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The Role of CD200 in Differentiating B-Chronic Lymphocytic Leukemia from Mantle Cell Lymphoma and its Prognostic Value

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ABSTRACT

Background: There is a wide range of disease feature overlap between B-cell lymphomas as Mantle Cell Lymphoma (MCL), especially when presented in leukemic phase, and B-Chronic Lymphocytic Leukemia (B-CLL). Both arise from CD5+ve B-cells; their distinction is critical as MCL is a more aggressive neoplasm.

Objective: To determine whether there is a role for CD200 in the differentiation between B-CLL and MCL andits prognosticvalue.

Patients and Methods: A retrospective study was conducted onarchival material of 61 patients (48 with B-CLL and 13 with MCL) with a well defined diagnosis with fifteen age and sex matched healthy subjects as control group. Detection of t(11:14) was done by Fluorescent in Situ Hybridization (FISH) and CD200 was done by Immunohistochemistryon stored fixed paraffin embedded bone marrow biopsies.

Results: CD200 expression was significantly higher in B-CLL patients (47.4±28.9) compared to MCL patients (6.2±5.3) (p=<0.001).

Conclusion: Adding CD200 in routine monoclonal antibody panels could be of diagnostic utility in differentiating B-CLL from MCL. Also, anti CD200 targeted therapy may carry a promising treatment option to CD200 expressing cancers in the future.

Key Words: *B-cell lymphomas – B-Chronic lymphocytic leukemia – Mantle Cell Lymphoma – CD200.*

INTRODUCTION

There is a wide range of B-cell tumors present in leukemic phase that may be misdiagnosed as B-Chronic Lymphocytic Leukemia (B-CLL) because of overlapping disease features especially in the presence of CD5 positive lymphocytosis **[1]**. So, it is important to differentiate between B-CLL and Mantle Cell Lymphoma (MCL) by immunophenotypic analysis-particularly CD23 because of its positivity in B-CLL and negativity in MCL; however some cases of B-CLL are CD23 negative **[2-4]**.

The diagnosis of MCL should be confirmed by demonstration of cyclin D1 or by the presence of the t(11:14) detected by cytogenetics, fluorescence in situ hybridization (FISH) and Western blot or polymerase chain reaction (PCR) analysis. Even though these represent reliable methods, they are expensive, time-consuming and not available in all Centers **[5-8]**.

Cyclin D1 negative MCL do actually exist **[9]**, also MCL without t(11:14) have been reported and the same translocation can be found in B-CLL and other lymphoproliferative disorders **[10,11]**. Therefore, there is a need for new markers that allow an easier differential diagnosis between CLL and MCL.

CD200 is a transmembrane glycoprotein with inhibitory immunoregulation role. It has a relatively broad distribution as it is expressed on thymocytes, activated T cells, B cells, dendritic cells, endothelial cells, and neurons **[12-15]**.

El Desoukey et al., **[16]** reported that CD200 is expressed in B-CLL and Hairy Cell Leukemia (HCL) versus negative expression in MCL, follicular lymphoma (FL), and splenic marginal zone lymphoma (SMZL). The expression of CD200 was also reported in multiple myeloma, lymphoblastic lymphoma/leukemia, mediastinal large B cell lymphoma, lymphoplasmacytic

lymphoma, angioimmunoblastic T-cell lymphoma, acute myeloid leukemias, and other non-hematologic malignancies **[17-20]**.

The different expression of CD200 in B-CLL than in MCL could be explained by the different activation of the AKT, also known as Protein Kinase B (PKB), which is a serine/threonine-specific protein kinase that plays a key role in multiple cellular processes such as glucose metabolism, apoptosis, cell proliferation, transcription and cell migration; also, by activation of Mitogen-activated protein kinases, originally called ERK, Extracellular signal-regulated kinases (MEK/ERK), pathways. It has been reported in melanoma that CD200 mRNA expression correlates with ERK activation **[21,22]** and this pathway is also activated in B-CLL **[23,24]** where CD200 is in fact present. While, in MCL the well-known activation of AKT might lead to a downstream down regulation of active ERK **[25,26]** and this could contribute to the absence of CD200 in this disease.

Starting from these data, we addressed the question of whether CD200 expression can be a guiding marker in the differential diagnosis of CLL and MCL.

PATIENTS AND METHODS

The retrospective study included 61 patients, 48 patients with B-CLL (21 males and 27 females) and 13 patients with MCL (9 males and 4 females) with fifteen age and sex matched healthy subjects as control group. Cases have been selected from patients who were attendingthe Clinical Hematology Unit of Assiut University Hospital and South Egypt Cancer Institute between years 2005 and 2012. The study was approved by the Ethics committee of the Faculty of Medicine, Assiut University. Patients with liver dysfunction, other CD5+ve lymphoproliferative disorders and autoimmune diseases were excluded from the study population. Diagnosis of B-CLL was made by flow cytometry according to the latest version of the Matutes score **[27]**. Diagnosis of MCL was based on morphology and immunohistochemical detection of CD5+ve lymphocytes and FISH detection of t(11:14) in bone marrow biopsies.

The collected retrospective data were: Full history with clinical examination stressing on anemic manifestations, bleeding tendency, B-symptoms, presence of hepatomegaly, splenomegaly and lymphadenopathy; also, the laboratory results of peripheral hemogram, Coomb's test, lactic dehydrogenase, uric acid and bone marrow aspirate with immunophenotyping.

Bone marrow biopsies were selected from stored fixed paraffin embedded samples of study-included patients for detection of t(11;14) by Fluorescent in situ hybridization (FISH) technique in 5μ thick paraffin sections cut on positive charge slides. While, detection of CD200 was done by immunohistochemistry using a rabbit anti-human CD200 IgG affinity purified polyclonal antibody (SIGMA, U.S.A.) number HPA03119 and K5361/EnVision™ G/2 Doublestain System, Rabbit/Mouse (DAB+/Permanent Red) (Dako, USA) using 3μ thick paraffin sections.

Statistical analyses:

The collected data were analyzed by using SPSS/PC (version 17). Descriptive statistics: Mean, standard deviation, frequencies, percentage were calculated. Test of significances: Chi square test was used to compare the difference in distribution of frequencies among different groups. One-way Analysis of Variance (ANOA) was calculated to test the mean differences in continuous variables between groups. A significant *p*-value was considered when it is <0.05. Correlation analysis between CD200 and different variables of CLL was calculated. Validity statistics for CD200 test was calculated with a cutoff of 20%.

RESULTS

The study included 61 patients, 30 males (49.2%) and 31 were females (50.8%) with 15 halthy matched controls 8 males (53.3%) and 7 females (46.7%). The patient's age ranged from 35-85 with a median of 55 years while that of the controls was 31-78 with a median of 44 years. Forty eight patients (78.7%) were diagnosed as chronic lymphocytic leukemia through detection of (CD5+ve) by flow cytometryand 13 patients (21.3%) were diagnosed as mantle cell lymphoma by immunohistochemical detection of CD5+ve lymphocytes and FISH detection of t(11:14) in bone marrow biopsies.

Table (1); summarize the clinical findings and peripheral Hemogram data of B-CLL and MCL patientswith only statistically significant difference in WBCs count in B-CLL in relation to MCL patients ($p<0.01$). While, Table (2), illustrates the percent of positivity in different diagnostic markers in both patient's groups including CD200 activity that appears positive in 72.9% of B-CLL patients and only in 7.6% of MCL patients ($p<0.001$). The ability of the test to detect positive cases (sensitivity) was 72.9% and the ability to detect negative cases (specificity) was 92.3%. The ability to predict positive cases (PPV) was 97.2% and that to predict negative cases (NPV) was 48% with 77% accuracy.

CD200 expression was significantly higher ($p<0.001$) in B-CLL patients compared to MCL and control with a Mean±SD in CLL (47.4± 28.9), in MCL (6.2±5.3) and in control (8.7±2.1) with insignificant difference between MCL and control group (Fig. 1).

Also, CD200 expression in B-CLL was not correlated with any of the clinical data (anemia, bleeding tendency, B-symptoms and organomegaly), laboratory data (total leucocytic count, lymphocyte percentage in bone marrow aspirate, hemoglobin level, or platelet count).

Table (1): Clinical and peripheral hemogram criteria of booth B-CLL and MCL patients.

Item	B-CLL (n=48)	MCL (n=13)	*p* value
Clinical Findings:			
Bleeding	8 (16.3%)	1 (7.7%)	
Pallor	17 (35.4%)	3 (23.1%)	
Fever	15 (31.2%)	2 (15.4%)	NS
Hepatomegaly	18 (37.5%)	4 (30.4%)	
Splenomegaly	34 (70.8%)	10 (76.9%)	
Lymphadenopathy	39 (81.2%)	11 (84.6%)	
Peripheral Hemogram:			
Hb	9.9±201	9.7±201	NS
WBCs	95.8±104.9	19.7±21.9	<0.01
Platelets	139.5±70	113.7±56.2	NS

B-CLL: B- Chronic Lymphocytic Leukemia.
MCL : Mantle Cell Lymphoma.
Hb : Hemoglobin.
WBCs : White Blood Cells.
$p<0.05$ = *
$p<0.01$ = **
$p<0.001$ = ***
NS = Not significant.

Table (2): Percent of diffferent diagnostic criteria of booth B-CLL and MCL patients.

Diagnostic markers	B-CLL (n=48)	MCL (n=13)	*p* value
CD23	48 (100%)	0 (0.0%)	<0.001
FMC7	2 (4.2%)	12 (92.3%)	<0.001
SIg	28 (58.3%)	11 (84.6%)	<0. 05
t(11:14)	0 (0.0%)	13 (100%)	<0.001
CD200	35 (72.9%)	1 (7.6%)	<0.001

B-CLL: B- Chronic Lymphocytic Leukemia.
MCL : Mantle Cell Lymphoma.
CD : Cluster of differentiation.
$p<0.05$ = * $p<0.01$ = ** $p<0.001$ = ***

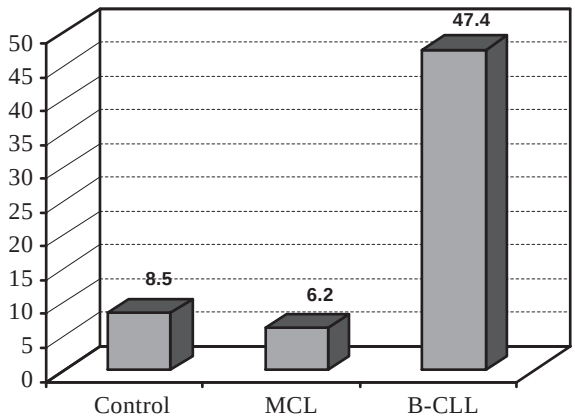

Fig. (1): Mean CD 200 expression in B-CLL, MCL patients and controls.

B-CLL: B- Chronic Lymphocytic Leukemia.
MCL : Mantle Cell Lymphoma.

DISCUSSION

It is important to differentiate B-CLL from MCL as the latter is a more aggressive disease and generally treated differently than B-CLL **[13]**. Differential diagnosis between B-CLL and MCL is assessed by immunophenotyping analysis on freshly isolated cells and in this respect CD23 is considered a reliable marker because of its positivity in B-CLL and negativity in MCL **[28,29]**.

The diagnosis of MCL should be confirmed by demonstration of Cyclin D1 positivity or by presence of chromosomal translocationt (11:14) **[30]** which is not pathgnomonic for MCL. In fact some MCL cases have been reported without t(11:14) **[31]**. The same translocation can be found in B-CLL and other lymphoproliferative disorders **[10,32]**.

In the current study, the frequency of CD200 positivity in B-CLL patients is significantly higher ($p<0.001$) than in MCL patients. The frequency of CD200 positivity in B-CLL patients is less when compared to the results of Palumbo et al., **[13]** who reported a statistically highly significant value in the expression of CD200 between CLL and MCL. The antigen was expressed in the neoplastic cells of all the B-CLL patients (100%) they studied but not in MCL. This is especially true for immunohistochemistry technique used in the current study that probably has a lower sensitivity when compared to flow cytometry as the flow cytometry can detect CD200 positive cells in a freshly prepared blood sample while immunohistochemical technique depends on staining of stored fixed tissue samples.

