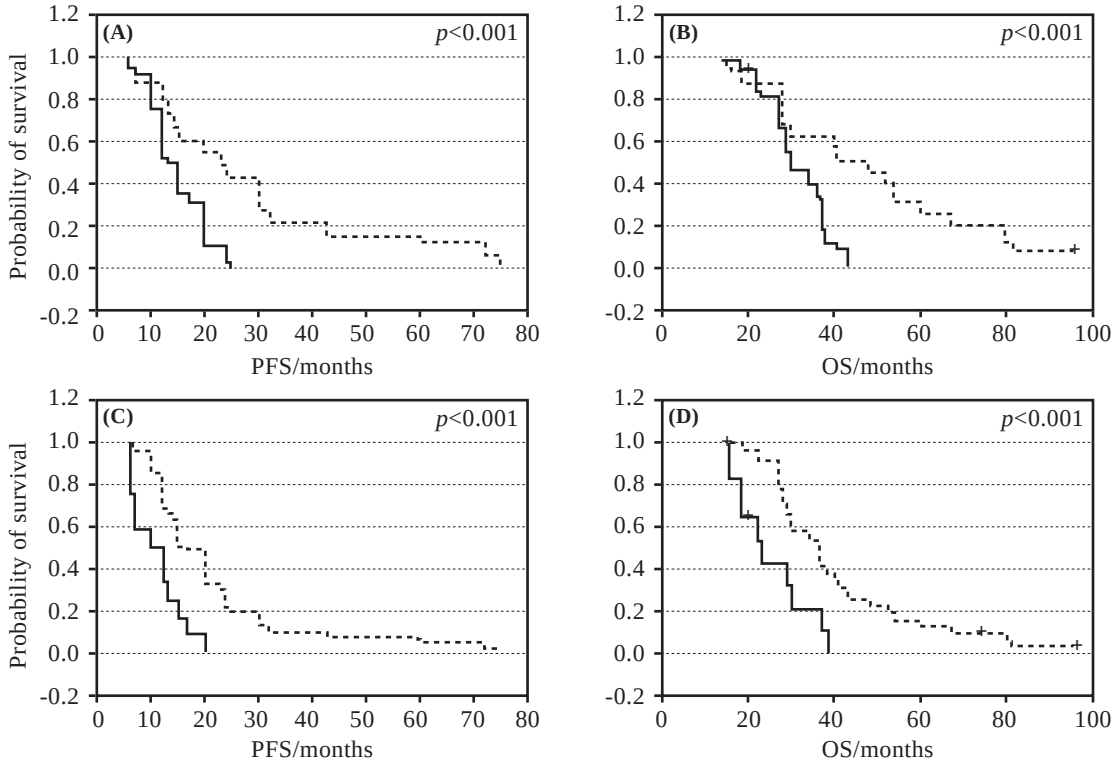

Fig. (2): Progression free survival (PFS) and overall survival (OS) in 83 DLBCL patients according to IPI score (A, B) (---- low IPI, – high IPI, + censored) and treatment outcome (C, D) (---- complete remission, – partial remission, + censored

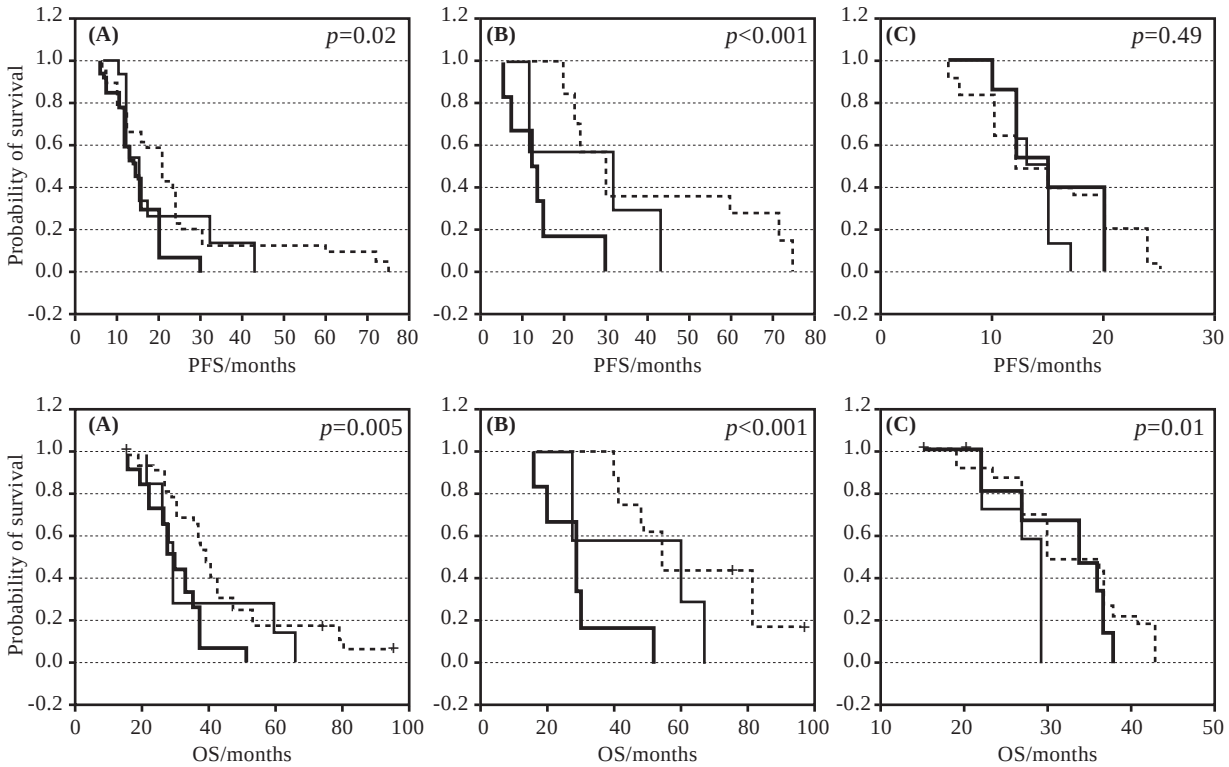

Fig. (3): Progression free survival (PFS) and overall survival (OS) in 83 DLBCL patients (A), in low IPI (<3) patient group (B) and in high IPI (≥3) patient group (C) according to GSTM1 and GSTT1 genotypes (---- one GST deletion, – double-null genotype, **–** wild genotype, + censored).

Table (4): Multivariate Cox regression analysis of variables affecting progression free survival and overall survival in DLBCL patients.

	Hazard ratio	95% CI	*p*-value
A- *Progression free survival:*			
IPI (≥3 vs. <3)	1.98	(1.43 – 2.75)	<0.001
Treatment outcome (PR vs. CR)	2.81	(1.37 – 5.75)	0.005
GST (any deletion vs. wild genotype)	0.40	(0.23 – 0.69)	0.001
B- *Overall survival:*			
IPI (<3 vs. ≥3)	2.29	(1.60 – 3.28)	<0.001
Treatment outcome (PR vs. CR)	3.37	(1.57 – 7.24)	0.002
GST (any deletion vs. wild genotype)	0.29	(0.16 – 0.51)	<0.001

GST = Glutathione S-transferase gene.
IPI = International prognostic index.
p-value = Probability of error.
$p \leq 0.05$ (significant).
OR = Odds ratio.
CI = Confidence interval.

DISCUSSION

GST genotyping was applied in a total of 172 unrelated Egyptian subjects for GSTM1 and GSTT1 genes. Subjects were categorized into 83 DLBCL patients and 89 age and gender matched control subjects. Our hospital (Menofiya University Hospital, Sheben El-Kom, Egypt) is a university hospital serving a rural geographical area. Controls were randomly selected from the same region. This is a farming locality with a known insecticide exposure for inhabitants.

