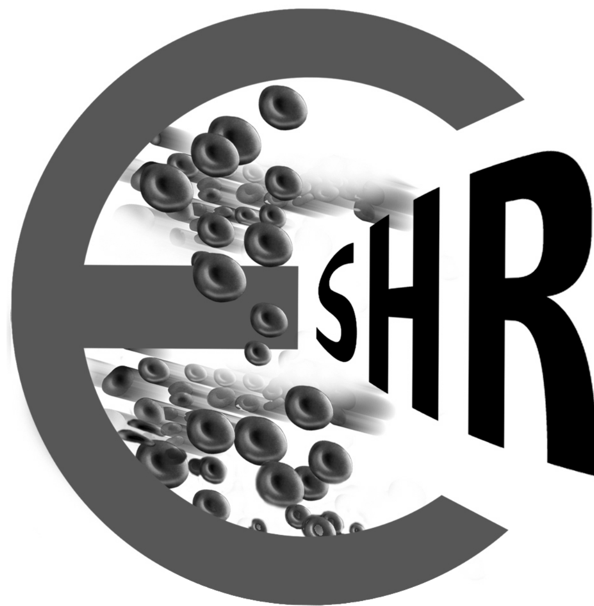
The Journal of the Egyptian Society of Haematology & Research



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The Effect of Hydroxyurea on Adhesion Receptor Integrin-Associated Protein (CD47) Expression in Patients with Sickle Cell Disease

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ABSTRACT

Background: Patients with sickle cell disorders (SCD) are prone to episodes of micro vascular obstruction. Integrin-associated protein (CD47) is one of the important participating adhesion molecules in this process.

Aim of the Work: To study the role of red cells (RBCs) CD47 expression as a predictor for severity of sickle cell disease and its relation to Hb F% and hydroxyurea therapy.

Patients and Methods: The study included 48 sickle cell disease children, divided into three groups: G1; patients not on hydroxyurea (HU) therapy in steady state, G2; patients on HU in steady state, and G3; patients in painful crisis at the time of evaluation. Twenty normal children were included as a control group. For all individuals % expression of CD47 on RBCs was evaluated by flowcytometry.

Results: The mean RBCs CD47 expression was significantly higher in the patient group compared to the control group ($p<0.001$). The mean CD47 expression in G1 (87.25±8.46) was lower than in G3 (89.04±5.14) but higher than in G2 (85.74±18.37) yet the difference was not significant ($p>0.05$). CD47expression was significantly positively correlated with both total leucocytic count and absolute neutrophilic count with non significant negative correlation with HbF%.

Conclusion: The adhesion molecule CD47 expression could be a contributing factor to acute and chronic vaso-occlusion characteristic of SCD.

Key Words: *Sickle Cell Disease – Adhesion molecules – Integrin-associated protein CD47 – Hydroxyurea.*

INTRODUCTION

Sickle cell disease is an inherited hemolytic disorder caused by abnormal hemoglobin (Hb S) [1], transmitted as incomplete autosomal dominant trait that results from a missense mutation in β-globin chain determined gene [2]. It is characterized by the crescent-like or sickle shape that red blood cells acquire in response to decreased oxygen concentration or other physiologic stresses [3].

One of the hallmarks of sickle cell disease is episodic occurrence of painful crises which are believed to originate from vascular occlusion and lead ultimately to organ failure [4]. Sickle red blood cells (RBC) were found to be more adhesive than normal [5]. This makes interactions between the sickle cells and endothelial cells central to the pathophysiology of the disease [4].

Adhesion events are mediated by: (i) membrane-bound receptors at the circulating-cell surface; (ii) membrane-bound counter receptors at the vascular endothelial cell (VEC) surface; (iii) sub-endothelial matrix elements exposed after VEC injury, and finally by (iv) soluble proteins in the plasma forming bridges between the red cells and endothelial cells [6].

Hydroxyurea is an important major advance in the treatment of sickle cell disease. Strong evidence supports the efficacy of hydroxyurea to decrease severe painful episodes, hospitalization, number of blood transfusions and the acute chest syndrome [7,8].

Unfortunately the more understanding of the pathophysiology of sickle cell disease is not accompanied with advances in therapy for this disease [9,10]. However, hydroxyurea therapy is often associated with clinical improvement

before any measurable rise in fetal hemoglobin, suggesting that hydroxyurea could also act through other mechanisms, such as a decrease of VCAM-1 expression and release of endothelin-1 from human endothelial cells [11]. Hydroxyurea also decreases the adhesion of sickle red cells and reticulocytes to endothelial cells [11] and to the sub-endothelial matrix proteins, thrombospondin and laminin [12]. In vivo, hydroxyurea decreases the percentage of reticulocytes expressing $\alpha 4\beta 1$, CD36 [13], soluble VCAM-1 [14] and plasma endothelin-1 levels [15]. Hydroxyurea could also exert its impact through a reduction in neutrophil activation [16]. Thus, adhesion receptors on both red blood cells and endothelial cells seem to be a target for hydroxyurea.

There are several cell surface receptors that appear to be involved in mediating the interaction between HbS erythrocytes and the endothelium. These include Lutheran blood group antigen (BCAM/Lu, CD239), CD147, intercellular adhesion molecule-4 (ICAM-4), CD36 on reticulocytes, very late activation antigen 4 (VLA-4) on reticulocytes, and sulfated glycolipids [17]. Membrane damage to the HbS erythrocyte also leads to the exposure of phosphatidylserine, which is normally restricted to the inner surface of the membrane lipid bilayer. This phosphatidylserine exposure is also thought to contribute to the adhesiveness of HbS erythrocytes to the endothelium [18,19].