Also, CD200 expression is significantly higher in CLL than control group; this is in agreement with the results of Mc-Whirter et al., 2006 **[33]** who reported that CLL patients exhibited 1.6 to 5.4 fold up-regulation of CD200 relative to normal B cells. The up-regulation of CD200 may be a mechanism used by CLL tumors to evade eradication by the immune system.

The different expression of CD200 could be explained by the different activation of the Mitogen-activated protein kinases, originally called ERK, Extracellular signal-regulated kinases (MEK/ERK) pathways in these two diseases. It has been reported that CD200mRNA expression correlates with ERK activation in melanoma as reported by **[21,22]**. This pathway is also activated in B-CLL as confirmed by **[23,24]**, where CD200 is in fact present. On the contrary, in MCL the well known activation of AKT might lead to a down regulation of active ERK and this could contribute to the absence of CD200 according to **[25,26]**.

In this study the optimum cut-off point of CD200 detection was selected at 20%, with a sensitivity of 72.9%, specificity 92.3%, positive predictive value 97.2%, negative predictive value 48% and accuracy of 77% to differentiate the B-CLL cases from the MCL cases. On the other hand Bhatnagar et al., 2010 **[34]** reported that at the same cutoff sensitivity was 100%, specificity was 98.7%, and positive predictive value was 98.7%. These differences may be attributed to the difference in the technique as they used flow cytometry and we used immunohistochemistry on archived paraffin-embedded blocks.

In the current study, CD200 expression in B-CLL was not correlated with any of the clinical and laboratory data; this is in agreement with El Desoukeyand his colleagues in 2012 **[16]** as they found that CD200 expression in B-CLL was significantly higher than other lymphoproliferative disease and was not correlated with any of the clinical and laboratory data.

CD200 could be very useful for the differential diagnosis between B-CLL and MCL especially in patients with CD5 positive lymphocytes. Although the B-CLL and MCL have very different behavior towards therapy and prognosis, they share many immunophenotyping and morphological features that make the differentiation exceedingly difficult as described by Bosch and his colleagues in 1998 **[31]**. So we recommend adding CD200 in immunohistochemistry and flow cytometry routine panels as it would be of great diagnostic value especially in cyclin D1 negative MCL cases as those reported by Fu et al., 2005.

Performing CD200 by immunohistochemistry might be useful in revising old cases or those referred by peripheral centers when fresh cells are not available. Also, further studies are needed to prove CD200 usefulness in differentiating B-CLL from other B-cell low grade lymphomas as it is considered a simple applicable, reliable, non expensive and accurate marker.

We recommend further studies of CD200 expression levels on B-CLL cells in relation to treatment and prognosis and we hope that anti-CD200 targeted therapy in CD200 expressing cancers would be promising treatment in the future.

REFERENCES

1- Nelson BP, Variakojis D, Peterson LC. Leukemic phase of B-cell lymphomas mimicking chronic lymphocytic leukemia and variants at presentation. Mod Pathol. 2002; 15 (11): 1111-20.

2- Dorfman DM, Pinkus GS. Distinction between small lymphocytic and mantle cell lymphoma by immunoreactivity for CD23. Mod Pathol, 1994; 7: 326-31.

3- Barna G, Reiniger L, Tatrai P, Kopper L, Matolcsy A. The cut-off levels of CD23 expression in the differential

diagnosis of MCL and CLL. Hematol Oncol. 2008; 26 (3): 167-70.

4- Jurisic V, Colovic N, Kraguljac N, Atkinson HD, Colovic M. Analysis of CD23 antigen expression in B-chronic lymphocytic leukaemia and its correlation with clinical parameters. Med Oncol. 2008; 25 (3), 315-322.

5- Banks PM, Chan J, and Cleary ML. Mantle cell lymphoma: A proposal for unification of morphologic, immunologic, and molecular data. Am J Surg Pathol. 1992; 16 (7): 637-40.

6- Li JY, Gaillard F, Moreau A, Harousseau JL, Laboisse C, Milpied N, Bataille R, Avet-Loiseau H. Detection of translocation t(11;14) (q13;q32) in mantle cell lymphoma by fluorescence in situ hybridization. Am J Pathol. 1999; 154 (5): 1449-52.

7- Jain P, Giustolisi GM, Atkinson S, Elnenaei MO, Morilla R, Owusu-Ankomah K. Detection of cyclin D1 in B cell lymphoproliferative disorders by flow cytometry. J Clin Pathol. 2002; 55: 940-5.

8- Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J, Vardiman JW (eds). WHO classification of tumours of haematopoietic and lymphoid tissues. IARC Press, Lyon, France. 2008.

9- Fu K, Weisenburger DD, Greiner TC, Dave S, Wright G, Rosenwald A. Cyclin D1-negative mantle cell lymphoma: A clinicopathologic study based on gene expression profiling. Blood. 2005; 106: 4315-21.

10- Swerdlow SH, Zukerberg LR, Yang WI, Harris NL, Williams ME. The morphologic spectrum of non-Hodgkin's lymphomas with BCL1/cyclin D1 gene rearrangements. Am J Surg Pathol. 1996; 20: 627-40.

11- Ruchlemer R, Parry-Jones N, Brito-Babapulle V, Attolico I, Wotherspoon AC, Matutes E, Catovsky D. B-prolymphocyticleukaemia with t(11;14) revisited: A splenomegalic form of mantle cell lymphoma evolving with leukaemia. Br J Haematol. 2004; 125 (3): 330-6.

12- Kawasaki BT, Farrar WL. Cancer stem cells, CD200 and immunoevasion. Trends Immunol. 2008; 29: 464Y468.

13- Palumbo GA, Conticello C, Villari L, Di Raimondo F., Parrinello N, Fargione G, Cardillo K, Chiarenza A, Berretta S. CD200 expression may help in differential diagnosis between mantle cell lymphoma and B-cell chronic lymphocytic leukemia. Leuk Res. 2009; 33 (9): 1212-6.

14- Dorfman DM, Shahsafaei A. CD200 (OX-2 membrane glycoprotein) expression in b cell-derived neoplasms. Am J Clin Pathol. 2010; 134 (5): 726-33.

15- Hoeller S, Zhou Y, Kanagal-Shamanna R, Zijun Y, Hoehn D, Bihl M, Swerdlow SH, Rosenwald A, Ott G, Said J, Dunphy CH., Young KH. Composite Mantle Cell Lymphoma and Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma: A Clinicopathologic and Molecular Study. Hum Pathol. 2013; 44 (1): 110-121.

16- El Desoukey NA, Afify RA, Amin DG, Mohammed RF. CD200 expression in B-cell chronic lymphoproliferative disorders. J Investig Med. 2012; 60 (1): 56-61.

17- Moreaux J, Hose D, and Reme T. CD200 is a new prognostic factor in multiple myeloma. Blood. 2006; 108: 4194-7.

18- Tonks A, Hills R, White P. CD200 as a prognostic factor in acute myeloid leukemia. Leukemia. 2007; 21: 566-8.

19- Moreaux J, Veyrune JL, Reme T, De VJ, Klein B. CD200: A putative therapeutic target in cancer. Biochem Biophys Res Commun. 2008; 366:117-122.

20- Dorfman DM, Shahsafaei A. CD200 (OX-2 membrane glycoprotein) expression in b cell-derived neoplasms. Am J Clin Pathol. 2010; 134 (5): 726-33.

21- Petermann KB, Rozenberg GI, Zedek D, Groben P, McKinnon K, Buehler C, Kim WY, Shields JM, Penland S, Bear JE. CD200 is induced by ERK and is a potential therapeutic target in melanoma. J Clin Invest. 2007; 117: 3922-3929.

22- Shields JM, Thomas NE, Cregger M, Berger AJ, Leslie M, Torrice C. Lack of extracellular signal-regulated kinase mitogen-activated protein kinase signaling shows a new type of melanoma. Cancer Res. 2007; 67: 1502-12.

23- Smal C, Lisart S, Maerevoet M, Ferrant A, Bontemps F, Van Den, Neste E. Pharmacological inhibition of the MAPK/ERK pathway increases sensitivity to 2-chloro-2_-deoxyadenosine (CdA) in the B-cell leukemia cell line EHEB. Biochem Pharmacol. 2007; 73: 351-8.

24- Longo PG, Laurenti L, Gobessi S, Sica S, Leone G, Efremov DG. The Akt/Mcl-1 pathway plays a prominent role in mediating antiapoptotic signals downstream of the B-cell receptor in chronic lymphocytic leukemia B cells. Blood. 2008; 111: 846-55.

25- Ghobrial IM, McCormick DJ, Kaufmann SH, Leontovich AA, Loegering DA, Dai NT. Proteomic analysis of mantle-cell lymphoma by protein microarray. Blood. 2005; 105: 3722-30.

26- Jares P, Colomer D, Campo E. Genetic and molecular pathogenesis of mantle cell lymphoma: Perspectives for new targeted therapeutics. Nat Rev Cancer. 2007; 7: 750-62.

27- Matutes E, Owusu-Ankomah K, and Morilla R. The immunological profile of B-cell disorders and proposal of a scoring system for the diagnosis of CLL. Leukemia. 1994; 8: 1640-5.

28- Schlette E, Fu K, Medeiros LJ. CD23 expression in mantle cell lymphoma: Clinicopathologic features of 18 cases. Am J Clin Pathol. 2003; 120: 760-6.

29- Jurisic V, Colovic N, Kraguljac N, Atkinson HD, Colovic M. Analysis of CD23 antigen expression in B-chronic lymphocytic leukaemia and its correlation with clinical parameters. Med Oncol. 2008; 25 (3), 315-322.

30- Chen YH, Gao J, Fan G, Peterson LC. Nuclear expression of sox11 is highly associated with mantle cell lymphoma but is independent of t(11;14) (q13;q32) in non-mantle cell B-cell neoplasms. Mod Pathol. 2010; 23 (1): 105-12.

31- Bosch F, Lopez-Guillermo A, Campo E. Mantle cell lymphoma: Presenting features, response to therapy, and prognostic factors. Cancer. 1998; 82: 567-75.

32- Fegan C, Robinson H, and Thompson P. Evolution in CLL: Identification of a new sub-group of patients with deletions of 11q and advanced or progressive disease. Leukemia. 1995; 9: 2003-8.

33- McWhirter JR, Kretz-Rommel A, Saven A, Maruyama T, Potter KN, Mockridge CI, Ravey EP, Qin F, Bowdish KS. Antibodies selected from combinatorial libraries block a tumor antigen that plays a key role in immunomodulation. Proc Natl Acad Sci USA. 2006; 103: 1041-1046.

34- Bhatnagar N., Wright F., Aleese D Mc, Namara C Mc. The utility of CD200 expression in the differentiating of Mantle cell lymphoma from B-cell chronic lymphocytic leukemia: a single centre experience. The Royal Free Hospital, London, UK F1000 Posters. 2010; 1: 719.

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The Predisposing Role of NAD (P) H:Quinine Oxidoreductase Gene Polymorphisms in the Development of Pediatric Acute Lymphoblastic Leukemia

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ABSTRACT

Objectives: NAD (P) H:Quinone Oxidoreductase (NQO1) protects cells against oxidative stress and toxic quinines which protects cells against mutagenicity of free radicals and toxic oxygen metabolites. In fact, low level of NQO1 activity is often associated with increased risk of developing different types of tumors and with toxic effects linked to environmental quinines. Acute lymphoblastic leukemia (ALL) is the most common pediatric cancer. C to T base substitution at nucleotides 609 and 465 of NQO1 cDNA, results in loss of enzyme activity. Low NQO1 activity may play a role in etiology of ALL. In the present study, we investigated the association between the NQO1 polymorphisms and increased risk of ALL in children.

Methods: C609T and C465T polymorphisms of NQO1 were explored using polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) assay in 100 pediatric ALL patients and 135 healthy controls.

Results: Although C609T polymorphism is very common among the general population, we found no association between this variant and increased risk for pediatric ALL [odds ratio (OR) = 0.95; 95% confidence interval (95% CI) = 0.55–1.64]. Interestingly the other polymorphic allele of NQO1 (C465T) was strongly associated with pediatric ALL (OR = 7.83; 95% CI = 3.27-18.75).