Two studies **[12,13]** investigated GST genotypes on Egyptian subjects. The frequencies of the tested genes in our control Egyptian subjects showed that the frequency of GSTM1-null genotype was 58.4% and that of GSTT1-null genotype was 11.2%. GSTM1-null genotype frequency was a little higher than that reported by Hamdy et al. **[13]** (55.5% among 200 subjects) and was also higher than that reported by Abdel-Rahman et al. **[12]** (44% among 34 subjects). GSTT1-null genotype frequency was a little lower than that reported by Abdel-Rahman et al. **[12]** (15% among 34 subjects) and was considerably lower than that reported by Hamdy et al. **[13]** (29.5% among 200 subjects). This difference could be explained by the older ages of our control subjects. As GST deletions were proved to be associated with a variety of cancers, one can suspect decreasing frequencies of GST deletions with increasing ages of healthy control subjects. The frequencies of GST deletions in our controls were very near to that reported in one study **[7]** performed on Caucasian population; their ages was matched with the ages of our controls. Those were 54% for GSTM1-null genotype and 14% for GSTT1-null genotype (among 205 subjects).

Previous studies on the role of GST polymorphisms in lymphoma risk [NHL overall and/or subtypes including follicular lymphoma (FL), DLBCL & gastric marginal zone lymphoma (GMZL) and Hodgkin's lymphoma (HL)] have given conflicting results, particularly concerning the role of GSTT1-null genotype. Three studies **[14,15,16]** reported no effect of GSTT1-null genotype on lymphoma risk, whereas four **[7,9,8,17]** showed positive associations of this genotype with lymphoma development. Kerridge et al. **[7]** (OR 4.3 for NHL), Al Dayel et al. **[9]** (OR 11.9 for DLBCL), Rollinson et al. **[8]** (OR 9.51 for GMZL) and Hohaus et al. **[18]** (OR 1.9 for HL). Our results also support a significant association between both GSTT1-null and double-null genotypes and an increase in DLBCL susceptibility. GSTT1-null conferred a 2.35-fold increase in DLBCL risk. The double-null genotype conferred a greater risk of 3.06 fold increase in DLBCL (OR was comparable with that reported by Kerridge et al. **[7]**; OR 3.6 and with that reported by Al Dayel et al. **[9]**; OR 3.09). The differences in studies' results could be attributed to racial heterogeneity, controls not selected from the appropriate at risk populations (in Sarmanova et al. **[14]** study) and sample size variability.

As with previous lymphoma studies **[7,8,9,14-17]**, we did not find a significant association between GSTM1-null genotype and DLBCL susceptibility. This together with the finding of GSTT1-null associated DLBCL risk support the importance of GSTT1 gene compared to GSTM1 gene in anti-carcinogenesis though larger studies are required for confirmation.

We found no associations between GST genotypes and IPI score. This is in concordance with Hohaus et al. **[18]** study on FL. However, Cho et al. **[19]** study on DLBCL found that GST1-null genotype was associated with high IPI score. We also, found no association between GST genotypes and treatment outcome. This is

in concordance with Cho et al. **[19]**. However, Hohaus et al. **[18]** reported that Patients with a GSTM1-null genotype had a lower complete remission rate after initial treatment when compared with patients with wild type GSTM1 and the GSTT1 genotype had no effect on the probability to achieve complete remission. Dieckvoss et al. **[20]** found that the presence of at least one GSTM1 allele was of significant beneficial effect on treatment outcome in pediatric patients with non-Hodgkin's lymphoma.

Modulation of prognosis of DLBCL according to genetic polymorphisms has been previously reported in 2 studies **[19,21]**. Both reported that no GST genotype had any impact on OS or event free survival (EFS) **[19]** or on risk of death, risk of relapse or secondary cancer **[21]**. However, in Han et al. study **[21]**, reduced risk of death (HR 0.66) and reduced risk of relapse, secondary cancer or death (HR 0.79) seemed likely. Here, we show that patients with GSTM1/T1-double null genotype or with one GST-null genotype had a significantly better PFS and OS when compared with those with both GST wild genes. Multivariate analysis using Cox regression showed that the presence of at least one GST-null genotype was associated with a 60% reduced risk of relapse/progression and 71% reduced risk of death i.e. the presence of at least one GST-null genotype tended to have a positive prognostic value independent from both IPI score and treatment outcome.

The prognostic impact of GST polymorphisms appears to vary according to the cancer type. Both favorable and unfavorable outcomes have been associated with null genotype of GST enzymes. Our results are in line with previous studies on lymphoma prognosis: Han et al. **[21]** observed that GSTT1-null genotype was associated with a 67% reduced risk of death and a 63% reduced risk of relapse, secondary cancer or death in patients with FL, Hohous et al. **[16]** found that GSTT1-null genotype was more prevalent in patients with low-stage HL than in patients with high-stage HL and Stanulla et al. **[22]** found that GSTT1-null genotype was associated with a reduced risk of relapse in patients with childhood acute lymphoblastic leukemia. On the other hand: Hohaus et al. **[18]** found that GSTT1-null genotype was associated with worse EFS of FL patients. Takanashi et al. **[23]** found that the simultaneous deletion of both GSTM1 and GSTT1 genes was more predictive of early relapse, than any other parameter in childhood precursor-B ALL.

It is assumed that the impact of the GST genotypes on disease control stems from the role of these enzymes in the metabolism of chemotherapeutic drugs. Deletion of GST enzymes could reduce or delay the metabolism of the chemotherapeutic drugs used for DLBCL treatment and may be expected to lead to improved efficacy. However lack of significant association between GST genotypes and treatment outcome could indicate that treatment efficacy may be affected by many other variables such as tumor burden and patient performance.

Conclusion:

Our results have shown a role for the GSTT1-null genotype and the GSTM1/T1-double null genotype as risk factors for DLBCL. The study also, shows that the presence of at least one GST-null genotype tended to have a positive prognostic value for DLBCL patients independent from both the IPI score and the treatment outcome. This might be useful in prediction of DLBCL prognosis and treatment selection. Large-scale studies on rituximab plus CHOP (R-CHOP) treated patients will be needed to either support or modify these findings. A better understanding of the underlying biological mechanisms should be pursued.

REFERENCES

1- Jaffe ES, Harris NL, Stein H, Vardiman JW (Eds.). World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues, IARC, Lyon. 2001.

2- Blinder V, Fisher SG. The role of environmental factors in the etiology of lymphoma. Cancer Invest. Apr-May. 2008; 26 (3): 306-316.

3- Zahm SH, Weisenburger DD, Saal RC, Vaught JB, Babbit PA, Blair A. The role of agricultural pesticide use in the development of non-Hodgkin's lymphoma in women. Archives of Environmental Health. 1993; 48: 353-358.

4- The International Non-Hodgkin's Lymphoma Prognostic Factors Project. A predictive model for aggressive non-Hodgkin's lymphoma. N Engl J Med. 1993; 329: 987-994.

5- Kelada SN, Eaton DL, Wang SS, Rothman NR, Khoury MJ. The role of genetic polymorphisms in environmental health. Environ Health Perspect. 2003 Jun; 111 (8): 1055-64.

6- Strange RC, Fryer AA. The glutathione S-transferases: Influence of polymorphism on cancer susceptibility. IARC Scientific Publications. 1999; 231-249.

7- Kerridge I, Lincz L, Scorgie F, Hickey D, Granter N, Spencer A. Association between xenobiotic gene polymorphisms and non-Hodgkin's lymphoma risk. Br J Haematol. 2002 Aug; 118 (2): 477-81.

8- Rollinson S, Levene AP, Mensah FK, Roddam PL, Allan JM, Diss TC, et al. Gastric marginal zone lymphoma is associated with polymorphisms in genes involved in inflammatory response and antioxidative capacity. Blood. 2003 Aug; 1, 102 (3): 1007-11. Epub 2003 Apr 3.

9- Al-Dayel F, Al-Rasheed M, Ibrahim M, Bu R, Bavi P, Abubaker J, et al. Polymorphisms of drug-metabolizing enzymes CYP1A1, GSTT and GSTP contribute to the development of diffuse large B-cell lymphoma risk in the Saudi Arabian population. Leuk Lymphoma. 2008 Jan; 49 (1): 122-9.