Integrin-associated protein (IAP), known as CD47 is an erythrocyte adhesion receptor expressed by both normal and HbS erythrocytes. For unclear reason, it was found that the HBS erythrocytes are more adherent to thrombospondin (TSP). It was suggested that in patients with SCD there are abundance of reticulocytes which express $\alpha 4\beta 1$ integrins, and CD47 is thought to function only in association with integrins [20,21].

In this study we examined the status of CD47 on Red blood cells from children with SCD and the effects of hydroxyurea therapy on modulation of CD47 expression on erythrocytes. Our aim was to study the role of RBCs CD47 expression as a predictor for severity of sickle cell disease and its relation to Hb F% and hydroxyurea therapy.

PATIENTS AND METHODS

This study included 48 sickle cell disease children (The patient group) selected from inpatient and outpatient clinics of Menoufiya University Hospital and Abou El-Rish University Hospital. Egypt. Their ages ranged between 1 and 18 with a mean of 9±4.27 and a median of 8.5 years. They included 26 males and 22 females. This group was subdivided into 2 subgroups 1- Group A: Included 28 patients not on hydroxyurea (HU) treatment, their ages ranged from 1 to 18 with a median of 7.5 yrs. 2- Group B: Included 20 patients on HU treatment for at least one year with good compliance. The treatment duration period ranged from 1 to 12 yrs. Their ages ranged between 6 and 18 with a median of 10.5 yrs. To study the effect of CD47 expression, all patients (48) were divided into another three groups according to the disease status and HU therapy at the time of evaluation: Group 1 (G1) 10 patients in steady state (that was maintained for at least 6 months before the time of evaluation) not on HU, group 2 (G2) 12 patients in steady state (that was maintained for at least 6 months before the time of evaluation) on HU therapy and group 3 (G3) 26 patients with frequent painful attacks and in acute painful crisis at the time of evaluation. Twenty normal children and adolescents of matched age, gender, and socio-economic standard were enrolled as the control group. Their ages ranged from 4 to 18 with a mean of 9.67±4.53 and a median of 9 years. They were 10 males and 10 females. Any individual with pallor, abnormal CBC findings, history of blood transfusion, or family history of SCD or any hematological disorder were excluded from this group. The Ethics Committee approved the study and an informed consent was obtained from all the parents.

For all individuals the following was done:

1- Full history taking including the pain rate (in patient group) defined according to Bonds [10] as the average number of days of hospital stay due to painful episodes or days of extreme relevant illness at home from patient own calendar during the last year.

2- Clinical examination.

3- Laboratory investigations including complete blood picture, reticulocytic count, Liver enzymes (ALT and AST), renal function tests, Hb electrophoresis and serum ferritin level.

4 - CD47 expression on RBCs by flow cytometry (BECKTON, DICKINSON FACS caliber, BD immune cytometry systems, San Jose, CA). The expression of CD47 was evaluated by using FITC labeled mouse monoclonal antibodies against human CD47 (clone B6H12 BD Pharmingen). CD71 PE (clone: M- A712 BD Pharmingen) was used in some cases.

In short 10ul of each monoclonal antibodies were added to 100ul of PB diluted 1:200 in PBS. After 30-45 minutes incubation at room temperature, cells were washed twice and resuspended in 200-400ul PBS and analyzed on Flow Cytometer. Hundred ul of diluted PB served as auto-control [22].

Flow cytometric analysis: Data were acquired on a FACS caliber flow cytometer (BD immune cytometry systems, San Jose, CA).

The instrument was checked weekly using QC windows beads (flowcytometry standard, San Juan, PR). Forward scatter and side scatter measurements were made using linear amplifiers, whereas fluorescence measurements were made with logarithmic amplifiers and flow cytometric parameters dot plots were generated by cell quest software [23].

In some case we use mouse monoclonal antibodies against human CD71 PE for gating on CD47 positive cells (data not presented).

Statistical analysis:

Data were collected, tabulated, and analyzed by using SPSS (11) statistical package for windows XP. Quantitative data were analyzed by student test for comparison of the means of two normally distributed variables and Mann-Whitney (U) test for comparison of the means of two non normally distributed variables. Kruskal Wallis test is a test of significance used for comparison between three groups not normally distributed having quantitative variables. ANOVA (f) is a test of significance used for comparison between three groups having quantitative variables normally distributed. Pearson correlation coefficient (r) is used to test association between two normally distributed quantitative continuous variables. Qualitative data were analyzed by X^2 test. The level of significance was set as 95% confidence interval so p-value was <0.05 [24].

RESULTS

Clinical and laboratory characteristics of patients with sickle cell disease.

Our data (Table 1) showed that patients on HU therapy are significantly taller than those not on HU therapies. The frequency of pain episodes was significantly lower in patients on hydroxyurea therapy compared with those not on HU therapies with no significant difference between the two groups regarding the transfusion index yet it was lower in the HU group. The HU group (GB) had significantly lower reticulocytic count%, total leucocytic count and absolute neutrophilic count compared to that not on HU (GA). No significant difference was found between the two groups regarding Hb level (yet higher in patients on HU therapy), platelets count, Hb F% (at the time of the study), serum ferritin, liver or renal function tests (Table 1).

Table (1): Comparison between Sickle Cell Disease patients not on HU therapy and those on HU therapy.