Conclusion: These findings do not support the predisposing role of NQO1 C609T polymorphism for pediatric ALL. However, The C465T polymorphism was associated with increased risk of pediatric ALL. Further studies with larger sample including evaluating multiple gene-gene interactions seem necessary to validate the exact role of these mutations.

Key Words: *Acute lymphoblastic leukemia – polymorphism – NQO1 – RFLP.*

INTRODUCTION

As a heterogeneous disease that disrupts normal hematopoiesis in acute or chronic form, leukemia accounts for one-third of all cancer cases among patients under the age of 15 years and constitutes the most common type of pediatric cancer **[1]**. Among patients younger than 15 years of age with childhood leukemia, acute lymphoblastic leukemia (ALL) comprises 80% of cases; acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) account for 18% and 2% of cases, respectively. Chronic lymphocytic leukemia (CLL) rarely occurs in children **[2]**. Various studies **[3-5]** have demonstrated polymorphisms that contribute to detoxification of carcinogens are related to the development of various types of cancer. NAD (P) H: Quinone Oxidoreductase 1 (NQO1), (OMIM: 125860), protects cells against oxidative stress and toxic quinines **[6]**. Moreover, it has been shown **[7,8]** that this protein interacts with and stabilizes the tumor suppressor protein p53. Asher et al., **[8]** suggested that exposure to carcinogenic substrates of NQO1 could lead to increased genotoxic damage at lower p53 levels in individuals with lower NQO1 activity (compared with individuals with normal NQO1 activity). NQO1 is expressed in most tissues, including bone marrow, in which expression is thought to be highly inducible and up-regulated during the oxidative process **[9]**. The NQO1 can contribute to the formation of reactive oxidation species via oxidative cycling; therefore, it can act as a pro-oxidant **[10]**. A number of single nucleotide polymorphisms (SNPs) have been identified in NQO1 but only 2 of them, namely, NQO1*2 and NQO1*3, have been implicated as signifying risk of a variety of cancers **[11]**. The NQO1*2 polymorphism (C609T) causes a change in the amino-acid sequence (Pro187Ser)

and a low activity variant enzyme [12]. The prevalence of the NQO1 C609T polymorphism is 4.4% in non-Hispanic whites, 5.2% in African Americans, 12.2% in ethnic Japanese, 15.5% in ethnic Mexican-Hispanics, 17.9% in Native Americans, 18.8% in ethnic Koreans, and 22.4% in ethnic Chinese [9,13,14]. Another polymorphic variant of NQO1, namely, NQO1*3 (ie, C465T that results in Arg139Trp), causes alternative messenger RNA splice sites that can lead to deletion of exon 4 and the creation of a protein that lacks the quinone binding site [15,16]. Heterozygotes for the variant alleles of NQO1 (ie, the C/T genotypes of NQO1 C609T and C465T) display intermediate enzymatic activity, whereas homozygotic alleles (ie, T/T genotypes) display essentially no NQO1 activity [17]. The frequency of the NQO1 C465T polymorphism is generally low and ranges from 0% to 5% among different ethnic populations [18]. Several reports [19-21] about the role of NQO1 in childhood ALL were summarized in a recent meta-analysis [22]. Despite the results of these studies, it has been shown [23,24] that NQO1 C609T is associated with an elevated risk of nonhematologic malignancies such as urologic and basal cell carcinomas. Association of the NQO1 C465T polymorphism, the other variant form of NQO1, with ALL has been reported in 2 studies [20,25]. Herein, we assessed common polymorphisms of NQO1 (C609T and C465T) in relation to pediatric ALL in a case control study. The basic questions in our study were whether these polymorphisms play an important role in susceptibility to ALL and if these SNPs in our population have a high degree of heterozygosity.

MATERIAL AND METHODS

Patient and Control Samples We performed a case-control study with 100 patients with ALL [male/female: 0.72, mean age 6.5 (±5.0) years] and 135 healthy age- and sex-matched individuals without leukemia as the control group [male/female: 0.84, mean age 6.0 (±6.0) years]. Patient samples were diagnosed as ALL through morphological and immunophenotypic assessments and were randomly collected at the time of diagnosis from October, 2010 through November, 2013, at Dammam University of Medical Sciences in Saudi Arabia. Immunophenotypic subtypes of patients with ALL according to French-American-British (FAB) classification were as follows: 41 patients with pre-B ALL; 37 patients with early pre-B ALL; 15 patients with T ALL, and 7 patients with pro-B ALL. The Medical Ethics Committee of the Dammam University of Medical Sciences (DUMS) approved the study; written informed consent was obtained from all patients and healthy control individuals who participated in this study.

Genotyping analysis:

Blood and bone marrow samples were collected into tubes containing ethylenediaminetetraacetic acid (EDTA); subsequently, mononuclear cells were purified by Ficoll-Hypaque (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) centrifugation, then their DNA was extracted through the standard method [26]. Genotyping was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), as described by Eguchi-Ishimae et al., [25] Twenty nmol of each primer for NQO1-C609T, forward primer: 5'-CCTCTC TGTGCTTTCTGTATCC-3' and reverse primer: 5'-GATGGACTTGCCCAAGTGATG-3'; for NQO1-C465T, forward primer: 5'-CTAGCTTT ACTCGGACCCACTC-3' and reverse primer: 5'-GCAACAAGAGGGAAGCTCCATC-3' were mixed with 60ng of DNA, 50mM KCl, 10mM tris (hydroxymethyl) aminomethane-hydrogen chloride (Tris-HCl) (pH, 8.3), 2.5 pmol of each deoxynucleoside triphosphate (dNTP), and 1.25 units of Taq polymerase (GeNet Bio, Nonsan, South Korea) in a total volume of 25μL. These samples were subjected to PCR using a TC-512 Techne Thermal Cycler (Bibby Scientific Limited, Staffordshire, England) with initial denaturation at 95ºC for 5 minutes, followed by 35 cycles (94ºC for 1 minute, 60ºC for 45 seconds, and 72ºC for 1 minute), and finally, an extension phase at 72°C for 10 minutes. In the next step, digestion of the PCR products for the NQO1 C609T polymorphism using HinfI (Thermo Fisher Scientific Inc, Waltham, MA) produced 2 bands for homozygous wildtypes (CC; 85 and 214 bp), 4 bands for heterozygotes (CT; 63, 85, 151, and 214 bp), and 3 bands for homozygous mutants (TT; 63, 85, and 151 bp). The PCR products were also digested by HpaII (Thermo Fisher Scientific Inc) to be assessed for NQO1 C465T polymorphism, which generated 2 bands for homozygous wild types (CC; 111and 353 bp), 3 bands for heterozygotes (CT; 111, 353, and 464 bp), and 1 band for homozygous mutants (TT; 464 bp).

Statistical analysis:

Statistical analysis for a different genotype distribution in case individuals versus controls was performed via the C2-test. The same analysis was also used to calculate the significance of differences in allele frequencies between the control and patient groups. Odds ratios (ORs) with 95% confidence intervals (CIs) were estimated by logistic regression. All statistical analyses were performed with SPSS software, version 16.0 (SPSS, Inc, Chicago, IL).

Table (1): Distribution of the NQO1 C609T & NQO1C 465T Genotypes Among ALL Patients and Controls

	CI (95%)	OR	Controls	Patients	Variants
NQO1 C609T	–	1.00*	89 (65.92%)	67 (67.0%)	CC
	(0.56-1.8)	1.01	38 (28.14%)	29 (29.0%)	CT
	(0.19-2.29)	0.66	8 (5.92 %)	4 (4.0%)	TT
	(0.55-1.64)	0.95	46 (34.07%)	33 (33.0%)	CT/TT
Allele-wise comparison (%)	–	1.00	108 (80.00%)	81 (81.0%)	C
	(0.48-1.82)	0.93	27 (20.00%)	19 (19.0%)	T
NQO1 C465T	–	1.00*	128 (94.82%)	70 (70.0%)	CC
	(3.06-23.09)	8.41	5 (3.70%)	23 (23.0%)	CT
	(1.29-31.64)	6.40	2 (1.48%)	7 (7.0%)	TT
	(3.27-18.75)	7.83	7 (5.18%)	30 (30.0%)	CT/TT
Allele-wise comparison (%)	–	1.00	130 (96.30%)	81 (81.0%)	C
	(2.19-16.97)	6.09	5 (3.70%)	19 (19.0%)	T

OR: Odds ratio. CC: Wild type. CI : Confidence interval. CT: Heterozygous. TT: Homozygous mutant.

Table (2): Combined Effects of NQO1 C609T and NQO1 C465T Genotypes on ALL Risk.

CI (95%)	OR	Controls	Patients	Combination
–	1.00*	86 (63.70%)	46 (46.0%)	CC609/ CC465
3.51-44.16	12.46	3 (2.22%)	20 (20.0%)	CC609/ CT465
0.33-42.34	3.73	1 (0.74%)	2 (2.0%)	CC609/ TT465
0.58-2.14	1.12	35 (25.92%)	21 (21.0%)	CT609/ CC465
1.52-36.68	7.47	2 (1.5%)	8 (8.0%)	CT609/ CT465
–	0.00	1 (0.74%)	0 (0.0%)	CT609/ TT465
0.12-3.21	0.62	6 (4.44%)	2 (2.0%)	TT609/ CC465
0.11-30.58	1.87	1 (0.74%)	1 (1.0%)	TT609/ CT465
–	0.00	0 (0.0%)	0 (0.0%)	TT609/ TT465

CI (95%) = Confidence interval. OR = Odds ratio.

RESULTS

All 235 DNA samples (from patients and controls) were successfully genotyped using the PCR-RFLP technique. NQO1 variants genotyping among 135 healthy controls indicated mutant allele frequencies of 20.0% and 3.7% for NQO1 C609T and C465T, respectively, which are comparable with the percentage of NQO1 variants in the control group of a previous study by some of us27 about NQO1 polymorphisms in adult AML patients (21.25% and 2.5% for C609T and C465T variants, respectively). The association between NQO1 variants and ALL risk were assessed by logistic regression. The NQO1 C609T genotypes for the 135 controls were distributed as follows: 65.92% wild type (CC), 28.14% heterozygous (CT) and 5.92% homozygous mutants (TT; the frequencies for CC, CT, and TT genotypes among the 100 patients with ALL were 67.0%, 29.0% and 4.0%, respectively. Regarding NQO1 C465T, genotype distribution among 135 controls was as follows: 94.8% wild type (CC), 3.7% heterozygous (CT), and 1.5% homozygous mutants

(TT), whereas CC, CT, and TT genotype frequencies in the patient group were 70.0%, 23.0% and 7.0%, respectively. The mutant allele frequency between patients was 19.0% for NQO1 C609T and C465T (Table 1).

In addition to evaluation of independent associations between NQO1 C609T and C465T polymorphisms and ALL, we assessed the joint effects of these 2 polymorphisms. Table (2) shows that CC609/CT465 and CT609/CT465 genotypes have significant correlation with pediatric ALL.

DISCUSSION

NQO1 may play a crucial role in protecting cells against cancer. For instance, it seems to not only stabilize the p53 protein 28 but also to contribute to anticancer signaling pathways that are activated by tumor necrosis factor and other inflammatory stimuli **[29]**. In this study, the distribution of alleles and genotype frequencies of NQO1 variants were compared with ALL and controls to find a possible association between these variants and elevated risk of developing ALL. We found no statistically significant association between the NQO1 C609T polymorphism and risk of childhood ALL (CT/TT versus CC; OR, 0.95; 95% CI, 0.55-1.64), whereas the mutant genotypes of NQO1 C465T showed a significant association with risk of ALL (CT/TT versus CC; 7.83; 3.27-18.75).

Chi-square analysis also showed a significant difference in the allele frequencies between the control and patient groups for the C465T polymorphism only (f2 =14.65, df = 1, $p<0.001$ for the C465T variant; f2 = 0.036, df = 1, $p=0.85$ for the C609T variant).

Regarding NQO1 C609T, our results contrast with those of previous studies **[20,30,31]** in other populations. Our results indicate that the NQO1 C609T polymorphism is associated with the elevated risk of childhood ALL.