10- Cheson BD, Horning SJ, Coiffier B, Shipp MA, Fisher RI, Connors JM, et al. Report of an international workshop to standardize response criteria for non-Hodgkin's lymphomas. NCI Sponsored International Working Group. J Clin Oncol. 1999 Apr; 17 (4): 1244.

11- Arand M, Mühlbauer R, Hengstler J, Jäger E, Fuchs J, Winkler L, et al. A multiplex polymerase chain reaction protocol for the simultaneous analysis of the glutathione S-transferase GSTM1 and GSTT1 polymorphisms. Anal Biochem. 1996 Apr 5; 236 (1): 184-6.

12- Abdel-Rahman SZ, El-Zein RA, Anwar WA, Au WW. A multiplex PCR procedure for polymorphic analysis of GSTM1 and GSTT1 genes in population studies. Cancer Lett. 1996; Oct. 22; 107 (2): 229-33.

13- Hamdy SI, Hiratsuka M, Narahara K, Endo N, El-Enany M, Moursi N, et al. Genotype and allele frequencies of TPMT, NAT2, GST, SULT1A1 and MDR-1 in the Egyptian population. Br J Clin Pharmacol. 2003 Jun; 55 (6): 560-9.

14- Sarmanová J, Benesová K, Gut I, Nedelcheva-Kristensen V, Tynková L, Soucek P. Genetic polymorphisms of biotransformation enzymes in patients with Hodgkin's and non-Hodgkin's lymphomas. Hum Mol Genet. 2001 Jun 1; 10 (12): 1265-73.

15- Chiu BC, Kolar C, Gapstur SM, Lawson T, Anderson JR, Weisenburger DD. Association of NAT and GST polymorphisms with non-Hodgkin's lymphoma: A population-based case-control study. Br J Haematol. 2005 Mar; 128 (5): 610-5.

16- Kim HN, Kim NY, Yu L, Kim YK, Lee IK, Yang DH, et al. Polymorphisms of drug-metabolizing genes and risk of non-Hodgkin lymphoma. Am J Hematol. 2009 Dec; 84 (12): 821-5.

17- Hohaus S, Massini G, D'Alo' F, Guidi F, Putzulu R, Scardocci A, et al. Association between glutathione S-transferase genotypes and Hodgkin's lymphoma risk and prognosis. Clin Cancer Res. 2003 Aug 15; 9 (9): 3435-40.

18- Hohaus S, Mansueto G, Massini G, D'Alo F, Giachelia M, Martini M, et al. Glutathione-S-transferase genotypes influence prognosis in follicular non-Hodgkin's Lymphoma. Leuk Lymphoma. 2007 Mar; 48 (3): 564-9.

19- Cho HJ, Eom HS, Kim HJ, Kim IS, Lee GW, Kong SY. Glutathione-S-transferase genotypes influence the risk of chemotherapy-related toxicities and prognosis in Korean patients with diffuse large B-cell lymphoma. Cancer Genet Cytogenet. 2010 Apr 1; 198 (1): 40-6.

20- Dieckvoss BO, Stanulla M, Schrappe M, Beier R, Zimmermann M, Welte K, et al. Polymorphisms within glutathione S-transferase genes in pediatric non-Hodgkin's lymphoma. Haematologica. 2002 Jul; 87 (7): 709-13; discussion 713.

21- Han X, Zheng T, Foss FM, Lan Q, Holford TR, Rothman N, et al. Genetic polymorphisms in the metabolic pathway and non-Hodgkin lymphoma survival. Am J Hematol. 2010 Jan; 85 (1): 51-6.

22- Stanulla M, Schrappe M, Brechlin AM, Zimmermann M, Welte K. Polymorphisms within glutathione S-transferase genes (GSTM1, GSTT1, GSTP1) and risk of relapse in childhood B-cell precursor acute lymphoblastic leukemia: A case-control study. Blood. 2000 Feb 15; 95 (4): 1222-8.

23- Takanashi M, Morimoto A, Yagi T, Kuriyama K, Kano G, Imamura T, et al. Impact of glutathione S-transferase gene deletion on early relapse in childhood B-precursor acute lymphoblastic leukemia. Haematologica. 2003 Nov; 88 (11): 1238-44.

Journal of the Egyptian Society of Haematology & Research, Vol. 6, No. 2, September: 55-59, 2010

Fetal Globin Induction with Oral Butyrates in ß-Thalassemia Major

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ABSTRACT

Background: The ß-thalassemias are characterized by insufficient or absent production of ß-globin chains. L-carnitine (L-3-hydroxy-4-N-trimethyl amino butyric acid) is an oral butyrate derivative that can stimulate Hb F production.

Objectives: The aim of this work is to study the hematological effects of short-term therapy of L-carnitine on the induction of fetal hemoglobin (Hb F) production in a group of ß-thalassemia major patients.

Patients and Methods: L-carnitine was given orally (50 mg/kg/day) to 27 patients (mean age 12.26±4.25 years) for 45 days. Complete blood count, hemoglobin electrophoresis and F cell percentages (by immuno-histochemical technique) were estimated before and after L-carnitine therapy.

Results: Fourteen patients (51.85%) were responders showing a significant increase in mean HbF and F cells (p=0.00001, 0.00006 respectively) after L-carnitine therapy. The transfusion intervals were significantly prolonged among responders and non-responders (p=0.0028 for both).

Conclusion: L-carnitine is a physiologic, well tolerated and safe drug that can stimulate Hb F production in thalassemia patients. Further studies with a larger number of patients and a higher dose of L-carnitine are required to evaluate its overall role in thalassemia.

Key Words: *L-carnitine – Butyrates – HbF induction – ß-thalassemia.*

INTRODUCTION

The ß-thalassemias are characterized by insufficient or absent production of ß-globin chains leading to imbalanced ß and non ß-globin chain synthesis. This causes accumulation and precipitation of unpaired ß-globin chains and consequently, to ineffective erythropoiesis and hemolysis **[1,2]**. Induction of fetal hemoglobin (HbF) is a novel therapeutic strategy that has been hypothesized for ß-thalassemia, based on the observation that the co-existence of hereditary persistence of fetal hemoglobin (HPFH) in patients with ß-thalassemia reduces the severity of the disease and the need for blood transfusion **[3,4,5]**. This suggested that agents that would mimic the HPFH mutation by preventing binding of "transcription factors" to the ß-globin promoter would favour expression of the ß-globin gene and ameliorate the severity of ß-thalassemia and sickle cell disease **[6]**.

Hb F enhancement would be beneficial especially for ß-thalassemia patients in developing countries that cannot sustain the high cost of maintaining regular transfusion regimen and chelation therapy. L-carnitine (L-3-hydroxy-4-N-trimethyl amino butyric acid) is an oral butyrate derivative that can stimulate Hb F production and stabilize the erythrocyte membrane against oxidative stress. Some studies reported its beneficial effects on physical fitness, cardiac status and pubertal development in thalassemic patients **[7,8,9]**. The aim of this work is to study the hematological effects of short-term therapy of L-carnitine on the induction of fetal hemoglobin production in a group of ß-thalassemia major patients.

MATERIAL AND METHODS

This is a prospective, single-arm study that included 27 transfusion-dependent homozygous ß-thalassemia patients, after their or their caregivers' consent and approval of our Institutional Ethical Committee. All patients were previously

diagnosed and followed-up at the Hematology Clinic of Cairo University New Children Hospital. Among the recruited patients, 12 were males and 15 females. Their age ranged between 6 and 22 years (mean of 12.26±4.25 years). Twenty-two patients had splenectomy.

The patients' medical records were reviewed and frequency of blood transfusion was recorded. Complete blood count, hemoglobin electrophoresis (by densitometry following cellulose acetate) and F cell percentages (by immunohistochemical technique using monoclonal anti-Hb A antibodies) were estimated before and after 45 days of oral L-carnitine therapy (50mg/kg/day). The Hematology Clinic provided the drug to all patients free of charge on an outpatient basis.

Statistical methods:

Numerical data were presented as mean and standard deviation. Student's *t*-test was used to compare numerical data between groups. Statistical significance was considered if *p*-value was <0.05.