Parameters	Patients not on hydroxyurea (GA) No=28	Patients on hydroxyurea (GB) No=20	*p* value
Height (cm)	120.8±20.85*	134.75±19.40	<0.05
Pain episodes	3.89±2.28	2.3±0.88	<0.05
(days/year)	89.64±16.88	84.0±16.35	>0.05
Transfusion index (ml/kg/yr)			
Hb (g/dl)	7.43±1.09	7.98±1.59	>0.05
Reticulocytic (%)	5.78±3.67	1.78±0.86	<0.001
WBC($x10^9$/L)	12.79±4.64	10.91±6.44	<0.05
ANC ($x10^9$/L)	6.0±2.94	3.98±1.75	<0.001
Platelets count ($x10^9$/L)	285.15±111.61	312.07±138.95	>0.05
Hb F%	20.16±6.27	23.0±2.77	>0.05
Ferritin (ng/ml)	484.6±330.77	414.35±210.37	>0.05
AST (IU/L)	30.86±11.21	34.15±14.87	>0.05
ALT(IU/L)	28.11±8.41	27.8±10.83	>0.05
BUN (mg/dl)	12.43±3.54	12.08±4.09	>0.05
Creatinine (mg/dl)	0.67±0.72	0.54±0.19	>0.05

* Mean±SD

The frequency of pain episodes was significantly lower in patients in steady state on HU therapy (G2) compared to patients in steady state not on HU therapy (G1). The transfusion index was significantly lower in G2 compared to G1 and to patients in acute painful crisis

(G3), while it was significantly higher in G3 compared to G1. Reticulocytic count% was significantly lower in G2 compared to G1 and G3. There was no significant difference between the three groups regarding Hb level, total leucocytic count, ANC, platelets count, or HbF% (Table 2). Comparing patients on HU therapy before and after at least one year of compliant therapy; the frequency of pain episodes, transfusion index, reticulocytic count% and the absolute neutrophilic count had significantly decreased after HU therapy with insignificant increase in Hb F% (Table 3).

CD47 expression on RBCs is summarized in (Table 4). There was significantly higher expression in the patient group compared to the control group. No significant difference between GA and GB was encountered, yet CD47 expression was lower in (GB). CD47% expression was found to be highest in G3 (patients in acute painful crisis) followed by that of G1 (patients without HU in steady state) and was least in G2 (patients on HU in steady state) but the difference between the three groups did not reach a significant level (Table 4). The frequency of pain episodes showed a significant negative correlation with Hb F% (r=–0.49, p=<0.05) and a significant positive correlation with Hb level (r=0.52, p=<0.05) There was a significant positive correlation between CD47% and both WBCs count and ANC (Table 5, Fig. 3).

Table (2): Comparison between different Sickle Cell Disease groups.

Parameter	Patients (G1) No=10	Patients (G2) No=12	Patients (G3) No=26	p1	p2	p3
Pain rate (days/year)	4.3±2.11	2.25±0.62	3.34±2.13	<0.05	>0.05	>0.05
Transfusion index (ml/kg/yr)	100±22.71	71±28.06	132.5±17.12	<0.05	<0.05	<0.05
Hb (g/dl)	7.84±0.84	7.5±0.92	7.83±1.76	>0.05	>0.05	>0.05
Reticulocytic (%)	4.5±3.66	1.98±0.87	5.65±3.91	<0.05	>0.05	<0.05
WBC (x10^9/L)	12.39±7.71	11.61±5.08	12.57±3.34	>0.05	>0.05	>0.05
ANC (x10^9/L)	5.49±3.14	4.13±1.77	5.52±2.11	>0.05	>0.05	>0.05
Platelets count (x10^9/L)	282.5±121.3	258.83±103.1	327.3±137.7	>0.05	>0.05	>0.05
HbF %	19±7.34	21.15±7.07	16.5±4.2	>0.05	>0.05	>0.05

p1: Between G1 & G2.
p2: Between G1 & G3.
p3: Between G2 &G3.
G1: Patients not on hydroxyurea (HU) therapy in steady state.
G2: Patients on HU in steady state.
G3: Patients in painful crisis.

Table (3): Comparison between Sickle Cell Disease patients on hydroxyurea before and after at least one year of treatment.

Parameter	Before therapy (No=20)	After therapy (No=20)	p value
Pain rate (days/year)	3.65±1.13*	2.3±0.88	<0.001
Transfusion index (ml/kg/yr)	123.5±24.97	84.0±16.35	<0.001
Hb (g/dl)	7.19±0.87	7.98±1.59	>0.05
Reticulocytic (%)	6.89±4.17	1.78±0.86	<0.001
WBCs (x10^9/L)	11.37±6.42	10.91±6.44	>0.05
ANC (x10^9/L)	6.13±3.63	3.98±1.75	<0.05
Platelets (x10^9/L)	301.55±111.5	312.07±138.95	>0.05
Hb F%	20.14±7.35	23±2.77	>0.05
AST (IU/L)	30.4±12.03	34.15±14.87	>0.05
ALT (IU/L)	33.65±10.99	27.8±10.83	>0.05
BUN (mg/dl)	11.25±3.59	12.08±4.09	>0.05
Creatinine (mg/dl)	0.49±0.16	0.54±0.19	>0.05

* Mean±SD

Table (4): Comparison between different Sickle Cell Disease patients groups regarding expression of CD47% on red cells.