Recently, a family-based study32 suggested that the NQO1 C609T variant was associated with the risk of developing childhood ALL; another study **[33]** performed in 2004 in Turkey did not support the role of the NQO1 C609T polymorphism in the increased risk of pediatric acute leukemia.

In Brazilian children, the NQO1 and myeloperoxidase (MPO) polymorphisms were shown to have a protective function against leukemogenesis **[34]**.

A French-Canadian study **[35]** showed that children carrying at least 1 mutant allele of the NQO1 C609T polymorphism had an increased risk of developing ALL, whereas individuals with wild-type homozygotes seem to be protected against ALL.

Lack of agreement between these studies might be due to differences in the duration of the exposure to the NQO1 substrates and small sample sizes, as well as the demographic stratification that exists in these kinds of studies.

In a HUGE net literature review and meta-analysis **[36]**, it was shown that the NQO1 C609T variant appeared to have no strong association with childhood ALL or AML but may be associated with mixed lineage leukemia–positive childhood leukemia.

In our study, it is noteworthy that the NQO1 C609T variant did not show any effect on ALL in univariant analysis; however, in multivariant analysis, the heterozygous genotype (CT) of NQO1 C609T in combination with the heterozygous genotype (CT) of NQO1 C465T showed an increased risk for ALL (OR, 7.47; 95% CI, 1.52-36.68), in which the main effect might have been created by the CT465 allele. Also, the CC609/CT465 combined genotype was significantly associated with risk of ALL; again, this supports the results of our univariant analysis.

The effect of these polymorphisms in NQO1 may be modified by polymorphisms in other carcinogen metabolizing genes, such as glutathione S-transferase (GST), cytochrome P450 2E1 (CYP2E1), and MPO. Therefore, it is important to study the effects of gene-gene and gene-environment interactions in the development of childhood ALL. In a study in Japan,13 variant alleles of NQO1 C465T also showed a striking positive association with infant ALL, especially in individuals with the chromosomal translocation of t(4;11)(q21;q23).

Other studies support the concept that the etiology of ALL in children is related to genetic variability at more than one gene locus and may

be related to the equilibrium between the metabolic activation and detoxification processes **[37]**. Therefore, it is crucial to study other polymorphisms that could possibly affect the susceptibility to ALL, such as MPO and CYP1E2.

Nevertheless, to yield more findings about the role of NQO1 polymorphisms in the etiology of childhood leukemia, further research, particularly using a larger sample size and sound design, seems to be necessary.

In summary, our study suggests that the mutant allele of the NQO1 C609T polymorphism is not associated with increased risk of pediatric ALL, whereas the NQO1 C465T variant showed a significant association with increased risk of ALL in children. Previous findings **[36]** have suggested that the etiology of ALL cannot be explained by polymorphism at a single locus, perhaps because of complexity in the metabolism of diverse xenobiotic compounds. Therefore, multiple gene-gene interactions should be investigated to enable prediction of the risk of ALL.

REFERENCES

1- M Krajinovic, H Sinnett, C Richer, D Labuda, D Sinnett. Role of NQO1, MPOand CYP2E1 genetic polymorphisms in the susceptibility to childhood acute lymphoblastic leukaemia, Int J Cancer. 2002; 230-236.

2- Pui C-H, Schrappe M, Ribeiro RC, Neimayer CM. Childhood and adolescent lymphoid and myeloid leukemia. ASH Education Book. 2004; 118-145.

3- Yamaguti G, Lourenço G, Silveira V, Tone LG, Lopes LP, Lima CP. Increased risk for acute lymphoblastic leukemia in children with cytochrome P450A1 (CYP1A1)- and NAD (P) H: Quinone Oxidoreductase 1 (NQO1)-inherited gene variants. Acta Haematol. 2010; 124 (3): 182-184.

4- Gallegos-Arreola M, Batista-González CM, Delgado-Lamas JL, et al. Cytochrome P4501A1 polymorphism is associated with susceptibility to acute lymphoblastic leukemia in adult Mexican patients. Blood Cells Mol Dis. 2004; 33 (3): 326-329.

5- Joseph T, Kusumakumary P, Chacko P, Abraham A, Pillai MR. Genetic polymorphism of CYP1A1, CYP2 D6, GSTM1 and GSTT1 and susceptibility to acute lymphoblastic leukaemia in Indian children. Pediatr Blood Cancer. 2004; 43 (5): 560-567.

6- Ernster L. DT-diaphorase: Its structure, function, regulation, and role in antioxidant defence and cancer chemotherapy. In: Yagi K, ed. Pathophysiology of Lipid Peroxides and Related Free Radicals. Basel, Switzerland: S Karger AG. 1998; 149.

7- Anwar A, Dehn D, Siegel D, Sachs L, et al. Interaction of human NAD (P) H: Quinone Oxidoreductase 1 (NQO1) with the tumor suppressor protein p53 in cells and cell-free systems. J Biol Chem. 2003; 278 (12): 10368-10373.

8- Asher G, Lotem J, Kama R, Sachs L, Shaul Y. NQO1 stabilizes p53 through a distinct pathway. Proc Natl Acad Sci USA. 2002; 99 (5): 3099-3104.

9- Ross D, Siegel D. NAD (P) H: Quinone Oxidoreductase 1 (NQO1, DT-diaphorase), functions and pharmacogenetics. Methods Enzymol. 2004; 382: 115-144.

10- Metodiewa D, Jaiswal AK, Cenas N, Dickancaité E, Segura-Aguilar J. Quercetin may act as a cytotoxic prooxidant after its metabolic activation to semiquinone and quinoidal product. Free Radic Biol Med. 1999; 26 (1-2): 107-116.

11- Larson RA, Wang Y, Banerjee M, et al. Prevalence of the inactivating 609C-T polymorphism in the NAD (P) H: Quinine Oxidoreductase (NQO1) gene in patients with primary and therapy-related myeloid leukemia. Blood. 1999; 94:803-807.

12- Traver RD, Horikoshi T, Danenberg KD, et al. NAD (P) H: Quinine oxidoreductase gene expression in human colon carcinoma cells: Characterization of a mutation which modulates DT-diaphorase activity and mitomicina sensitivity. Cancer Res. 1992; 52: 797-802.

13- Kiyohara C, Yoshimasu K, Takayama K, Nakanishi Y. NQO1, MPO, and the risk of lung cancer: A HuGE Review. Genet Med. 2005; 7: 463-478.

14- Kelsey KT, Ross D, Traver RD, et al. Ethnic variation in the prevalence of a common NAD (P) H quinone oxidoreductase polymorphism and its implications for anti-cancer chemotherapy. Br J Cancer. 1997; 76: 852-854.

15- Gasdaska PY, Fisher H, Powis G. An alternatively spliced form of NQO1 (DT-diaphorase) messenger RNA lacking the putative quinine substrate binding site is present in human normal and tumor tissues. Cancer Res. 1995; 55: 2542-2547.

16- Pan S-S, Han Y, Farabaugh P, Xia H. Implication of alternative splicing for expression of a variant NAD (P) H: Quinone oxidoreductase-1 with a single nucleotide polymorphism at 465C>T. Pharmacogenetics. 2002; 12: 479-488.

17- Malik E, Cohen SB, Dvorah C, Dann EJ, Rund D. The frequencies of NAD (P) H quinone oxidoreductase (NQO1) variant allele in Israeli ethnic groups and the relationship of NQO1*2 to adult acute myeloid leukemia in Israeli patients. Haematologica. 2006; 91: 956-959.

18- Greaves MF, Alexander FE. An infectious etiology for common acute lymphoblastic leukemia in childhood? Leukemia. 1993; 7: 349-360.

19- Clavel J, Bellec S, Rebouissou S, et al. Childhood leukaemia, polymorphisms of metabolism enzyme genes, and interactions with maternal tobacco, coffee

and alcohol consumption during pregnancy. Eur J Cancer Prev. 2005; 14: 531-540.

20- Wiemels JL, Pagnamenta A, Taylor GM, et al. A lack of a functional NAD (P) H: Quinone oxidoreductase allele is selectively associated with pediatric leukemias that have MLL fusions. Cancer Res. 1999; 59: 4095-4099.

21- Lanciotti M, Dufour C, Corral L, et al. Genetic polymorphism of NAD (P) H: Quinone oxidoreductase is associated with an increased risk of infant acute lymphoblastic leukemia without MLL gene rearrangements. Leukemia. 2005; 19: 214-216.

22- Guha N, Chang JS, Chokkalingam A, et al. NQO1 polymorphisms and de novo childhood leukemia: A HuGE review and meta-analysis. Am J Epidemiol. 2008; 168: 1221-1232.

23- Schulz WA, Krummeck A, Rösinger I, et al. Increased frequency of a null-allele for NAD (P) H: Quinone oxidoreductase in patients with urological malignancies. Pharmacogenetics. 1997; 7: 235-239.

24- Clairmont A, Sies H, Ramachandran S, et al. Association of NAD (P) H: Quinone Oxidoreductase (NQO1) null with numbers of basal cell carcinomas: Use of a multivariate model to rank the relative importance of this polymorphism and those at other relevant loci. Carcinogenesis. 1999; 20: 1235-1240.

25- Eguchi-Ishimae M, Eguchi M, Ishii E, et al. The association of a distinctive allele of NAD (P) H: Quinone oxidoreductase with pediatric acute lymphoblastic leukemias with MLL fusion genes in Japan. Haematologica. 2005; 90 (11): 1511-1515.

26- Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. 1982.

27- Zaker F, Safaei A, Hashemi M, et al. The frequency and association of C609T and C465T polymorphisms of NAD (P) H: Quinine oxidoreductase gene with adult acute myeloid leukemia. LabMedicine. 2011; 42 (11): 18-21.

28- Anwar A, Dehn D, Siegel D, et al. Interaction of human NAD (P) H: Quinone oxidoreductase 1 (NQO1) with the tumor suppressor protein p53 in cells and cell-free systems. J Biol Chem. 2003; 278: 10368-10373.

29- Kwang S, Gautam S, Abhinav J , et al. Genetic deletion of NAD (P) H: Quinone oxidoreductase 1 abrogates activation of nuclear factor-jB, IjBa kinase, c-Jun N-terminal kinase, Akt, p38, and p44/42 mitogenactivated protein kinases and potentiates apoptosis. J Biol Chem. 2006; 281: 19798-19808. QR code generated on http://qrcode.littleidiot.be.

30- Smith MT, Wang Y, Skibola CF, et al. Low NAD (P) H: Quinine oxidoreductase activity is associated with increased risk of leukemia with MLL translocations in infants and children. Blood. 2002; 100: 4590-4593.

31- Vijayakrishnan J, Houlston RS. Candidate gene association studies and risk of childhood acute lymphoblastic leukemia: A systematic review and meta-analysis. Haematologica. 95 (8): 1405-1414. 32. Infante-Rivard C, Vermunt JK, Weinberg CR. Excess transmission of the NAD (P) H: Quinone oxidoreductase 1 (NQO1) C609T polymorphism in families of children with acute lymphoblastic leukemia. Am J Epidemiol. 2007; 165 (11): 1248-1254.

33- Sirma S, Agaoglu L, Yildiz I, et al. NAD (P) H: Quinone oxidoreductase 1 null genotype is not associated with pediatric de novo acute leukemia. Pediatr Blood Cancer. 2004; 43 (5): 568-570.

34- Silveira Vda S, Canalle R, Scrideli CA, et al. Role of the CYP2D6, EPHX1, MPO, and NQO1 genes in the susceptibility to acute lymphoblastic leukemia in Brazilian children. Environ Mol Mutagen. 2010; 51 (1): 48-56.

35- Krajinovic M , Sinnett H, Richer C, et al. Role of NQO1, MPO and CYP2E1 genetic polymorphisms in the susceptibility to childhood acute lymphoblastic leukemia. Int J Cancer. 2002; 97 (2): 230-236.

36- Jeffrey N, Chang JS, Chokkalingam PA, et al. NQO1 Polymorphisms and De Novo Childhood Leukemia: A HuGE Review and Meta-Analysis. Am J Epidemiol. 2008; 168: 1221-1232.