RESULTS

According to the increase in the percentage of Hb F and F cells after treatment, the patients were divided into 2 subgroups; Group 1 (responders) who showed an increase in Hb F and F cells percentage and group 2 (non responders) who showed either decrease or constant Hb F and F cells percentages after treatment.

In group 1 (n=14, 51.85%), mean Hb F increased significantly from 19.3±14.62 % to 45.05±17.45% (*p*=0.00001) and mean F cells increased from 19.72±14.45% to 43.89±16.85% (*p*=0.00006) after L-carnitine therapy. In group 2 (n=13, 48.15%), mean Hb F significantly decreased from 35.92±34.92 % to 32.1±34.16% (*p*=0.012) and mean F cells from 36.53±35.73% to 32.5±33.96% (*p*=0.014) (Fig. 1). The mean intervals between blood transfusions were significantly prolonged in groups 1 and 2 (*p*=0.0028 for both). This was observed in 55.6% (n=15/27) of patients within 2 weeks. Hemoglobin, hematocrit and all red cell indices increased, but non-significantly, after therapy in both groups except for the red cell distribution width (RDW) which decreased significantly in groups 1 and 2 (*p*=0.001, *p*=0.004 respectively) (Table 1). The drug was well tolerated by all patients without any adverse reactions.

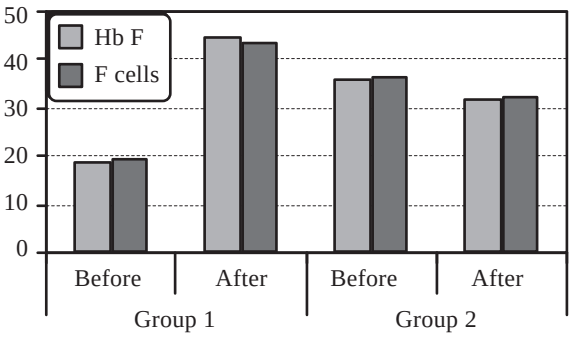

Fig. (1): Mean Hemoglobin F and F cells percentages before and after L-carnitine therapy in group 1 (responders) and group 2 (non-responders) *ß*-thalassemia patients.

Table (1): Clinical and laboratory parameters of *ß*-thalassemia Patients (n=27) before and after 45 days of L-carnitine therapy.

Parameter		Group 1 (n=14)	*p*-value	Group 2 (n=13)	*p*-value
Interval of blood transfusion (weeks)	Before	3.36±1.22	0.0028*	3.08±1.04	0.0028*
	After	4.07±1.27		3.62±1.12	
Hemoglobin (g/dl)	Before	7.21±0.58	0.23	7.33±1.01	0.73
	After	7.46±0.96		7.42±0.92	
Hematocrit (%)	Before	20.79±2.07	0.12	22.52±5.44	0.98
	After	21.96±2.74		22.57±3.51	
MCV (fl)	Before	71.01±7.86	0.23	72.92±7.38	0.10
	After	72.89±7.95		74.65±6.45	
MCH (pg)	Before	24.58±2.22	0.84	24.95±2.62	0.28
	After	24.66±2.33		25.45±2.65	
RDW	Before	29.01±7.10	0.001*	28.82±7.99	0.004*
	After	24.54±5.74		23.03±6.35	

* Statistically significant. MCV: Mean Cell Volume. MCH: Mean Cell Hemoglobin. RDW: Red cell Distribution Width.

DISCUSSION

Butyrate is a well-known histone deaceytylase inhibitor (HDACi) that was shown to induce HbF synthesis after intravenous infusion in patients with ß-thalassemia **[10,11]**, and in a majority of patients with sickle cell disease **[12]**. Other butyrate analogues (e.g. phenylbutyrate, isobutyramide and other short chain fatty acids) that also induce HbF expression in human erythroid cells were only used in limited clinical trials in ß-thalassemia **[13]**. Butyrate was shown to displace histone deaceytylase 3 (HDAC3), which might be primarily responsible for ß-globin silencing **[14]**. In addition, exposure to butyrate was shown to alter the DNA methylation status of the ß-globin locus **[15]** and to increase the efficiency of the translation of the globin mRNA **[16]**.

In this study, a response rate of 51.85% was noted among our patients after L-carnitine therapy, with a statistically significant increase in mean Hb F and F cells percentages (p=0.00001 and 0.00006 respectively). The oral route of administration of L-carnitine is an additional advantage especially being well tolerated by all our patients. On the other hand, non-responders (48.15%) showed a significant decrease in mean Hb F and F cells percentages (p=0.012 and 0.014 respectively). This is difficult to be explained, it might be due to the wide variation in individual responses to pharmacological stimulation of fetal hemoglobin by butyrates. This variability of response can be replicated in vitro when erythroid progenitors from the same patients are cultured in the presence of butyrate **[15]**. It was suggested that the responsiveness of a patient to butyrate may be determined by the epigenetic configuration of the ß-globin gene cluster. The elucidation of the role of this epigenetic variability is a major challenge for the effective use of these compounds in the treatment of patients with hemoglobin disorders. The chromatin structure of the locus is determined by the acetylation state of the histones and the state of its DNA methylation **[17]**. Furthermore, low dose and short duration of L-carnitine therapy in our work may contribute to the reduced response rate.

Even though L-carnitine therapy was administered for 45 days only, it seemed to be beneficial for most homozygous thalassemia patients as shown by prolongation of transfusion intervals in 55.6% of cases within 2 weeks with a significant increase in the mean transfusion interval in both groups (p=0.0028 for both). This was previously reported and was explained by the protective effect of L-carnitine on the red blood cells from oxidative stress and the stabilization of their membranes where latent peroxidative damage has occurred **[8,7,18,19,20]**. In ß-thalassemia, increased oxidative stress is probably due to auto-oxidation of globin chains and iron overload **[21,22]**. The counteracting effect of antioxidants on lipid peroxidation and their protective effect against oxidative damage of erythrocytes were previously demonstrated **[23]**.

The non-significant increase in hemoglobin, hematocrit, MCV and MCH values after L-carnitine therapy among our responders group may be due to the low dose and short duration of therapy. On the other hand, improvement of the hematological parameters in the non-responders could be attributed to the other physiologic effects of L-carnitine on the red cells of thalassemia patients. One study demonstrated no alteration in hematological picture after one month of L-carnitine therapy despite of improvement of RBC quality including RBC lipid peroxidation, cytosolic calcium concentrations and RBC deformability **[20]**. Maintaining normal RBC mechanical properties was suggested to be an important objective in therapeutic approach to thalassemia patients **[17]**.

Red cell distribution width (RDW) is a quantitative parameter of variation in the red cell volume; it is equivalent to the microscopic assessment of the degree of anisocytosis. This study showed a significant decrease in RDW in both groups which denotes that red cells became more homogenous. The effect of L-carnitine on red cell membrane may explain this finding. Previous studies supported the effects of L-carnitine on membrane stability and function. Altered sodium-potassium pump activity, activation of enzymes for lipid incorporation in RBC membranes and interacting membrane skeleton proteins were suggested as possible mechanisms **[24,25,26]**.

In conclusion, L-carnitine as an oral butyrate is a physiologic, well-tolerated and safe drug that can stimulate Hb F production in homozy-

gous thalassemia patients. Further longer studies with a larger number of patients and a higher dose of L-carnitine are required to evaluate its overall role in the course and prognosis of thalassemia.

REFERENCES

1- Thein SL. Beta-thalassaemia prototype of a single gene disorder with multiple phenotypes. Int J Hematol. 2002; 76: 96-104. [PubMed: 12430908].

2- Fucharoen S, Winichagoon P. Thalassemia and abnormal hemoglobin. Int J Hematol. 2002; 76: 83-89. [PubMed: 12430905].

3- Gambari R, Fibach E. Medicinal chemistry of fetal hemoglobin inducers for treatment of beta-thalassemia. Curr Med Chem. 2007; 14: 199-212. [PubMed: 17266579].