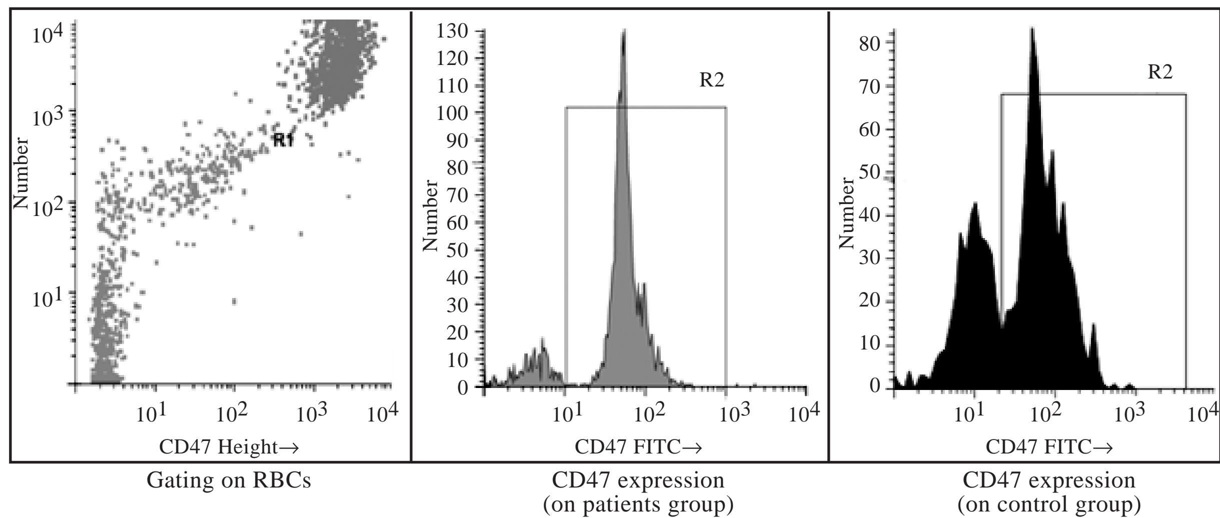
Group	CD47%	p-value
Patients	86.8±14.4*	
Controls	59.1±14.18	<0.001
Patients not on HU (GA)	89.64±6.91	
Patients on HU (GB)	82.98±20.25	>0.05
Patients in Steady state without HU (G1)	87.25±8.46	
Patients in Steady state with HU (G2)	85.74±18.37	>0.05
Patients with Painful crisis (G3)	89.04±5.14	

* Mean±SD.
HU:Hydroxyurea.

Table (5): Correlation between Hb F%, the pain rate and CD47% and different parameters in Sickle Cell Disease patients.

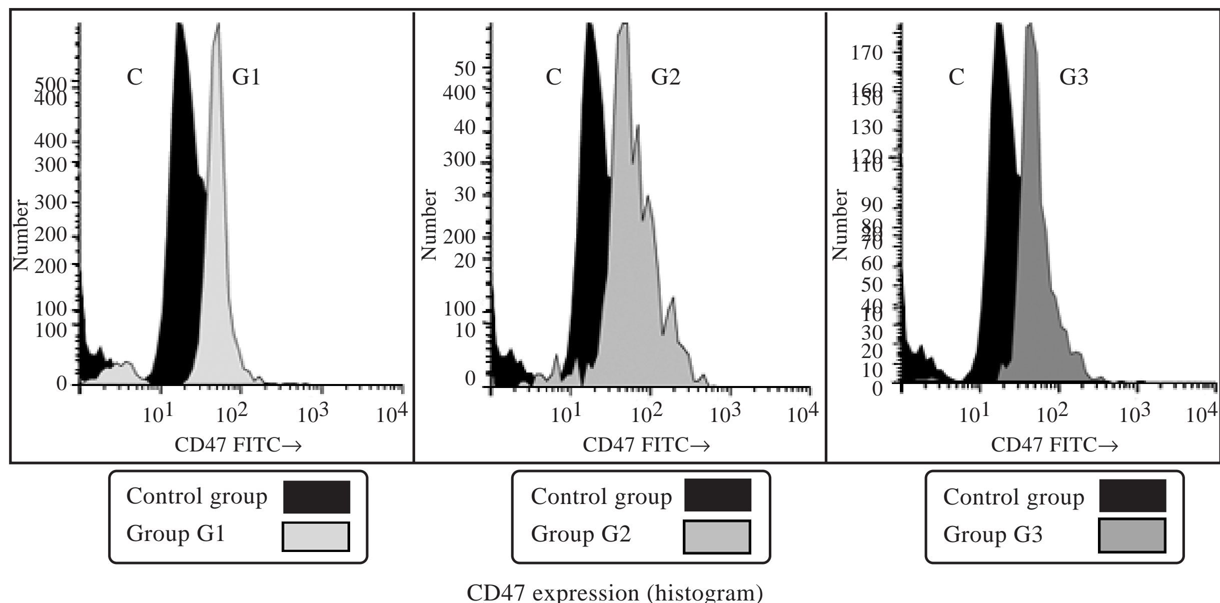
Parameter	Hb F%		Pain rate		CD47	
	r	*p*-value	*r*	*p*-value	*r*	*p*-value
Pain rate (days/year)	–0.49	<0.05			0.20	>0.05
Duration of HU in yrs	0.24	>0.05			0.29	>0.05
Transfusion index (ml/kg/yr)	0.23	>0.05			0.32	>0.05
Hb (g/dl)	–0.12	>0.05	<0.05	0.52	0.09	>0.05
WBCs ($x10^9$/L)	–0.14	>0.05	>0.05	0.21	0.39	<0.05
ANC ($x10^9$/L)	–0.09	>0.05			0.39	<0.05
Reticulocytic count %	0.32	>0.05			–0.22	>0.05
Platelets ($x10^9$/L)	–0.33	>0.05			–0.04	>0.05
HbF%					–0.46	>0.05

HU: Hydroxyurea.



Gating on RBCs | CD47 expression (on patients group) | CD47 expression (on control group)

Fig. (1): CD47 expression on normal control and Sickle Cell Disease patients.



CD47 expression (histogram)

Fig. (2): Comparing of CD47 expression on different groups.