37- Tuimala J, Szekely G, Gundy S, et al. Genetic polymorphisms of DNA repair and xenobiotic-metabolizing enzymes: Role in mutagen sensitivity. Carcinogenesis. 2003; 23: 1003-1008.

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The Potential Role of Annexin V on RBCs and P-Selectin on Activated Platelets in the Hypercoagulable State in Egyptian Patient with Thalassemia

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ABSTRACT

Background: The presence of a high incidence of thromboembolic events has led to the identification of a hypercoagulable state in thalassemia. Several etiologic factors may play a role in the pathogenesis of the hypercoagulable state in thalassemia.

Objectives: The aim of this study was to assess the existence of a chronic hypercoagulable state in Egyptian thalassemic patients and study the potential role of thalassemic RBCs and activated platelets in the hypercoagulable state.

Patients and Methods: Thirty-nine patients with thalassemia (27 with thalassemia major (TM) and 12 with thalassemia intermediate (TI) were used as the study group and 20 healthy volunteers were used as control group. Flowcytemerty was used to study the expression of anionic phospholipids (Annexin V) on the RBCs and CD62p (P-selectin) on the activated platelets.

Results: Annexin V positive RBC in TM and TI patients were significantly over expressed compared to control group ($p<0.001$) with no significant difference between patients with and without splenectomy. The expression of activation-dependent platelets neoantigen, P-selectin, was significantly higher ($p<0.001$) in thalassemic patients compared to the control. There was a strong association between the expression of Annexin V on the RBCs and P-selectin on the activated platelets.

Conclusion: There is a strong association between levels of RBCs expressing Annexin V and levels of platelets expressing P-selectin. The strong association between the expressions of these two cellular markers in the context of the known tendency of the hypercoagulable state observed in patients with thalassemia may help to predict and to avoid the development of this state in patients at risk.

Key Words: *Hypercoagulable state – Thalassemia – Annexin V – P-selectin.*

INTRODUCTION

The thalassemia are a heterogeneous group of genetic disorders of hemoglobin synthesis, all of which result from a reduced rate of production of one or more of the globin chains of hemoglobin. They are divided into α- ß- δ ß-, or γδß - thalassemia, according to which globin chain is produced in reduced amounts **[1]**.

The thalassemia represents the most common monogenetic disorder worldwide **[2]**, there is a particularly high incidence of thalassemia (2.5%-25%) in the Mediterranean basin, the Middle East, the tropical and subtropical regions of Africa, the Asian subcontinent, and Southeast Asia, where milder forms of the disease are most commonly seen. Cases of thalassemia also occur sporadically in virtually every ethnic group and geographic location **[3]**.

Several clinical and laboratory finding suggest the presence of chronic hypercoagulable state in patients of thalassaemia major (TM) and thalassaemia intermedia (TI) including transient ischemic attacks, pulmonary embolism and deep venous thrombosis. Diverse factors contributing to the hypercoagulable state in patients with thalassemia have been identified. In most cases, a combination of these abnormalities leads to clinical thrombosis **[4]**.

Among cellular factors, platelet activation contributes, to a significant extent. Much evidence suggests that patients with thalassemia have activated platelets. First, there is evidence of increased platelet aggregation **[5]**. Moreover,

flow cytometric studies have also confirmed the chronic platelet activation status manifested by an increased proportion of platelets expressing CD62P (P-selectin) and CD63 **[6-7]**, in addition to shortened platelet survival due to enhanced platelet consumption (especially in splenectomized patients) **[8-9]**.

Alteration in RBCs due to oxidation of globin subunits in thalassemia erythroid cells, leading to the formation of red-cell "senescence" antigens such as phosphatidylserine and phosphatidyletholamine causes thalassemic red cells to be rigid and deformed. These changes cause RBCs to aggregate, resulting in premature cell removal **[10]**. So, thalassemic RBCs expressing these negatively charged phospholipids are used as a source of phospholipids, enhancing and eventually increasing thrombin generation in a prothrombinase assay where normal RBC had no effect **[11]**.

This was evident by the finding that annexin V, a protein with high affinity and specificity for anionic phospholipids, could block the procoagulant effect of isolated thalassemic RBCs **[12]**. The procoagulant effect of thalassemic RBCs was suggested to contribute to the hypercoagulable state in thalassemia by amplifying thrombin generation and initiating platelet activation in vivo as one aspect of hypercoagulable state **[7]**. These abnormalities have been reduced to normal range after the patients have received a blood transfusion **[11]**.

The finding of elevated levels of endothelial adhesion proteins E-selectin (ELAM-1), intercellular adhesion molecule-1 (ICAM-1) and von Willebrand factor (VWF) and vascular cell adhesion molecule-1 (VCAM-1) in thalassemic patients suggested that endothelial injury or activation may be a feature of this genetic disease which also plays an important role in the recruitment of white blood cells and RBCs and promote thrombosis at vascular inflammation sites, vessel obstruction, tissue hypoxia and death **[13]**. More recently, it was shown that microparticles of red blood cell origins were elevated in patients with TI versus controls; these have a potential to aggravate thrombotic events **[14]**.

Clinical observations have suggested that splenectomy in thalassemia can contribute to an increased susceptibility to thrombosis **[15,16]**. The development of these complications has been ascribed to the presence of high platelet counts following splenectomy and/or to increased number of abnormal RBCs **[6,10,17]**. Also, in splenectomized TI patients, thrombin generation was significantly higher than in control subjects and patients who had not undergone splenectomy **[15,17]**.

The presence of a persistent hypercoagulable state combined with the infrequent occurrence of significant thrombotic events suggests that thrombosis is largely a subclinical process in thalassemia and has been associated with autopsy findings of platelet and fibrin thrombi in the microvasculature in the lungs **[18]** and the brain **[19]**. Management of this hypercoagulable state has two arms: Prevention and treatment. Prevention consists of proper anticoagulation to high-risk patients with thalassemia who are exposed to transient thrombotic risk factors (e.g. surgery, immobilization, pregnancy); treatment entails the adequate use of anticoagulation according to the recommendation for hypercoagulable state **[20-21]**.

The aim of this study is an attempt to confirm the presence of a chronic hypercoagulable state in Egyptian patients with ß-thalassaemia major and intermedia due to the expression of anionic red cell phospholipids leading to platelet activation; and to estimate the magnitude of the problem to be able to plan for prophylactic measures.

SUBJECTS AND METHODS

Ethical approval:

This study was approved by the ethical committee review board of "The General Organization for Teaching Hospitals and Institutes (GOTHI)" in accordance with the Helsinki guidelines for the protection of human subjects. Written informed consent from all participants (above 18 years old) and from the legal guardians (for those below 18 years old) involved in the study were taken.

Subjects:

The present study included 39 patients with Thalassemia (27 with TM, and 12 with TI), presented to the outpatient Clinic of Ahmed Maher Hospital, who were compared to 20 age and gender matched healthy volunteers as a control group.

Patients and control were subjected to:

• *Complete history:* Including age, sex, age of disease onset, height, weight, splenomegaly, hepatomegaly, onset and number of blood units received, splenectomy, history of complications, and treatment with iron chelating agent.

• In all patients the results of complete blood pictures, hemoglobin electrophoresis, hemoglobin A2, hemoglobin F and the clinical course were used to classify our patients as TM or TI. The blood samples required for the study were taken immediately before a blood transfusion (at least 4 weeks after the last transfusion) and samples were obtained by standard venous puncture using a light tourniquet, where the first 2mL of blood were discarded to avoid platelet activation. The blood was drawn into EDTA tubes and the test has been strictly done within 4-6 hours.

• *Annexin V binding to RBCs:* The sample was mixed well and 10µl of EDTA blood were added to 40µl of HEPES buffer, mixed well then a saturating concentrations of specific monoclonal antibodies namely Annexin V (FITC, BD Bioscience Pharmingen, San Diego) and anti-human CD235a (Glycophorin A/RPE, clone JC159, Dako) were added then incubated at room temperature in dark for 20min and the read on a Coulter EPICS XL-MCL flow cytometer system (Coulter Corporation, Hialeah, USA) after re-suspension by isoton without lysis of RBCs. Light scatter fluorescent data were obtained with a gain setting in the logarithmic mode. The results were expressed as percentage (%) of positive cells for the co-expression of Annexin V and Glycophorin on the surface of RBCs and mean fluorescent intensity (MFI) **[22]**.

• *Circulating activated platelets:* Blood was immediately centrifuged at 750g for 5 minutes at 22ºC. The supernatant was separated as Platelet Rich Plasma (PRP) and diluted 1:10 in HEPES buffer saline. Fifty-µL aliquot from each diluted PRP sample was added to a tube containing saturating concentrations of specific monoclonal antibodies, the platelets activation marker P-Selectin (CD62p) FITC (clone CLB Thromb/6, Dako) and the normal platelet marker CD41 RPE (clone 5B12, Dako). Samples were incubated in the dark at room temperature for 20 minutes. After immunolabelling, the samples were diluted 1:10 HEPES buffer and analysed on a Coulter EPICS XL-MCL flow cytometer system (Coulter Corporation, Hialeah, USA). Platelets are distinguished by the characteristic light scatter; results were expressed as percentage (%) of positive cells for the co-expression of CD41 and CD62p on the activated platelets and MFI **[23]**.

Statistical analysis:

A statistical analysis was performed using SPSS version 14. Nonparametric Kruskal-Wallis test was used to compare between studied markers in each group. ANOVA test was used to compare between variance of each marker level among two different groups of TM and TI and the control **[24]**.

RESULTS

This study included 39 patients with thalassemia (27 TM and 12 TI) in addition to 20 healthy age and sex matched control. The age of the patients with TM ranged from 4-19 with a median of 14; for TI, it ranged from 5-18 with a median of 15 and for the control group, it ranged from 6-16 with a median of 13 years. Male to female ratio was 1:1 in TM and 1.2:1 in TI.

Patients with TM showed an early onset of the disease at age 0.9±0.2 months (within the first year of life); while those with TI showed a late onset of disease at age 5.3±1.7 years ($p<0.001$). Also, patients with TM showed growth retardation; their height and weight were 128.6±21.1cm and 27.8±8.5kg respectively, compared to both patient with TI (148.3±23.3cm and 42.9±14.9kg) and the control group (149.7±15.9cm and 44.5±14.6kg) ($p<0.001$).

Splenomegaly was reported in all cases (100%) of TM and in 8/12 (66.7%) of patients with TI, while splenectomy was done for 5/27 (18.5%) of TM and none of the TI patients.

All patients with both TM and TI needed to receive blood transfusion and most of patients with TM (97%) received at least one unit of blood or packed RBCs every month, while patients with TI needed to receive one unit of blood or packed RBCs every four to six months. A significant early onset of transfusion was encountered in patients with TM, within the first year of life (0.98±0.27 year) as compared

to those with TI who needed to receive blood later on (5.79±2.13 year), (p<0.001). Also, a significant difference regarding the need to receive iron chelating agent as 25/30 (83%) of TM and none of TI patients received Desferol.

The results showed a significant reduction in the Hb level in patients with TM and TI compared to the control group, as the Hb was 6.1±0.5gm/dl for TM, 7.4±0.3 for TI and 12.3±0.6 for healthy controls (p<0.001). Ferritin level was markedly higher in patients with TM (2626±1094) compared to (636±172) for TI and (220±59) for control group, (p<0.001).

Expression of platelets activation markers:

The percentage expression of activation-dependent platelets neoantigen CD62p (P-Selectin) was 50.1±20.1% in thalassemic patients compared to 10.9±7.9% in the control (p<0.001) (Fig. 1). There was higher co- expression of CD 41 and CD62p on the activated platelets in patients with spleenectomy (58.1± 15.8%) compared to (44.6±21.4%) on patient without Splenectomy but this difference was not statically significant (p>0.05).

The Mean Florescent Intensity (MFI) for the co-expression of CD 41 and CD62p (P-Selectin) on the platelets was 19.0±12.7 in all thalassemic patients compared to 6.1±1.1 in the control (p<0.001), but the MFI was higher in our patients with TI (19.3±9.7) compared to those with TM (18.9±8.5); the difference was in (p>0.05).