4- Feriotto G, Salvatori F, Finotti A, et al. A novel frameshift mutation (+A) at codon 18 of the beta-globin gene associated with high persistence of fetal hemoglobin phenotype and delta beta-thalassemia. Acta Haematol. 2008; 119: 28-37. [PubMed: 18230963].

5. Bianchi N, Zuccato C, Lampronti I, et al. Fetal hemoglobin inducers from the natural world; a novel approach for identification of drugs for the treatment of beta-thalassemia and sickle-cell anemia. Evid Based Complement Altern Med. 2009; 6: 141-51. [PubMed: 18955291].

6- Atweh GF, Schechter AN. Pharmacologic induction of fetal hemoglobin: Raising the therapeutic bar in sickle cell disease. Curr Opin Hematol. 2001; 8: 123-130. [PubMed: 11224687].

7- El-Beshlawy A, El Accaoui R, Abd El-Sattar M, Gamal El-Deen MH, Youssry I, Shaheen N, Hamdy M, El-Ghamrawy M, Taher A. Effect of L-carnitine on the physical fitness of thalassemic patients. Ann Hematol. 2007; 86: 31-34. [PubMed: 17031691].

8- El-Beshlawy A, Ragab L, Fattah AA, Ibrahim IY, Hamdy M, Makhlouf A, Aoun E, Hoffbrand V, Taher A. Improvement of cardiac function in thalassemia major treated with L-carnitine. Acta Haematol. 2004; 111: 143-148 [PubMed: 15034235].

9- El-Beshlawy A, Mohtar G, Abd El Gafar E, Abd El Dayem SM, EL Sayed MH, Aly AA, Farok M. Assessment of puberty in relation to L-carnitine and hormonal replacement therapy in beta-thalassemic patients. J Trop. 2008; 54: 375-381 Epub 2008 Jul 22 [PubMed: 18647801].

10- Perrine SP, Ginder GD, Faller DV, Dover GH, Ikuta T, Witkowska HE, Cai SP, et al. A short-term trial of butyrate to stimulate fetal-globin-gene expression in the ß-globin disorders. N Engl J Med. 1993; 328: 81-86. [PubMed: 7677966].

11- Sher GD, Ginder GD, Little J, Yang S, Dover GJ, Olivieri NF. Extended therapy with intravenous arginine butyrate in patients with ß-hemoglobinopathies. N Engl J Med. 1995; 332: 1606-1610. [PubMed: 7753139].

12- Atweh GF, Sutton M, Nassif I. Sustained induction of fetal hemoglobin by pulse butyrate therapy in sickle cell disease. Blood. 1999; 93: 1790-97. [PubMed: 10068649].

13- Cao H. Pharmacological induction of fetal hemoglobin synthesis using histone deacetylase inhibitors. Hematology. 2004; 9: 223-233. [PubMed: 15204104].

14- Mankidy R, Faller DV, Mabaera R, Lowrey CH, Boosalis MS, White GL, et al. Short-chain fatty acids induce ß-globin gene expression by displacement of a HDAC3-NCoR repressor complex. Blood. 2006; 108: 3179-186. [PubMed: 16849648].

15- Fathallah H, Weinberg RS, Galperin Y, Sutton M, Atweh GF. Role of epigenetic modifications in normal globin gene regulation and butyrate-mediated induction of fetal hemoglobin. Blood. 2007; 110: 3391-3397. [PubMed: 17638855].

16- Weinberg RS, Ji X, Sutton M, Perrine S, Galperin Y, Li Q, Liebhaber SA, Stamatoyannopoulos G, Atweh GF. Butyrate increases the efficiency of translation of gamma-globin mRNA. Blood. 2005; 105: 1807-1809. [PubMed: 15479724].

17- Migliaccio AR, Rotili D, Nebbioso A, Atweh G, Mai A. Histone deacetylase inhibitors and hemoglobin F induction in ß-thalassemia. Int J Biochem Cell Biol. 2008; 40: 2341-2347. [PubMed: 18617435].

18- Ye_ilipek MA, Hazar V, Yegin O. L-Carnitine treatment in beta thalassemia major. Acta Haematol. 1998; 100: 162-163. [PubMed: 9858797].

19- Meral A, Tuncel P, Sürmen-Gür E, Ozbek R, Oztürk E, Günay U. Lipid peroxidation and antioxidant status in beta thalassemia. Pediatr Hematol Oncol. 2000; 17: 687-693. [PubMed: 11127401].

20- Toptas B, Baykal A, Yesilipek A, Isbir M, Kupesiz A, Yalcin O, Baskurt OK. L-carnitine deficiency and red blood cell mechanical impairment in ß-thalassemia major Clin Hemorheol Microcirc. 2006; 35: 349-357. [PubMed: 16899956].

21- Kassab-Chekir A, Laradi S, Ferchichi S, Haj Khelil A, Feki M, Amri F, Selmi H, Bejaoui M, Miled A. Oxidant, antioxidant status and metabolic data in patients with beta-thalassemia. Clin Chim Acta. 2003; 338:79-86. [PubMeD: 14637270].

22- Naithani R, Chandra J, Bhattacharjee J, Verma P, Narayan S. Peroxidative stress and antioxidant enzymes in children with beta-thalassemia major. Pediatr Blood Cancer. 2006; 46: 780-785. [PubMed: 16317757].

23- Al-Quobaili FA, Abou Asali IE. Serum levels of lipids and lipoproteins in Syrian patients with beta-thalassemia major. Saudi Med J. 2004; 25: 871-875. [PubMed: 15235691].

24- de los Reyes B, Navarro JA, Pérez-García R, Liras A, Campos Y, Bornstein B, Arenas J. Effects of L-carnitine on erythrocyte acyl-CoA, free-CoA, and glycerophospholipid acyltransferase in uremia. Am J Clin Nutr. 199; 67: 386-390. [PubMed: 9497180].

25- Labonia WD, Morelli OH Jr., Gimenez MI, Freuler PV, Morelli OH. Effect of L-carnitine on sodium transport in erythrocytes from dialyzed uremic patients. Kidney Int. 1987; 32: 754-759. [PubMed: 2448515].

26- Arduini A, Rossi M, Mancinelli G, Belfiglio M, Scurti R, Radatti G, Shohet SB. Effect of L-carnitine and acetyl-L-carnitine on the human erythrocyte membrane stability and deformability. Life Sci. 1990; 47: 2395-2400. [PubMed: 2263166].

Journal of the Egyptian Society of Haematology & Research, Vol. 6, No. 2, September: 61-66, 2010

Low Prevalence of Cardiac Siderosis in Heavily Iron Loaded Egyptian Thalassemia Major Patients

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ABSTRACT

Background: Myocardial siderosis in thalassemia major remains the leading cause of death in developing countries. Once heart failure develops, the outlook is usually poor with precipitous deterioration and death. Cardiovascular magnetic resonance (CMR) technology can measure cardiac iron deposition directly using the magnetic relaxation time T2*. This allows earlier diagnosis and treatment and helps to reduce mortality from this cardiac affection.

Aims: To find out the prevalence of cardiac siderosis among our patients who are heavily iron loaded by CMR technology and its relation to liver iron concentration, serum ferritin and left ventricular ejection fraction.

Patients and Methods: Eighty nine ß-thalassemia patients (10 to 43 years, mean age of 20.78±6.36) were recruited in this study. All patients were receiving chelation therapy of subcutaneous desferroxamine. Evaluation of hemosiderosis was based on CMR, liver magnetic resonance R2 and serum ferritin.