G1: Patients not on hydroxyurea (HU) therapy in steady state.
G2: Patients on HU in steady state.
G3: Patients in painful crisis.

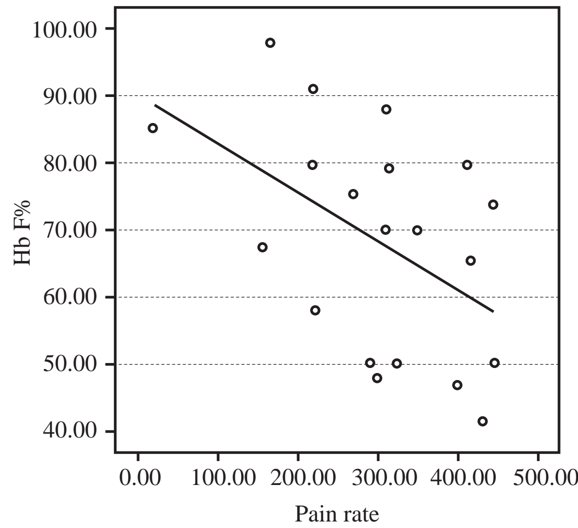


Fig. (3A): Correlation between Hb F% and the pain rate.

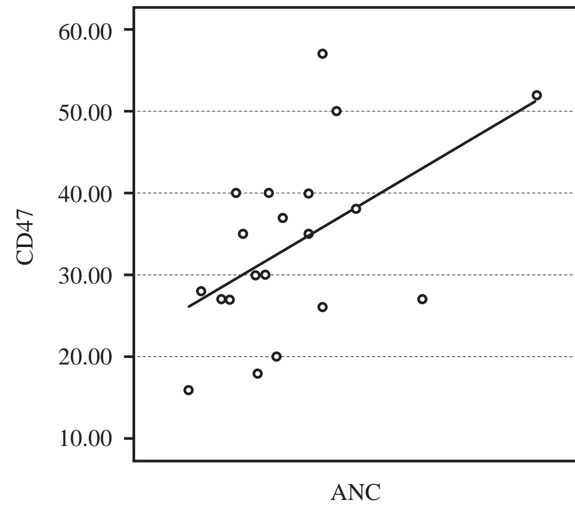


Fig. (3B): Correlation between CD47% and the ANC.

Fig. (3): Correlation studies in 20 sickle cell disease patients.

DISCUSSION

Hydroxyurea is the most prescribed therapy for sickle cell disease **[25]**. The clinical improvement after hydroxyurea therapy is out of question; there is reduction in painful vaso-occlusive crisis and adhesion of sickle cells to vascular endothelium that is a critical factor in pathogenesis of this event **[4]**.

Comparing the group of patients not on HU therapy (GA) and the patients on HU therapy (GB), the pain rate was significantly lower in the HU group than the other group with no significant difference between the two groups regarding the transfusion index (Table 1) indicating that the beneficial clinical effect of HU therapy was reflected on the decline of the pain rate before the effect on RBCs transfusion requirement. It is speculated that reduction in leukocytic count may be an important component of the beneficial effects of hydroxyurea therapy **[9,15,25]**.

The results of this study revealed that the reticulocytic count%, WBCs and ANC were significantly lower in (GB) compared to (GA) with insignificant difference between the two groups regarding the Hb level yet it was higher in (GB) (Table 1). This data is consistent with that obtained by Borba et al. **[16]**.

Hb F% did not significantly differ between the two groups yet was higher in the HU group (Table 1) indicating that HU treated patients had improved clinically and hematologically even before the peak level of Hb F was reached. This is consistent with results reported by Bachir et al. **[26]** who found no correlation between Hb F% and HU serum level but is not in agreement with results of other studies done by Rodgers **[27]** Maier-Redelsperger et al. **[28]** and Koren et al. **[29]**.

The multi-center phase I/II pediatric hydroxyurea trial (HUG-KIDS) confirmed a wide variability in the Hb F% response; a few children who reached the maximal tolerated dose (MTD) had Hb F% levels that were persistently below 10% whereas several others had levels that exceeded 25% **[30]**. So, apparently, the beneficial effect of HU may be exerted through reduction of the total leucocytic count, ANC and reticulocytic count even before the increase of Hb F%.

The pain rate and the transfusion index were significantly lower in patients in steady state on HU therapy (G2) compared to patients in steady state not on HU therapy (G1). The transfusion index was significantly higher in G3 (patients in acute painful crisis) compared to both G1 and G2. There was no significant difference between the three groups regarding the Hb level or Hb F%.

For the group of patients who received HU therapy (for at least one year), there was a significant decrease in the pain rate and transfusion index after HU therapy and this is going with several studies done by Charache et al.

[31], Al-Jam'a and Al-Dabbous [32] and Anderson [33].

The Hb level insignificantly increased after HU therapy and this is in contrast to Wang et al. [30] who reported a significant elevation of Hb level after HU therapy. There was a significant decrease in reticulocytic count and absolute neutrophilic count (ANC) after HU therapy and this is in agreement with Bagdasaryan et al. [34] and Debaun & Field [35]. In contrast, Koren et al. [29] found no significant effect of HU on reticulocytic count.

There was insignificant increase in HbF% after HU therapy. Again these results support that patients on HU therapy had improved clinically (reflected on decline in the pain rate and the transfusion index) even before the peak of Hb F% has been reached and reticuolocytic count improved before improvement of the Hb level. In contrast to what was reported by Al-Jam'a & Al-Dabbous [32], the result of this study revealed that the total leucocytic count had insignificant decrease after HU therapy.