Annexin V binding to RBCs:

The percentage expression of Annexin V on RBCs of thalassemic patients was 68.4±8.0% compared to 9.9±3.6% on normal RBCs (p< 0.001) (Fig. 2). The values were comparable on RBCs of patient with splenectomy and without splenectomy (69±8.3% vs. 66.9±8.6%, p> 0.05). Also, the MFI was 2.1±0.5 in all thalassemic patients compared to 1.6±0.1 in the control group (p=0.03), with comparable MFI of Annexin V on the RBCs of patients with TM and TI (1.9±0.4 and 2.1±0.8 respectively, p>0.05). Patients who receive regular blood transfusion showed a lower percentage of Annexin V expression on RBCs (55.4±7.3%) compared to (68.7±9.6%) in poorly transfused patients (p<0.05).

In this study a significant positive correlation between the expression of the platelet activation marker, CD62p, and the Annexin V binding to thalassemic RBCs were observed (r=0.42, p< 0.05).

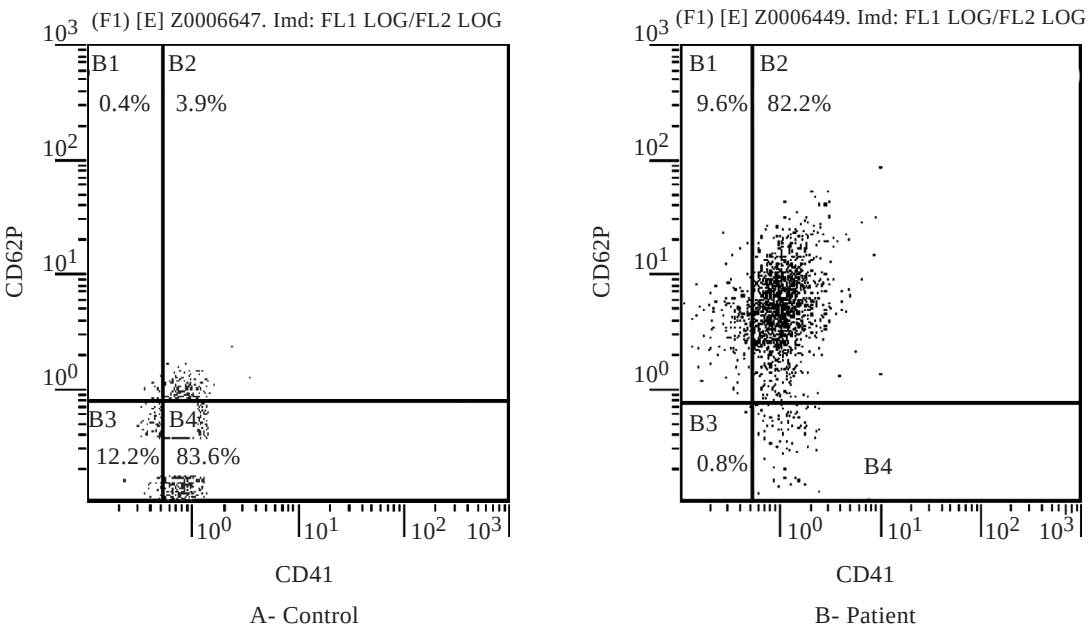

Fig. (1): The expression of CD41 and CD62p on the activated platelets of the control (A) and thalassemic patient (B).

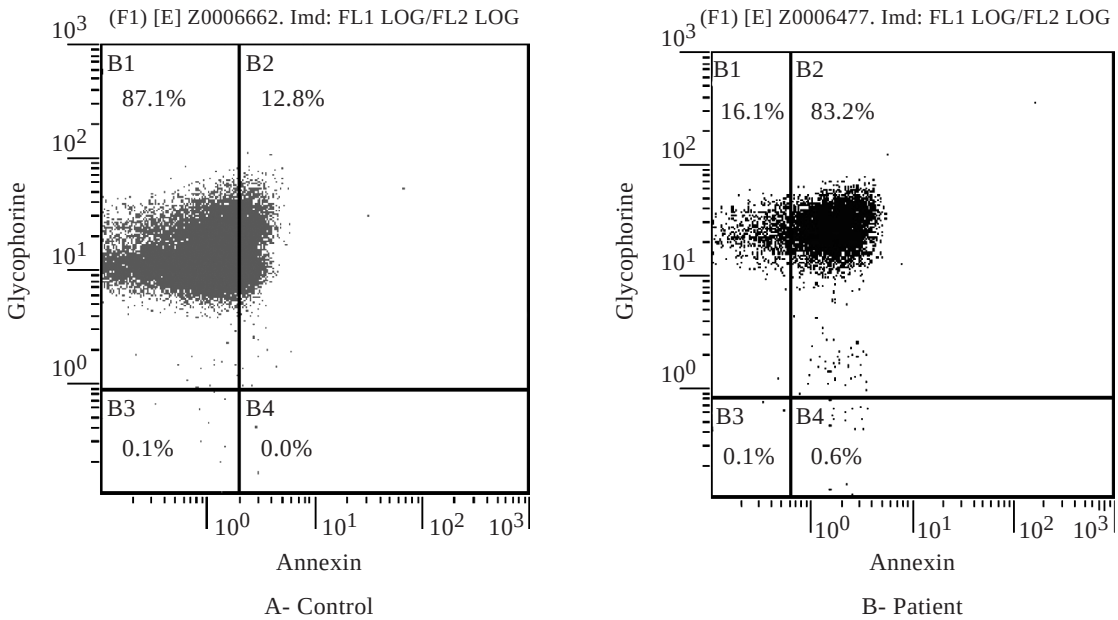

Fig. (2): The expression of Annexin V and Glycophorin on the RBCs of the control (A) and thalassemic patient (B).

DISCUSSION

There is increasing evidence that chronic hemolytic anemia such as sickle cell disease (SCD), thalassemia, paroxysmal nocturnal hemoglobinuria, autoimmune hemolytic anemia and unstable hemoglobinopathies, are characterized by a hypercoagulable state **[25-28]**. In addition to increased thrombin and fibrin generation, increased tissue factor activity, and increased platelet activation, patients with hemolytic anemias manifest thrombotic complications, including venous thromboembolism **[5]**, in situ pulmonary thrombosis **[20]** and stroke **[29]**. Furthermore, the risk of thromboembolic complications appears to be higher following splenectomy **[18,30]**.

The mechanism of coagulation activation in hemolytic anemia is likely multifactorial. Both SCD and thalassemia are characterized by red blood cell membrane abnormalities, with abnormal exposure of phosphatidylserine **[25]**. Abnormal phosphatidylserine exposure functions as both a recognition signal for cell removal during apoptosis of nucleated cells **[11]** and as a docking site for enzymatic complexes involved in coagulation and anticoagulation pathways **[31]**. External exposure of phosphatidylserine alters the adhesive properties of RBC **[32-33]** and appears to be involved in the hemostatic changes observed in hemolytic anemia **[34-36]**.

In this study there was a significantly increased fraction of Annexin V labelled RBC in TM and TI patients compared to RBC from healthy individuals. These findings are consistent with an abnormal membrane phospholipids asymmetry and exposure of phosphatidylserine (PS) in thalassemic patients, which may increase thrombin generation and may enhance the hypercoagulable state in thalassemic patients.

These findings are in line with those reported by many authors **[5,8,32,33]** who stated that the hypercoagulable state in TM and TI may result from procoagulant effect of abnormal RBCs of thalassemic patients, by amplifying thrombin generation and initiating platelet activation. Furthermore it was found that Annexin V, a protein with high affinity and specificity for anionic phospholipids, could block the procoagulant effect of isolated thalassemic RBCs and that these abnormalities have been reduced to normal range after the patients have received a blood transfusion **[13]**.

Similar results were reported by Capellini who found that thalassemic RBCs expressing these negatively charged phospholipids may be used as a source of phospholipids, enhancing and eventually increase thrombin generation in a prothrombinase assay where normal RBC had no effect **[12]**. The persistent hypercoagulable state in thalassemic patients was explained by

the abnormal exposure of some phospholipids, especially Annexin V, on the surface of these RBCs **[11,15,25,34,35]**.

Despite of the fact that none of our TM or TI patients in this study had an overt thrombotic event, a chronic hypercoagulable state was evident by the increased fraction of circulating platelets expressing activation dependent neoantigen, P-Selectin (CD62p). In consistence with the current results several authors demonstrated that overt thromboembolic events (TEE) occur only rarely in thalassaemia patients; however, laboratory tests have provided evidence for a chronic hypercoagulable state which already exists in early childhood **[7-9,12,37]**.

On the other side higher, incidence of thrombotic events were observed in 4% of 683 patients with TM and in 9.6% of 52 patients with TI presented with TEE **[37]**. The same group showed six years later lower incidence as only 1.1% of 720 patients from seven Italian centers with TM, had thrombosis **[38]**. In a large clinical study among 8860 thalassemia patients (6670 TM and 2190 TI), the cumulative prevalence of thromboemolic events was estimated at 1.65%, with thrombosis occurring 4.38 times more frequently in TI than TM **[39]**.

In the current work the lower chance for developing overt thrombosis may be due to the young age as all patients were below 20 years old and spleenectomy was done for only 18.5% of thalassemic patients. The main risk factors for developing thrombosis were described as: Age beyond 20 years, spleenectomy, family history of TEE and previous TEE **[39]**.

In this work we found a strong association between the expression of the platelet activation marker, CD62p, and the Annexin V binding to thalassemic RBCs and the two cellular anomalies are highly correlated and the two cellular anomalies are linked together. In consistence with our results Rulf et at., **[8]** reported a strong correlation between the cellular anomalies and assumed that the abnormal RBCs might enhance thrombin generation in vivo and thus trigger platelet activation in thalassemia.

In parallel with the results of this study, it was hypothesized that there is a causal relationship and a significant association between RBCs membrane anomaly and the degree of in vivo platelet activation **[40]**. More recently, Monnucci et al., **[41]** stated that the RBCs from thalassemic patients are an important player for the activation of platelets in patients with TM.

In this study we found that patients who received regular blood transfusion had lower percentage of Annexin V expression as markers for hypercoagulable state than poorly transfused patients.

The same results were found by Taher et al., **[17]**, who stated that a positive history of transfusion and hemoglobin level ≥9g/dl were found to be protective against thrombosis in patients with TI, while splenectomy, age above 35 years and serum ferrtin ≥1,000ug/l were associated with a higher risk of thrombosis.

In conclusion, we found a significantly higher number of circulating activated platelets expressing CD62p (P-Selectin) and increased fraction of RBCs expressing Annexin V in both TM and TI patients with a strong association between the expression of these two cellular markers. Although there are diverse factors contributing to the hypercoagulable state observed in patients with thalassemia, the strong association between the expression of these two cellular markers and the tendency of the hypercoagulable state observed in patients with thalassemia may help to predict and probably avoid the development of thromboembolic events in those patients.

REFERENCES

1- Steinberg MH, forget BG, Higgs DR, et al. Disorders of Hemoglobin. Cambridge University Press, Cambridge. 2001.

2- Weatherall DJ, Clegg JB. Inherited haemoglobin disorders, an increasing global health problem. Bulletin of the World Health Organization. 2001; 79: 704-1.

3- Weatherall DJ. Genetic variation and susceptibility to infection: the red cell and malaria. Br J Haematol. 2008; 141 (3): 276-286.

4- Weatherall DJ. The inherited diseases of hemoglobin are an emerging global health burden. Blood. 2010; 115 (22): 4331-4336.

5- Taher AT, Otrock ZK, Uthman I, Cappellini MD. Thalassemia and hypercoagulability. Blood Rev. 2008; 22: 283-292.

6- Atichartakarn V, Angchaisuksiri P, Aryurachai K, Chuncharunee S, Thakkinstian A. In vivo platelet activation and hyperaggregation in hemoglobin E/beta-thalassemia: A consequence of splenectomy. Int J Hematol. 2003; 77: 299-303.

7- Del Principe D, Menichelli A, Di Giulio S, De Matteis W, Cianciulli P, Papa G. PADGEM/GMP-140 expression on platelet membranes from homozygous beta thalassaemic patients. Br J Haematol. 1993; 84: 111-117.