Results: Cardiac T2* values ranged between 4.3 to 53.8 ms with a mean of 28.5±11.7ms. The left ventricular ejection fraction (LVEF) as measured by CMR ranged between 55 and 78% with a mean of 67.7±4.7% and liver iron concentration (LIC) ranged between 1.5 to 56mg/g dry weight with a mean of 26.1±13.4mg/g. Serum ferritin varied from 533 to 22360ng/ml; mean=4510±2847ng/ml with 83.2% above 2500ng/ml. The prevalence of myocardial siderosis (T2*<20ms) among our patients was 22/89 patients (24.7%) whose mean age was 20.9±7.5 years with a mean T2* value of 12.7±4.4ms and LVEF of 68.6±5.8%, LIC and serum ferritin level of 30.9±13.5mg/g and 6120±4190ng/ml respectively. There was no correlation between T2* results and the age, LVEF, LIC and serum ferritin of this group (p=0.65, p=0.085, p=0.99 and p=0.63 respectively). Patients with severe cardiac siderosis (T2*<10ms) constituted 7/89 (7.9%) with a mean age of 18.4±4.4 years. Although these patients had a mean T2* of 7.8±1.7ms, the LVEF value was 65.1±6.2% and only one patient had clinical cardiac disease (T2*=4.3 ms and LVEF=55%). LIC and serum ferritin levels were 29.8± 17.0mg/g and 7200±6950ng/ml respectively. In this group of severe cardiac siderosis, T2* was not correlated to age (p=0.5), LVEF (p=0.14), LIC (p=0.97) or serum ferritin (p=0.82).

Conclusion: There is a low prevalence of myocardial siderosis in the Egyptian thalassemia patients in spite of very high serum ferritin. Cardiac T2* is the best test that can identify at risk patients who can be treated with optimization of their chelation protocols. The possibility of a genetic component for the resistance to cardiac iron loading in our population should be considered.

Key Words: *Cardiac siderosis – Thalassemia major.*

INTRODUCTION

Myocardial siderosis remains the leading cause of death in thalassemia major in developing countries. Once heart failure develops, the outlook is often poor with precipitous deterioration and death **[1,2]**.

Methods for predicting heart failure have been developed that are based on established measures of iron loading, most importantly, serum ferritin >2500µg/L **[2]** and liver iron concentration >15mg/g dry weight **[3]**. However, the persistently high mortality rate from heart failure indicates that high risk patients are not being identified in time for effective intervention.

Measurement of ventricular function such as alteration over time in ejection fraction has also been proposed in thalassemia, but it identifies patients at relatively late stage **[4]** and dysfunction may be masked because of supranormal left ventricular function in thalassemia

patients in absence of myocardial iron loading **[5]**.

Most recently, assessment of myocardial iron with magnetic resonance (CMR) relaxation time T2* has been used. The measurement of T2* is fast, robust and the most sensitive to iron deposition **[6]**. The classification of patients is that those with T2*>20ms are regarded as not having cardiac iron, those with T2* between 10-20ms have mild to moderate cardiac iron load and those <10ms are considered to have heavy cardiac iron load **[7]**.

Our objectives were to find out the prevalence of cardiac siderosis among our patients using T2* CMR and relate the findings to liver iron concentration, serum ferritin and left ventricular ejection fraction.

PATIENTS AND METHODS

Eighty nine multiply transfused ß–thalassemia major patients, following at the Hematology Clinic of the New Children's Hospital of Cairo University, were selected to participate in this study after the approval of the ethical committee and after signing a written informed consent. Their age ranged between 10 to 43 years; (mean age = 20.78±6.36 years).

All patients were receiving chelation therapy in the form of subcutaneous desferroxamine.

Patients were evaluated for:

Cardiac siderosis and left ventricular ejection fraction (LVEF) by cardiovascular magnetic resonance (CMR), using relaxation parameter T2* **[8]**: A single 10 mm thick short-axis mid-ventricular slice of left ventricle was acquired at 8 echo times with standard shimming in a single breath-hold. For analysis, a full thickness region of interest was chosen in the LV septum.

Liver iron concentration (LIC) measurements were conducted on a Philips Intera (Netherlands) 1.5-T MRI scanner. LIC measurements were made using SDPA R2-MRI (FerriScan®). Detailed methodology is described elsewhere **[9,10,11]**. Axial images were acquired with a multislice single spin-echo (SSE) pulse sequence, with a pulse repetition time TR of 2500ms, spin echo times TE of 6, 9, 12, 15, and 18ms, and slice thickness of 5mm. For each subject, the largest axial slice of the liver was selected for R2 image analysis and LIC calculation.

Serum ferritin was performed by microparticle enzyme immunoassay (Abott AXSYM System).

Statistical methods:

Data were statistically described in terms of mean ± standard deviation (± SD), median and range, or frequencies (number of cases) and percentages when appropriate. Comparison of numerical variables between the study groups was done using Student *t*-test for independent samples in comparing 2 groups when normally distributed and Mann Whitney U test for independent samples when not normally distributed. Correlation between various variables was done using Pearson moment correlation equation. *p*-values less than 0.05 was considered statistically significant.

RESULTS

T2* values of all patients ranged between 4.3 to 53.8ms with a mean of 28.5±11.7ms. The left ventricular ejection fraction (LVEF) as measured by CMR ranged between 55 and 78%; mean = 67.7±4.7%,.and liver iron concentration (LIC) ranged between 1.5 and 56mg/g dry weight with a mean of 26.1±13.4mg/g. Serum ferritin varied from 533 to 22360ng/ml; mean = 4510±2847ng/ml with 83.2% above 2500ng/ml (Fig. 1).

Myocardial siderosis (T2*<20ms) was encountered in 22/89 patients (24.7%) whose mean age was 20.9±7.5 years with a mean T2* value of 12.7±4.4ms and LVEF of 68.6±5.8%. LIC and serum ferritin levels were 30.9± 13.5mg/g and 6120±4190ng/ml respectively (Table 1).

There was no correlation between T2* results and age, LVEF, LIC or serum ferritin of this group (p=0.65, p=0.085, p=0.99 and p=0.63 respectively) (Figs. 2,3,4).

There were 7/89 patients (7.9%) with severe cardiac siderosis (T2*<10ms) with a mean age of 18.4±4.4 years. Although these patients had a mean T2* of 7.8±1.7ms, the LVEF value was 65.1±6.2% and only one patient had clinical cardiac disease (T2*=4.3 ms and LVEF=55%). LIC and serum ferritin values were 29.8± 17.0mg/g and 7200±6950ng/ml respectively. In this group of severe cardiac siderosis, T2* was not correlated to age (p=0.5), LVEF (p=0.14), LIC (p=0.97) or serum ferritin (p=0.82).

Table (1): ß-thalassemia major Patients with T2* >20ms versus patients with cardiac siderosis T2* <20ms.

	Patients T2* >20ms (n-67)		Patients T2* <20ms (n-22)	
	Mean	Std.D	Mean	Std.D
Age (yrs)	20.8	6.0	20.9	7.5
T2* (ms)	33.7	8.2	12.7	4.4
LVEF (%)	67.4	4.3	68.6	5.8
LIC (mg Fe/gdw)	24.6	13.1	30.9	13.5
Ferritin (ng/ml)	3986	2023	6120	4190

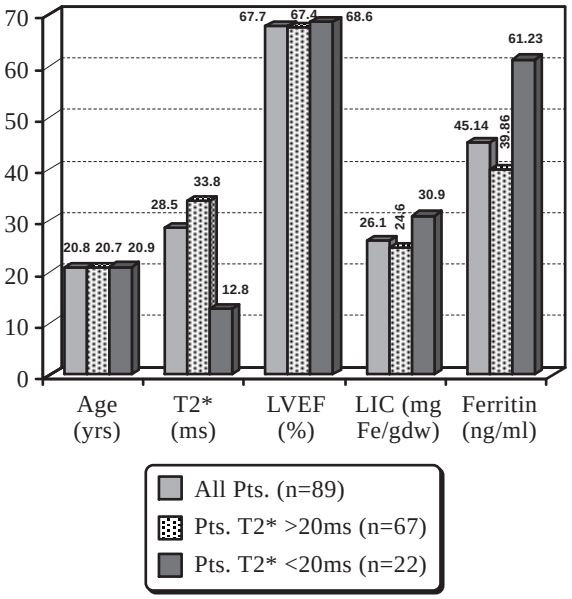

Fig. (1): Comparison of the mean age, T2*, LVEF, LIC and ferritin values in 89 thalassemia major patients and in relation to cardiovascular magnetic resonance (T2*).