It has been shown that CD47 on sickle red blood cells activates G-protein-dependent signaling, which promotes cell adhesion to immobilized thrombospondin through a receptor called $\alpha 4\beta 1$ [20].

Studying the role of CD47 as an adhesion molecule in the present study, the results revealed that the mean CD47 expression on the RBCs was significantly higher in patient group compared to the control group suggesting that this adhesion molecule could have a role in the pathology of SCD. A few studies have investigated the expression of CD47 in SCD. In contrast to the results of this work, Brittain et al. [36], had reported that CD47 levels are similar or identical on SS and AA RBCs. However, they stated that the structure of CD47 on SS RBCs is different from the structure of CD47 expressed on normal AA RBCs.

The role of adhesion molecules as mediators of HU effectiveness was studied by some researchers [37]; their results supported the hypothesis that HU reduces the adhesive properties of sickle cells and suggested that this decrease may be mediated, at least in part, by a decrease in the gene and consequently, surface protein expression of adhesion molecules such as VLA-4 and CD36.

In the present study, CD47 expression was lower in the group on HU therapy (GB) compared to those not on HU therapy (GA), however, the difference did not reach a significant level. This may suggest that HU produces clinical improvement through pathways not involving CD47 as the pain rate was significantly lower in patients on HU therapy. Also when comparing CD47 expression in patients in steady state not on HU (G1), patients in steady state on HU (G2) and patients in acute painful crises (G3); the mean of CD47 expression in G1 was lower than that in G3 but higher than that in G2, yet the difference was not significant. The last results may indicate that CD47 expression is lower in steady state in general than during the painful crisis. The results also indicate that HU therapy effect is not exerted through decreased CD47 expression.

Fetal Hb concentration is the most important disease modifier in SCD as it is protective to the HbS-containing RBCs. Increased Hb F% is associated with decreased mortality in children and adults with SCD [38] and it protects against painful episodes, acute chest syndrome (ACS) and leg ulcers [39].

In this study significant negative correlation between Hb F% and the pain rate was found. This is in agreement with numerous studies [27,33,40]. Thus, it is expected that patients with higher Hb F% level would have milder disease course and agents that increase Hb F% level would provide significant amelioration of disease severity.

Significant positive correlation between the pain rate and Hb level was found and this is consistent with Platt et al. [41] who stated that increased Hb concentration is a predictor of pain. Positive correlation between the pain rate and the WBCs count was found, yet insignificant. In this regard Redding-Lallinger & Knoll [42] reported that steady state WBCs count >20.000 is a risk factor for SCD complications including recurrent painful crises.

Studying the correlation between CD47 expression and some parameters significant positive correlations between CD47% and both WBCs count and absolute neutrophilic count were found supporting its role in vaso-occlusion as it is matched with other risk factor for vaso-occlusion (WBCs and ANC). This agrees with

Lindberg et al. [43] who stated that CD47 activates neutrophils and with Ticchioni et al. [44], who reported that CD47 activates mature T cells. The results of this work had revealed non significant positive correlation between CD47 expression and both the pain rate and the duration of HU therapy that could be a form of drug tolerance over time.

Chung et al. [45] had reported that CD47 activates platelets and increases platelet adhesion via a Gi-linked signal transduction pathway. In this study negative correlation was found between CD47% and both reticulocytic and platelet counts yet did not reach a significant level.

From the results of this study, it can be concluded that Hydroxyurea is an effective treatment for amelioration of the disease severity of sickle cell disease through its effect on the pain rate and transfusion index. This beneficial effect of hydroxyurea is probably achieved through reduction of total leucocytic and the absolute neutrophilic counts.

In conclusion, the adhesion molecule CD47 expression is increased in SCD patients compared to the normal control and it is positively correlated with WBC and absolute neutrophilic count which could be a factor contributing to acute and chronic vaso-occlusion characteristic of SCD. As CD47 has a potential role in the pathophysiology of vaso-occlusion in SCD, consequently, it should be a target of further research to establish this role as well as to search for useful therapeutic agents capable of antagonizing it and alleviating disease severity.

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The Impact of Cyclooxygenase-2 and Proliferating Cell Nuclear Antigen Over-Expressions in Multiple Myeloma Patients' Bone Marrow Biopsies and their Correlations with other Prognostic Parameters

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ABSTRACT

Background: The survival of patients with multiple myeloma ranges from few years to more than 10 years from the time of diagnosis. The prognosis depends on several clinical, laboratory and histological/cytological parameters. Proliferating cell nuclear antigen (PCNA) correlates with cellular proliferation in benign and malignant neoplasms, including hematological malignancies. Markers of angiogenesis correlate with clinical characteristics in hematologic malignancies, including multiple myeloma (MM). Also Cyclooxygenases (COX)-2 over expression is claimed to be associated with reduced estimated survival in MM.

Objectives: Our aim is to find if COX-2 and PCNA over expressions in MM patients affect their prognosis and survival and/or correlate with other prognostic parameters.

Material and Methods: The study population included 44 newly diagnosed MM patients. Pre-treatment bone marrow (BM) aspiration and biopsy samples were available from all the patients. In addition myeloma work up was done for all patients. BM biopsy samples from 15 patients who had no evidence of BM involvement upon work-up for lymphoma or metastatic tumor were used as normal controls. The biopsies of the myeloma cases and controls were immuno-stained with COX-2 antibodies and antibodies to PCNA and CD 138.