8- Rulf A, Pick M, Deutsch V, Patscheke H, Goldfarb A, Rachmilewitz EA, Guillin MC, Eldor A. In-vivo platelet activation correlates with red cell anionic phospholipid exposure in patients with beta-thalassaemia major. Br J Haematol. 1997; 98: 51-56.

9- Eldor A, Lellouche F, Goldfarb A, Rachmilewitz EA, Maclouf J. In vivo platelet activation in beta-thalassemia major reflected by icreased platelet-thromboxane urinary metabolites. Blood. 1991; 77: 1749-1753.

10- Cappellini MD, Grespi E, Cassinerio E, Bignamini D, Fiorelli G. Coagulation and splenectomy: An overview. Ann N Y Acad Sci. 2005; 1054: 317-324.

11- Kuypers FA, De Jong K. The role of phosphatidylserine in recognition and removal of erythrocytes. Cell Mol Biol (Noisy-le-grand). 2004; 50: 147-158.

12- Cappellini MD, Motta I, Musallam KM, Taher AT. Redefining thalassemia as a hypercoagulable state. Ann NY Acad Sci. 2010; 1202: 231-236.

13- Helley D, Rachmilewitz EA, Mosseri M, et al. Comparison of the procoagulant activity of red blood cells from patients with homozygous sickle cell disease and ß-thalassaemia. Thrombosis and Haemostasis. 1996; 76: 322-327.

14- Butthep P, Rummavas S, Wisedpanichkij R, Jindadamrongwech S, Fucharoen S, Bunyaratvej A. Increased circulating activated endothelial cells, vascular endothelial growth factor, and tumor necrosis factor in thalassemia. Am J Hematol. 2002; 70: 100-106.

15- Habib A, Kunzelmann C, Shamseddeen W, Zobairi F, Freyssinet JM, Taher A. Elevated levels of circulating procoagulant microparticles in patients with b-thalassemia intermedia. Haematologica. 2008; 93: 941-942.

16- Iolascon A, Giordano P, Storelli S, Li HH, Coppola B, Piga A, Fantola E, Forni G, Cianciulli P, Perrotta S, Magnano C, Maggio A, Mangiagli A, Devoto M. Thrombophilia in thalassemia major patients: Analysis of genetic predisposing factors. Haematologica. 2001; 86: 1112-1113.

17- Taher AT, Musallam KM, Karimi M, et al. Splenectomy and thrombosis: The case of thalassemia intermedia. J Thromb Haemost. 2010; 8: 2152-2158.

18- Cappellini MD, Robbiolo L, Bottasso BM, Coppola R, Fiorelli G, Mannucci AP. Venous thromboembolism and hypercoagulability in splenectomized patients with thalassaemia intermedia. Br J Haematol. 2000; 111: 467-473.

19- Taher AT, Musallam KM, Nasreddine W, Hourani R, Inati A, Beydoun A. Asymptomatic brain magnetic resonance imaging abnormalities in splenectomized adults with thalassemia intermedia. J Thromb Haemost Jan. 2010; 8 (1): 54-9.

20- Gillis S, Cappellini MD, Goldfarb A, Ciceri L, Fiorelli G, Rachmilewitz EA. Pulmonary thromboembolism in thalassemia intermedia patients. Haematologica. 1999; 84: 959-960.

21- Taher AT, Mussallam KM, Karimi M, et al. Overview on practices in thalassemia intermedia management aiming for lowering complication rates across a region of endemicity: The optimal care study. Blood. 2010; 115 (10): 1886-1892.

22- Freikman J, Amer I, Ringel E, Fibach A. Flow cytometry approach for quantitative analysis of cellular phosphatidylserine distribution and shedding, Anal Biochem. 2009; 393: 111-116.

23- Villagra J, Shiva S, Hunter LA, Roberto F, Machado MT, Kato GJ. Platelet activation in patients with sickle disease, hemolysis-associated pulmonary hypertension, and nitric oxide scavenging by cell-free haemoglobin. Blood. 2007; 110 (6): 2166-2172.

24- Snedecor G W, Cochran W. Statistical Methods, Eighth Edition, Iowa State University Press. 1989.

25- Ataga KI, Cappellini MD, Rachmilewitz EA. B-thalassemia and sickle cell anaemia as paradigms of hypercoagulability. Br J Haematol. 2007; 139: 3-13.

26- Stein PD, Beemath A, Meyers FA, Skaf E, Olson RE. Deep venous thrombosis and pulmonary embolism in hospitalized patients with sickle cell disease. Am J Med. 2006; 119: 7-11.

27- Ziakas PD, Poulou LS, Rokas GI, Bartzoudis D, Voulgarelis MJ. Thrombosis in paroxysmal nocturnal hemoglobinuria: sites, risks, outcome. Thromb Haemost. 2007; 5: 642-5.

28. Hendrick AM. Auto-immune haemolytic anaemia-a highrisk disorder for thromboembolism. Hematology. 2003; 8: 53-6.

29- Karimi M, Bagheri H, Rastgu F, Rachmilewitz EA. Magnetic resonance imaging to determine the incidence of brain ischaemia in patients with beta-thalassaemia intermedia. Thromb Haemos. 2010; 103 (5): 989-993.

30- Franchini M, Mannucci PM. Hypercoagulability in congenital haemolytic anaemias. Blood Transfusion. 2012; 10: 423-427.

31- Zwaal RF, Schroit AJ. Pathophysiologic implications of membrane phospholipids asymmetry in blood cells. Blood.1997; 89: 1121-1132.

32- Borenstain-Ben Yashar V, Barenholz Y, Hy-Am E, Rachmilewitz EA, Eldor A. Phosphatidylserine in the outer leaflet of red blood cells from b-thalassemia patients may explain the chronic hypercoagulable state and thrombotic episodes. Am J Hematol. 1993; 44: 63-65.

33- Chen S, Eldor A, Barshtein G, Zhang S, Goldfarb A, Rachmilewitz E, Yedgar S. Enhanced aggregability of red blood cells of beta-thalassemia major patients. Am J Physiol. 1996; 270: 1951-1956.

34- Setty BN, Kulkani S, Stuart MJ. Role of erythrocyte phosphatidylserine in sickle red cell-endothelial adhesion. Blood. 2002; 99: 1564-71.

35- Leventis PA, Grinstein S. The distribution and function of phosphatidylserine in cellular membranes, Annu Rev Biophys. 2010; 39: 407-427.

36- Eldor A, Rachmilewitz EA. The hypercoagulable state in thalassemia. Blood. 2002; 99: 36-43.

37- Borgna Pignatti C, Carnelli V, Caruso V, Dore F, De Mattia D, Di Palma A, Di Gregorio F, Romeo MA, Longhi R, Mangiagli A, Melevendi C, Pizzarelli G, Musumeci S. Thromboembolic events in beta thalassemia major: An Italian multicenter study. Acta Haematol. 1998; 76-79.

38- 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. Oct. 2004; 89 (10): 1187-93.

39- Taher A, Isma'eel H, Mehio G, Bignamini D, Kattamis A, Rachmilewitz EA, Cappellini MD. Prevalence of thromboembolic events among 8,860 patients with thalassaemia major and intermedia in the Mediterranean area and Iran. Thromb Haemost. 2006; 96: 488-91.

40- Atichartakarn V, Angchaisuksiri P, Aryurachai K, Onpun S, Chuncharunee S, Thakkinstian A, Atamasirikul K. Relationship between hypercoagulable state and erythrocyte phosphatidylserine exposure in splenectomized haemoglobin E/beta-thalassaemic patients. Br J Haematol. 2002; 118: 893-898.

41- Mannucci PM. Red cells playing as activated platelets in thalassemia intermedia. J Thromb Haemost. 2010; 8: 2149-2151.

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Case Report:
Blastic NK Cell Leukemia/Lymphoma

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ABSTRACT

Blastic natural killer (NK) cell lymphoma/leukemia is a rare NK cell malignancy of unknown etiology. It occurs in multiple sites and with a propensity for skin involvement. We report a 56-year-old male presented with nasopharyngeal mass, hepatosplenomegaly, lymphadenopathy and multiple skin lesions. Laboratory data revealed pancytopenia with, peripheral blood and bone marrow involvement with blast cells. These cells showed negative cytochemical staining for myeloperoxidase, α naphthyl butyrate esterase and positive cytochemical staining for acid phosphatase. By immunophenotyping, these cells were positive for CD4, CD56, CD7, CD38, cCD3 and HLA-DR. The patient was treated with adult ALL protocol; after 2 cycles of induction chemotherapy there was complete resolution of all masses but with persistent blast cells in bone marrow.

Key Words: *Blastic NK cell lymphoma/leukemia – CD4+/CD56+cells – Bone marrow.*

INTRODUCTION

Natural killer (NK) cells are lymphoid cells that mediate lysis of tumor cells and bacteria- or virus-infected cells and the production of immunomodulatory cytokines **[1,2]**. Natural killer (NK) cells are believed to arise in the bone marrow, thymus, and fetal liver from "common lymphocyte precursors," which are derived from pluripotent hematopoietic stem cells **[3,4]**. Mature NK cells constitute 10% to 20% of lymphocytes in normal blood **[5]**.

Morphologically, mature NK cells are large granular lymphoid cells, which are characterized by the presence of pale cytoplasm containing azurophilic granules. Unlike T cell large granular lymphocytes, they are negative for surface CD3 but characteristically express cytoplasmic CD3 epsilon (ε), CD56, and cytotoxic molecules. Furthermore, clonal rearrangement of the T-cell receptor (TCR) genes is also absent in NK cells **[6,7]**.

Blastic natural killer (NK) cell neoplasms are rare, highly aggressive malignancies with a poor prognosis. They have a predilection for the skin, and disseminate rapidly into the blood, bone marrow, lymph nodes, and extranodal organs **[1]**. This type of aggressive T-cell neoplasm is more common in Asia and Latin America but very rare in Middle East **[8,9]**. Epstein-Barr virus (EBV) is found in most cases of NK-cell leukemia/lymphoma, suggesting an oncogenic role **[10]**.

CASE PRESENTATION

A 56-year-old Egyptian male was admitted to Oncology Department of Cairo University Hospital on 6/9/2012 with bilateral diminution of hearing for 3 months with progressive dysphagia and dyspnea with no particular family or past history of medical illness. On admission, clinical examination revealed the presence of large nasopharyngeal mass, enlarged submandibular and right posterior cervical lymph nodes, hepatosplenomegaly and multiple cutaneous lesions (Fig. 1) of long duration.

A complete blood count showed mild leucopenia with white blood cell count of 3,600/μL and 30% peripheral blasts, mild anemia with hemoglobin concentration of 11g/dL, and marked thrombocytopenia with platelet count of 48,000/μL. Biochemical results revealed (aspartate aminotransferase, 55U/L (RR: 0-37);

alanine aminotransferase, 70U/L (RR: 10-40), lactate dehydrogenase; LDH, 485U/L (RR: 230-460) and uric acid, 6.1mg/dl (RR: 3-5.7). Hepatitis C virus (HCV) was screened by 3rd generation ELIZA and the result was negative.

Morphologic study of bone marrow aspirates on the patient's first admission demonstrated hypercellular marrow with dyserythropoiesis and high count of abnormal cells (90% blast cells). The cells were with almost eccentric nuclei, fine nucleolated nuclear chromatin and bluish occasionally vacuolated cytoplasm (Fig. 2); they were encroaching on all other hemopoietic elements which were depressed. Cytochemical tests revealed that the abnormal cells were negative for myeloperoxidase (Fig. 3-A) and α naphthyl butyrate esterase but positive for acid phosphatase reactions (Fig. 3-B). Bone marrow biopsy revealed hypercellular bone marrow with nodular collection of atypical lymphocytes.

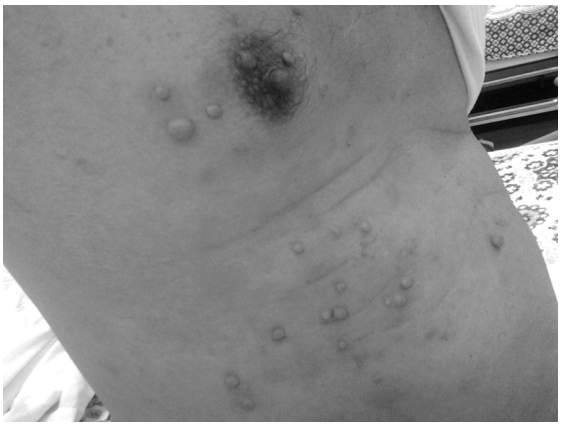

Fig. (1): Multiple well defined cutaneous lesions.