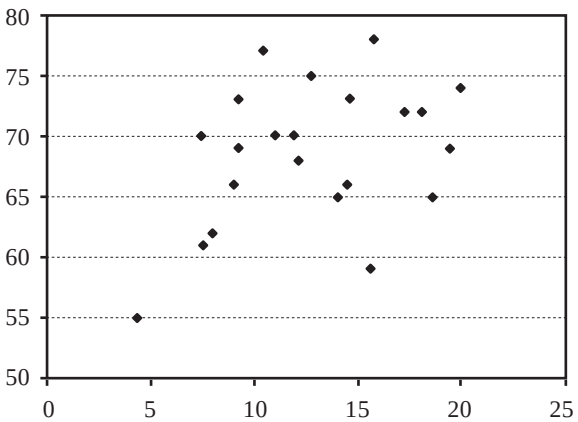

Fig. (2): Correlation between T2* and LVEF in 22 thalassemia major patients with cardiac siderosis (T2* <20ms).

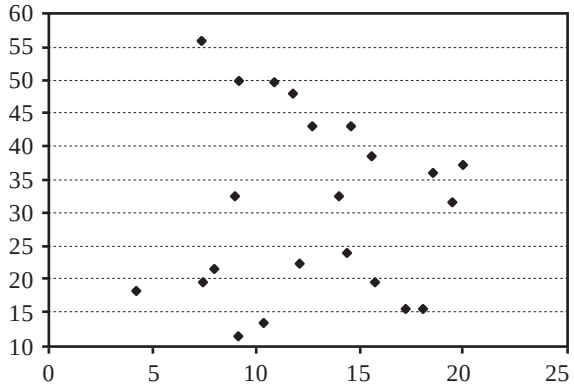

Fig. (3): Correlation between T2* and LIC in 22 thalassemia major patients with cardiac siderosis (T2* <20ms).

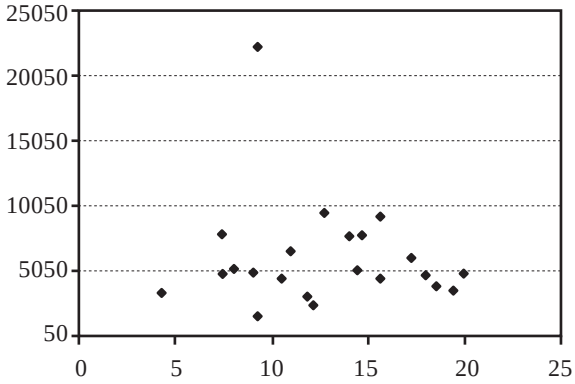

Fig. (4): Correlation between T2* and serum ferritin in 22 thalassemia major patients with cardiac siderosis (T2* <20ms).

DISCUSSION

Low values of liver iron or serum ferritin do not necessarily signify low risk of iron–induced cardiomyopathy **[6]**. Therefore, there is often a need to identify those thalassemia patients most at risk of cardiomyopathy even when serum ferritin and liver iron values are currently well controlled. Although sequential quantification of heart function identifies a patient group at increased risk of cardiac mortality, **[4]** it would be preferable to identify high-risk patients early before cardiomyopathy develops and use targeted treatment **[12,13]**. The estimation of heart iron by MR offers this possibility. We have used the MR relaxation parameter T2*, which is sensitive to the presence of storage iron microaggregates which disturb the homogeneity of the magnetic microenvironment.

The prevalence of myocardial siderosis (T2*<20ms) in this cohort study of Egyptian patients was 24.7%. These subjects were be-

lieved to have a broad range of compliance (ferritin 533-22360ng/ml, mean of 4510ng/ml) and as such are likely to be fairly representative of the thalassemia major population as a whole in Egypt. This rate was much lower than those previously reported by different studies around the world; 65%, 50%, 30%, 46%, 64%, 86% **[7,14-18]**.

There was no correlation between cardiac T2* and liver iron concentration among our studied group. It has been described in several prior studies **[6,7,15]** that on cross-sectional evaluation, there is marked disconnect between liver and heart iron values **[19]**. One reason for this disconnect is organ specific mechanisms of iron uptake/release. Liver, bone marrow and spleen are the natural reservoirs for iron and transferrin-bound iron is shuttled among these stores in a tightly regulated manner. The heart and endocrine glands also have well-regulated transferrin-mediated uptake, but pathologic iron deposition in these organs occurs through unregulated influx of non-transferrin-bound iron (NTBI) **[20,21]**. NTBI levels rise once transferrin is fully saturated and are modulated by the type and duration of chelator exposure **[22]**.

In addition to differences in iron uptake, there are differences in iron elimination as chelation therapy can remove iron more rapidly from the liver than from the heart, which may normalize liver iron while myocardial iron remains high **[6,23,19]**. Other mechanisms may be involved, including potential genetic variations in function of cardiac iron transport channels such as the L-type calcium channel and the divalent metal transporter 1 **[24,25]**. The discordance between liver and myocardial iron indicates that the risk of heart complications cannot be predicted solely from liver iron measurement **[7]**.

Myocardial T2* had no correlation with serum ferritin among our studied group. Cohort studies have failed to show any significant correlation between heart T2* and serum ferritin **[6,7,16,28]** or very weak relation **[26,27]**. The relation between serum ferritin and myocardial iron loading indicates that whilst a high ferritin may be bad, a low ferritin cannot be taken as reassuring **[7]** and total body iron stores have little immediate predictive value with respect to the presence or absence of cardiac iron **[28]**.

We observed no correlation between T2* and age of the patients, similar results were previously reported **[15,16]** denoting the possibility of a genetic component for the susceptibility of cardiac iron loading in some populations. However, Wood and colleagues showed that the relationship between T2* and age was fundamentally non-linear **[28]**.

There was a trend towards correlation between T2* and ejection fraction but this was not statistically significant (p=0.085). Positive correlation was observed in different studies **[6,15,17,29,30]**. There is significant variation in ejection fraction between patients with similar levels of iron loading. Thus a once-off ejection fraction measurement may be inadequate for assessing the cardiac risk resulting from myocardial siderosis **[7]**. The relationship between T2* and cardiac function is shallow until a critical level is reached, after which rapid deterioration occurs. This explains why identification of abnormal systolic function is a late sign of iron toxicity **[6]**. However, in the study of Daar and colleagues **[16]**, there was no correlation between ejection fraction and cardiac T2*. Ejection fraction in their group of patients was measured by routine echocardiography, which is a less accurate and reproducible technique than CMR **[31]**.

Among our patients, 7.9% had severe cardiac siderosis. Higher prevalence of severe cardiac siderosis was reported among different groups **[7,14,16,17]**. One patient had clinical heart failure (LVEF=55%) in contrary to previous reports of impaired ventricular function in 45%, 62% and 19% among severe cardiac siderosis **[7,14,17]**. Chirnomas and colleagues showed that systolic function has poor sensitivity for detecting elevated myocardial iron as 18% of their patients had elevated myocardial iron with a normal ejection fraction **[15]**. Left ventricular function falls with decreased myocardial T2*, and this is accompanied by left ventricular dilation and hypertrophy; all of which are classic cardiac responses of heart failure **[6,27]**.

What was of more concern was the fact that the other 6 patients in this group of severe cardiac siderosis were asymptomatic and had normal ejection fractions. These patients are at the highest risk of developing clinically significant myocardial complications such as cardiac

failure and life-threatening ventricular arrhythmias.

Conclusion:

There is a low prevalence of myocardial siderosis in the Egyptian thalassemia patients in spite of very high serum ferritin.

Cardiac T2* is the best test that can identify at risk patients who can be treated with optimization of their chelation protocols. The possibility of a genetic component for the resistance to cardiac iron loading in our population should be considered.

REFERENCES

1- Borgna-Pignatti C, Rugolotto S, De Stefano P, Zhao H, Cappellini MD, Del Vecchio GC, Romeo MA, Forni GL, Gamberini MR, Ghilardi R, Piga A, Cnaan A. Survival and complications in patients with thalassemia major treated with transfusion and deferoxamine. Haematologica. 2004; 89: 1187-1193.