Results: We studied 44 cases, the age of the patients ranged from 37 to 73 with a mean of 56.36±9.84 and a median of 57 years. They included 28 males and 16 females. Patients were followed for 24 months; 8 patients died during the follow-up period. Seven patients (15.9%) were in stage I, 26 (59.1%) in stage II and 11 (25.0%) in Stage III. The trephine biopsy showed >50% plasma cells in 35 cases (59.3%), while 21 cases (35.6%) showed a plasma cell percentage of >50% in the bone marrow aspirate ($p<0.001$). Positive COX-2 immuno-staining was found in 70.5% with 36.4% having weak to moderate positivity and 34.1% strongly positive. Poor prognostic factors as advanced stage, elevated beta-2 microglobulin (B2M) and reduced albumin were correlated with COX-2 expression. The overall survival estimate of those patients with negative or weak-moderate COX-2 immunoreactivity in myeloma cells was significantly better than that of patients with strong COX-2 immunoreactivity ($p=0.001$). We also found that PCNA expression increased with advancing disease stage and correlated significantly with prognostic factors such as elevated B2M, reduced albumin. Moreover, there was strong correlation with COX-2 over expression, response to treatment and survival. Conclusion: Bone marrow biopsy is better than aspirate in estimating the plasma cell burden in the marrow. COX-2 and PCNA expressions by immunohistochemistry are associated with shorter survival and poor prognostic factors such as advanced stage, elevated B2M and reduced albumin. Moreover, PCNA expression is associated with plasmablastic morphology and poor response to treatment.

Key Words: *MM – Bone marrow biopsy – COX-2 – PCNA.*

INTRODUCTION

Multiple myeloma is a malignant disorder of monoclonal plasma cells. Besides the serum or urinary M protein, patients also have increased plasma cells in bone marrow, lytic areas in bone and various other clinical and laboratory abnormalities characteristics of this disease. It has been mentioned that approximately 2% of patients with MM are younger than 40 years and it is still rarer in patients younger than 30 years **[1]**.

Multiple myeloma is a well-established clinical and immunological entity with considerable variability in biological behavior and survival **[2]**. Bone marrow examination continues to be the cornerstone for establishing a diagnosis in association with other clinical and laboratory parameters **[3]**.

Several clinical, laboratory and histological/cytological variables help us in determining the prognosis of the disease [4]. The first histological classification and staging of multiple myeloma, based on the bone marrow trephine biopsy, was put forward by Bartl et al., in 1987 [5]. The Durie and Salmon clinical staging system, proposed in 1977 [6], is still being used today, though it has been replaced by the International staging system (ISS) at many places [7].

Assessment of bone marrow involvement by malignant plasma cells is an important element in the diagnosis and follow-up of patients with multiple myeloma and other plasma cell dyscrasias. However, multiple myeloma is often a focal process, a fact that impacts the accuracy and reliability of the results of bone marrow plasma cell percentages obtained by differential counts of bone marrow aspirate smears. CD138 allows excellent assessment of plasma cell numbers and distribution in bone marrow biopsies. CD138 is a highly specific and sensitive marker of normal and neoplastic plasma cells [8].

Determining those patients with myeloma who will develop progressive disease is an important clinical issue. The $\beta 2$ microglobulin and plasma cell labelling index remain important independent laboratory markers of prognosis [9]. Immunohistological assessment of plasma cell differentiation, the volume of plasma cell infiltration, and the pattern of infiltration all have prognostic value [4]. An increased volume of myeloma in the bone marrow trephine is associated with shorter survival [10].

Proliferating cell nuclear antigen (PCNA) is a 36-KD auxiliary protein of DNA polymerase-delta, that has been found to be a useful marker in immunocytochemical studies of cell proliferation because its expression correlates with the proliferative state of the cell. Its expression increases from the late G1 phase through the S phase of the cell cycle [11].

Exposure of exponentially growing cells to antisense oligdeoxynucleotides to PCNA resulted in complete suppression of DNA synthesis and mitosis, indicating an important role for this protein in cell proliferation [12].

Cyclooxygenases (COX) are enzymes that are involved in the synthesis of prostaglandins (PGs) from arachidonic acid. They catalyze the insertion of molecular oxygen into arachidonic acid to form the unstable intermediate PG-G2 being rapidly converted to PGH2.

PGH2 is the source of several biological active PGs, thromboxanes, and prostacyclins, which contribute to many physiological and pathological processes like hemostasis, kidney and gastric functions, pain, inflammation and tumor defense, and also tumorigenesis [13].

Angiogenesis is defined as the formation of new capillaries from existing blood vessels and plays an important role in the progression of many cancer types. Markers of angiogenesis also correlate with clinical characteristics in hematologic malignancies, including multiple myeloma (MM), serving as predictors of poor prognosis as well as in solid tumors [14].

Forced over-expression of COX-2 stimulates angiogenesis in animal models [15]. Pharmacological inhibition of COX-2, but not COX-1, inhibits corneal neovascularization and experimental colon and lung tumor growth [16]. Giles et al. [17] showed that elevated bone marrow COX-2 levels are associated with reduced survival in chronic phase chronic myeloid leukemia (CML).

In this work, we evaluated cyclooxygenase-2 and PCNA expressions in MM patients' bone marrow biopsies by immunohistochemistry and investigated the relationship of their expression with other myeloma parameters.

MATERIAL AND METHODS

In the current study, we investigated 44 patients (28 males and 16 females) with newly diagnosed multiple myeloma during the period (2008-2011), presented to the Hematology Department, Medical Research Institute. The diagnosis of MM was established using WHO classification 2008 [18]. The patients were clinically staged according to Durie-Salmon staging system [6]. Treatment regimens were either VAD (vincristine, doxorubicin and dexamethasone) infusion chemotherapy or Thal-dex (thalidomide and dexamethasone) [19]. Patients taking medications that could affect the COX-2 metabolism, such as aspirin, and chemotherapeutic agents were excluded from the study. BM biopsy samples from 15 patients who had no evidence of

BM involvement upon investigational work-up for lymphoma or metastatic tumor were used as normal controls for COX2 and PCNA expressions after exclusion of those with significant drug history.