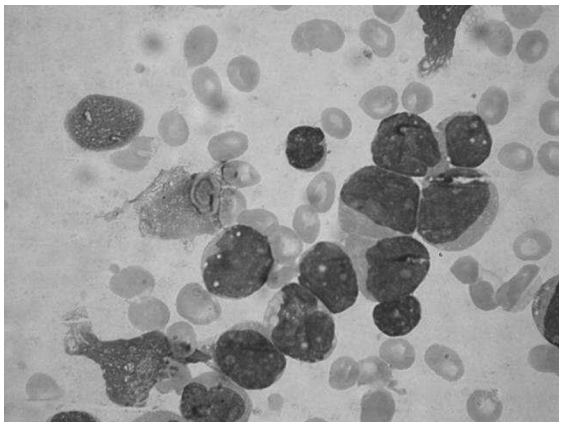

Fig. (2): Bone marrow aspirate showing mononuclear cells with eccentric nucleus and occasionally vacuolated cytoplasm (100 x).

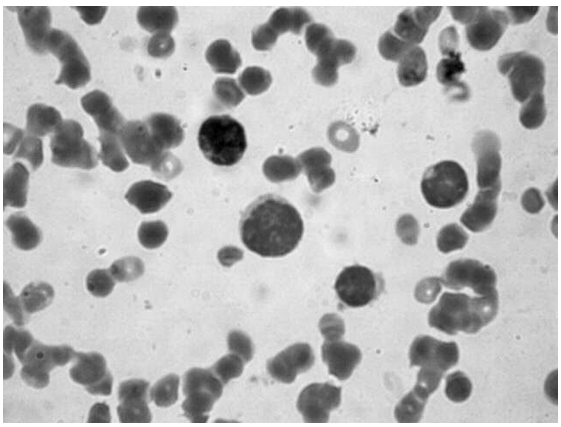

Fig. (3A): Blast cells were negative for myeloperoxidase (100 x).

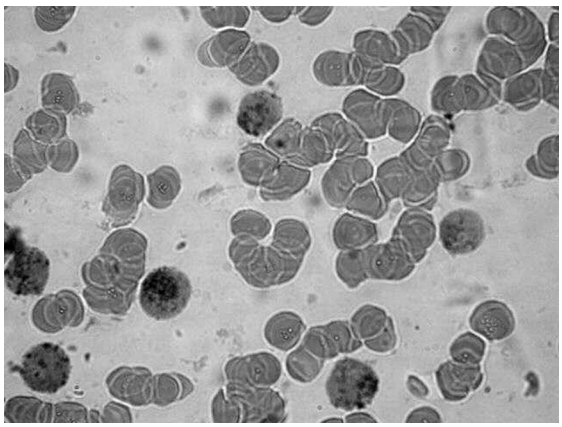

Fig. (3B): Blast cells were positive for acid phosphatase (100 x).

Flow cytometric analysis on bone marrow aspirate clearly highlighted an immunophenotypic feature: Positive for CD4 (51.7%), CD56 (71.8%), CD38 (58%), CD7 (48%), cCD3 (56.2%) and HLA-DR (69%), but negative for CD13, CD33, CD2, CD3, CD5, CD10, CD19, CD20, cCD22 and CD34.

Rhinoscpoic biopsy of the nasopharyngeal mass showed pieces of tissue lined by focally hyperplastic, focally keratinized stratified squamous epithelium and pseudo stratified columnar epithelium of respiratory type. The sub epithelial layer was expanded and densely infiltrated by atypical lymphoid tissue with diffuse and vague

nodularities. They were formed of atypical lymphocytes. Immunohistochemistry revealed that these atypical cells were positive for CD56, CD4 and CD3. Excision of one of skin nodule revealed diffuse infiltration by mononuclear cells in the dermis and subcutis. Immunohistochemistry revealed that the mononuclear cells were CD56 and CD45 positive.

The diagnosis of blastic NK-cell lymphoma/ leukemia stage IV was made with involvement of nasopharynx, lymph nodes, skin, liver, spleen and the bone marrow.

The patient started treatment with adult ALL protocol according to The International ALL trial (MRC UKALL XII/ECOG E2993). He received two induction cycles. After 2 months, reevaluation before starting maintenance phase revealed complete resolution of nasopharyngeal mass, cervical nodes, HSM and almost all cutaneous nodules. BM examination revealed residual blast cells (9%) compared with 90% at initial presentation. Then the patient achieved hematological remission at 5 months from presentation and remained in remission till relapse after 1 year and appearance of Liver metastasis.

The publication was approved by the IRB of the Clinical Oncology Department, Cairo University and an informed consent was taken from the patient.

DISCUSSION

Blastic natural killer (NK) cell lymphoma, a relatively rare NK-cell malignancy was first described by Suchi and Mori in 1994 **[11]**. It can occur at any age, but most commonly in the middle and older-aged males. This disease tends to involve multiple sites, with a tendency for the skin showing erythematous and purpuric indurated plaques or nodules. Moreover, lymph nodes, soft tissue, peripheral blood or bone marrow can be simultaneously involved. A majority of patients show widespread disease at the initial presentation **[12]**.

A special immunophentypic feature of blastic NK cell lymphoma is the positivity of CD56 antigen; it is expressed very early on committed NK precursor and on more than 95% of mature NK cells **[4,5]**. CD7 also is expressed early in NK cell development **[4]**. Markers found on developing and mature NK cells include variable expression of CD2, CD8, CD11b, CD25, CD16, CD57, TIA-1, perforin, and granzyme. Differentiation of “true NK cells” from CD56+ cytotoxic “NK-like T cells” can be difficult because they may express similar surface antigens **[5]**. NK cells are currently best distinguished from T cells by lack of TCR gene rearrangement and lack of CD3 expression on the cell surface **[13]**.

Blastic NK-cell lymphoma is a disease with poor prognosis, particularly when bone marrow involvement is seen at onset **[14]**. With the exception of few case reports **[15-17]**, chemotherapy alone seems to be inadequate to attain long-term complete remission in patients with disseminated blastic NK-cell lymphoma. Our patient reached partial response with treatment that is usually used for induction of remission in ALL. As reported previously, regimens for ALL could be effective for this disease. Even if complete remission is achieved by chemotherapy alone, the period of CR is usually short and most patients relapse within several months. Local irradiation to bulky diseases requires doses in the range of 50Gy **[14]**.

The median survival time when treated with chemotherapy alone is 12-20 months **[18]**; therefore, bone marrow transplantation is the only option for long-term remission **[19]**. A retrospective analysis by Suzuki and colleagues comparing stem cell transplantation to chemotherapy alone in patients with a variety of NK neoplasms revealed a significant increase in long-term survival in the transplant arm versus the chemotherapy alone arm (40% at median follow-up of 51 months vs. 25% at median follow-up 32 months) **[18]**. Patients who underwent an allogeneic transplant had higher transplant-related mortality (TRM), but a lower relapse rate compared to autologous transplant patients, suggesting a graft-versus-leukemia effect. For patients without a suitable matched donor, umbilical cord blood (UCB) has emerged as a viable alternative source of hematopoietic progenitor cells. In comparison to matched unrelated donor (MUD) bone marrow allografts, UCB demonstrates a greater degree of tolerance to human leukocyte antigen (HLA) mismatches with similar rates of severe acute GVHD. Additionally, cord blood has a high concentration of donor-derived NK cells that exhibit functional cytotoxic properties, which may confer protection through a graft-versus-leukemia effect **[20]**.

In conclusion, we present a typical case of blastic NK-cell lymphoma/leukemia. This disease is highly malignant and until now, has no definite curative treatment. Appropriate therapeutic approaches to this disease should be explored.

Conflicts of interest:

None

REFERENCES

1- Liang X, Graham DK. Natural killer cell neoplasms. Cancer. 2005; 104: 1022-1031.

2- Spits H, Lanier LL, Phillips JH. "Development of human T and natural killer cells", Blood. 1995; Vol. 85, No. 10, pp. 2654-2670.

3- Orkin SH. Diversification of haematopoietic stem cells to specific lineages. Nat Rev Genet. 2000; 1: 57-64.

4- Spits H, Blom B, Jaleco AC, et al. Early stages of the development of human T, natural killer and thymic dendritic cells. Immunol Rev. 1998; 165: 75-86.

5- Chan JKC. Natural killer cell neoplasms. Anat Pathol. 1998; 3: 77-145.

6- Chan JKC, Quintanilla-Martinez L, Ferry JA, Peh S C. "Extranodal NK/T-cell lymphoma, nasal type," in WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, pp. 285-288, International Agency for Research on Cancer, Lyon, France. 2008.

7- Chan JKC, Jaffe ES, Ralfkiaer E, Ko YH. "Aggressive NK-cell leukaemia", in WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, pp. 276-277, International Agency for Research on Cancer, Lyon, France. 2008.

8- Au WY, Ma SY, Chim CS, et al. "Clinicopathologic features and treatment outcome of mature T-cell and natural killer-cell lymphomas diagnosed according to the World Health Organization classification scheme: A single centerexperience of 10 years", Annals of Oncology. 2005; Vol. 16, No. 2, pp. 206-214.

9- Armitage JO. "A clinical evaluation of the International Lymphoma Study Group classification of non-Hodgkin'slymphoma," Blood. 1997; Vol. 89, No. 11, pp. 3909-3918.

10- Suzuki R. Leukemia and Lymphoma of Natural Killer Cells. J. Clin. Exp. Hematopathol. 2005; Vol. 45, No. 2, Dec.

11- Jaffe E, Chan JKC, Suchi J, et al. Report of the workshop on nasal and related extranodal angiocentric T/natural killer cell lymphomas. Am J Surg Pathol. 1996; 20: 103-111.

12- Lin CM, Wang SH, Kuo TT, Chi CC, Ho HC, Hong HS. Blastic NK-Cell Leukemia/Lymphoma-A Case Report-Dermatol Sinica. 2003; 21: 408-412.

13- Michael G Bayerl, Christiane K Rakozy, Anwar N Mohamed. Blastic Natural Killer Cell Lymphoma/ Leukemia. Am J Clin Pathol. 2002; 117: 41-50.

14- Yamaguchi M, Maekawa M, Nakamura Y, Ueda M. Long-Term Remission of Blastic Natural Killer-Cell Lymphoma After Autologous Peripheral Blood Stem-Cell Transplantation. Am J Hematol. 2005; 80: 124-127.

15- Shapiro M, Wasik MA, Junkins-Hopkins JM, et al. Complete remission in advanced blastic NK-cell lymphoma/leukemia inelderly patients using hyper-CVAD regimen. Am J Hematol. 2003; 74: 46-51.

16- Falcão RP, Garcia AB, Marques MG, et al. Blastic CD4 NK cellleukemia/lymphoma: A distinct clinical entity. Leuk Res. 2002; 26: 803-807.

17- Yamada O, Ichikawa M, Okamoto T, et al. Killer T-cell inductionin patients with blastic natural killer cell lymphoma/leukaemia: Implications for successful treatment and possible therapeutic strategies. Br J Haematol. 2001; 113: 153-160.

18- Suzuki R, Suzumiya J, Nakamura S, et al. Hematopoietic stem cell transplantation for natural killer-cell lineage neoplasms. Bone Marrow Transplant. 2006; 37: 425-431.

19- Yamaguchi M, Maekawa M, Nakamura Y, Ueda M. Long term remission of blastic natural killer-cell lymphoma after autologous peripheral blood stem-celltransplantation. Am J Hematol. 2005; 80: 124-127.

20- Jennifer M Duff, Michelle L Rossi, Jason Fromm, Jack W Hsu. A 21-Year-Old Woman With Blastic NK Cell Leukemia/Lymphoma Who Achieved Durable Remission With Hyper CVAD and Unrelated Umbilical Cord Blood Transplant. Clinical Advances in Hematology and Oncology. 2011; Volume 9, Issue 7 July.