2- Modell B, Khan M, Darlison M. Survival in beta thalassemia major in the UK; data from the UK Thalassemia Register. Lancet. 2000; 355: 2051-2052.

3- Brittenham GM, Griffith PM, Nienhuis AW, Mc Laren CE, Young NS, Tucker EE, Allen CJ, Farrell DE, Harris JW. Efficacy of deferoxamine in preventing complications of iron overload in patients with thalassemia major. N Engl J Med. 1994; 331: 567-573.

4- Davis BA, O'Sullivan C, Jarritt PH, Porter JB. Value of sequential monitoring of left ventricular ejection fraction in the management of thalassemia major. Blood. 2004; 104: 263-269.

5- Westwood MA, Anderson LJ, Maceira AM, Shah FT, Prescott E, Porter JB, Wonke B, Walker JM, Pennell DJ. Normalized left ventricular volumes and function in thalassemia major patients with normal myocardial iron. J Magn Reson Imaging. 2007; 25: 1147-1151.

6- Anderson LJ, Holden S, Davis B, Prescott E, Charrier CC, Bunce NH, Firmin DN, Wonke B, Porter J, Walker JM, Pennell DJ. Cardiovascular T2* magnetic resonance for the early diagnosis of myocardial iron overload. Eur Heart J. 2001; 22: 2171-2179.

7- Tanner MA, Galanello R, Dessi C, Westwood MA, Smith GC, Nair SV, Anderson LJ, Walker JM, Pennell DJ. Myocardial iron loading in patients with thalassemia major on deferoxamine chelation. J Card Magn Reson. 2006; 8: 543-547.

8- Westwood M, Anderson LJ, Firmin DN, Gatehouse PD, Charrier CC, Wonke B, Pennell DJ. A single breath-hold multiecho T2* cardiovascular magnetic resonance technique for diagnosis of myocardial overload. J Magn Reson Imaging. 2003; 18: 33-39.

9- St. Pierre TG, Clark PR, Chua-anusorn W, Fleming AJ, Jeffrey GP, Olynyk JK, Pootrakul P, Robins E, Lindeman R. Non invasive measurement and imaging of liver iron concentrations using proton magnetic resonance. Blood. 2005; 105: 855-861.

10- St Pierre TG, Clark PR, Chua-Anusorn W. Single spin-echo proton transverse relaxometry of iron-loaded liver. NMR Biomed. 2004; 17: 446-458.

11- Clark PR, Chua-anusorn W, St Pierre TG. Bi-exponential proton transverse relaxation rate (R2) image analysis using RF field intensity-weighted spin density projection: Potential for R2 measurement of iron-loaded liver. Magn Reson Imaging. 2003; 21: 519-530.

12- Pennell DJ, Berdoukas V, Karagiorga M, Ladis V, Piga A, Aessopos A, Gotsis ED, Tanner MA, Smith GC, Westwood MA, Wonke B, Galanello R. Randomized controlled trial of deferiprone or deferoxamine in beta thalassemia major patients with asymptomatic myocardial siderosis. Blood. 2006; 3738-3744.

13- Tanner MA, Galanello R, Dessi C, Smith GC, Westwood MA, Agus A, Roughton M, Assomull R, Nair SV, Walker JM, Pennell DJ. A randomized, placebo-controlled, double-blind trial of the effect of combined therapy with deferoxamine and deferiprone on myocardial iron in thalassemia major using cardiovascular magnetic resonance. Circulation. 2007; 115: 1876-1884.

14- Wing-Yang A, Winnie WL, Winnie WC, Hui-Leung Y, Alvin SL, Rever CL, Helen MC, Harold KL, Man-Fai L, Herman SL, Raymond L, Shau-Yin H. A cross-sectional magnetic resonance imaging assessment of organ specific hemosiderosis in 180 thalassemia major patients in Hong Kong. Haematologica. 2008; 93: 784-785.

15- Chirnomas SD Geukes-Foppen M, Barry K, Braunstein J, Kalish LA,Neufeld EJ, Powell AJ. Practical implications of liver and heart iron load assessment by T2*-MRI in children and adults with transfusion-dependent anemias. Am J Hematol. 2008; 83: 781-783.

16- Daar S, Pathare AV, Jain R, Al Zadjali S, Pennell DJ. T2* cardiovascular magnetic resonance in the management of thalassemia patients in Oman. Haematologica. 2009; 94: 140-141.

17- Patton N, Brown G, Leung M, Bavishi K, Taylor J, Lioyd J, Lee SH, Tay L, Worthley S. Obsevational study of iron overload asassessed by magnetic resonance imaging in an adult population of transfusion dependant patients with beta thalassemia: Significant association between low cardiac T2*<10ms and the occurrence of cardiac events. Int Med J. 2009.

18- Aypar E, Alehan D, Hazirolan T, Gumruk F. 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.

19- Noetzli LJ, Carson SM, Nord AS, Coates TD, Wood JC. Longitudinal analysis of heart and liver iron in thalassemia major. Blood. 2008; 112: 2973-2978.

20- Parkes JG, Hussein RA, Olivieri NF, Templeton DM. Effects of iron loading on uptake, speciation, and chelation of iron in cultured myocardial cells. J Lab Clin Med. 1993; 122: 36-47.

21- Randell EW, Parkes JG, Olivieri NF, Templeton DM. Uptake of non-transferrin-bound iron by both reductive and non reductive processes is modulated by intracellular iron. J Biol Chem. 1994; 269: 16046-16053.

22- Cabantchik ZI, Breuer W, Zanninelli G, Cianciulli P. LPI-labile plasma iron in iron overload. Best Pract Res Clin Haematol. 2005; 18: 277-287.

23- Anderson LJ, Westwood MA, Holden S, Davis B, Prescott E, Wonke B, Porter JB, Walker JM, Pennell DJ. Myocardial iron clearance during reversal of siderotic cardiomyopathy with intravenous desferrioxamine: a prospective study using T2* cardiovascular magnetic resonance. Br J Haematol. 2004; 127: 348-355.

24- Oudit GY, Trivieri MG, Khaper N, Liu PP, Blackx PH. Role of L-type Calcium channels in iron transport and iron-overload cardiomyopathy. J Mol Med. 2006; 84: 349-364.

25- Ludwiczek S, Theurl I, Muckenthaler MU, Jakab M, Mair SM, Theurl M, Kiss J, Paulmichl M, Hentze MW, Ritter M, Weiss G. Calcium channel blockers reverse iron overload by a new mechanism via divalent metal transporter-I. Nat Med. 2007; 13: 448-454.

26- Wood JC, Tyszka JM, Ghurge N, Carson S, Nelson MD, Coates TD. Myocardial iron loading in transfusion dependent thalassemia and sickle cell disease. Blood. 2004; 103: 1934-1936.

27- Tanner MA, Westwood MA, Galanello R, Pennell DJ. Baseline findings of CMR driven randomized contolled trial of iron chelation therapy in thalassemia major. J Cardiovas Magn Reson. 2005; 7: 31-32.

28- Wood JC, Origa R, Agus A, Matta G, Coates TD, Galanello R. Onset of cardiac loading in pediatric patients with thalassemia major. Haematologica. 2008; 93: 917-920.

29- Leonardi B, Margossian R, Colan SD, Powell AJ. Relationship of magnetic resonance imaging estimation of myocardial iron to left ventricular systolic and diastolic function in thalassemia. JACC Cardiovasc Imaging. 2008; 1: 579-581.

30- Pennell DJ. T2* magnetic resonance and myocardial iron in thalassemia. Ann N Y Acad Sci. 2005; 1054: 373-378.

31- Bellenger NG, Burgess M, Ray SG, Coats A, Lahiri A, Cleland JGF. Comparison of left ventricular ejection fraction and volumes in heart failure by two-dimensional echocardiography, radionuclide ventriculography and cardiovascular magnetic resonance: are they interchangeable? The CHRISTMAS steering committee and investigators. Eur Heart J. 2000; 21: 1387-1396.