Our patients were followed-up for 24 months or more from diagnosis. Pre-treatment bone marrow aspiration and biopsy samples were available from all the patients. Biopsies were taken from the posterior iliac crests. All patients were subjected to complete blood count, serum beta-2 microglobulin (B2M), serum albumin, serum lactate dehydrogenase (LDH), serum calcium, serum creatinine and serum protein electrophoresis. A radiological skeletal bone survey, including spine, pelvis, skull, humeri and femurs was carried out for all patients.

Bone marrow aspirate smears were stained with Leishman stain and examined using a 100x oil immersion objective. Percentages of plasma cells in the aspirates were estimated by a 500-cell count. The bone marrow aspirates were typed for plasma cell morphology as plasmacytic (well differentiated or intermediately differentiated) or plasmablastic (poorly differentiated plasma cells).

Trephine biopsies were decalcified, processed, paraffin embedded, sectioned and stained with hematoxylin and eosin (H&E) **[20]**. In bone marrow trephine biopsy sections, we estimated the percentages of cellular marrow occupied by plasma cells to the nearest 5% based on the examination of conventional H&E sections and CD138 stained sections. In addition, the pattern of infiltration in the trephine biopsies whether interstitial, mixed (nodular and interstitial) or diffuse was determined. All cases were independently reviewed by 2 hematologists without prior knowledge of the clinical data.

Immunohistochemistry:

Sections of bone marrow trephine biopsies of the myeloma cases and controls were immune-stained according to the manufacturer instructions with CD 138 Ab-2 (Catalog number MS-1793-S0, Thermo Scientific, UK), COX-2 antibody (Catalog number RM-9121-S0) (Thermo Scientific, UK) and antibody to PCNA (Catalog MS-106-R7, Thermo Scientific, UK), using the AEX080-IFU system (Econo Tek HRP Anti-Polyvalent (DAB) (Scy Tek, USA).

We utilized an immune-histochemical score (IHS) for COX-2 based on the German Immunoreactive score. The IHS is calculated by multiplying the quantity and staining intensity scores. The scores could range from 0 to 12 for three groups. An IHS score of (7-12) was considered strong immunoreactivity, (1-6) weak or moderate, and 0 was scored as negative. COX-2 [21].

Two observers, blinded to the clinical outcome of the patients, independently scored the myeloma cell staining for COX-2 and PCNA.

Statistical analysis:

Data were fed to the computer using the Predictive Analytics Software (PASW Statistics 18). Qualitative data were described using number and percent. Association between categorical variables was tested using Chi-square test. When more than 20% of the cells have expected count less than 5, correction for chi-square was conducted using Monte Carlo correction. Quantitative data were described using median, minimum and maximum as well as mean and standard deviation.

The distributions of quantitative variables were tested for normality using Kolmogorov-Smirnov test, Shapiro-Wilk test. D'Agstino test was used if there was a conflict between the two previous tests. If it reveals normal data distribution, parametric tests was applied. If the data were abnormally distributed, non–parametric tests were used. For normally distributed data, comparison between more than two population were analyzed by F-test (ANOVA). Correlations between two quantitative variables were assessed using Pearson coefficient. For abnormally distributed data, Mann-Whitney Test (for data distribution that was significantly deviated from normal) were used to analyze two independent population. If more than two population were analyzed, Kruskal Wallis test was used. Kaplan-Meier for Survival curve was used. Significance test results are quoted as two-tailed probabilities. Significance of the obtained results was judged at the 5% level.

RESULTS

During the period (2008-2011) we studied 44 cases, the age of the patients ranged from 37 to 73 years with a mean of 56.36±9.84 and a median of 57 years. They included 28 males and 16 females with a male to female ratio of

1.75:1. Bone pain and easy fatigability were the most common symptoms seen in 37/44 (84.1%) and 33/44 (75%) respectively. Patients were followed during the study period for a minimum of 24 months. Eight patients died during the study period. Serum creatinine more than 2mg/dl was detected in 20.4% and serum calcium more than 11.5mg/dl in 29.5% of the cases. LDH was elevated in 21/44 (47.7%) of the patients, B2M in 13/44 (29.5%) and albumin was reduced in 36/44 (81.8%).

Seven patients (15.9%) were in stage I, 26 (59.1%) in stage II and 11 (25.0%) in Stage III. The biopsy showed >50% plasma cells in 35 cases (59.3%), while only 21 (35.6%) showed a plasma cell percentage of >50% in the bone marrow aspirate ($p<0.001$) (Table 1).

Twenty-six cases (59.1%) had plasmablastic morphology, while 18 cases (40.9%) had plasmacytic morphology. Nearly 71% of the cases (31/44) had a diffuse pattern of infiltration in the bone marrow trephine biopsy, while interstitial and mixed (nodular + interstitial) patterns were found in 18.2% (8/44) and 11.4% (5/44) of patients respectively.

According to the international uniform response criteria for multiple myeloma, 28 cases (63.6%) responded to therapy in the form of partial or complete remission (Responders) and 16 (36.4%) showed no response (Non–responders).

On comparing the clinical stage of the disease with the response to treatment, it was found that 8/11(72.7%) of patients in stage III were non-responders versus 8/26 (30.7%) of patients in stage II, whereas all patients in stage I were responders ($p=0.005$). As regards the bone marrow histological features, 8/11 (72.7%) of patients in stage III showed plasmablastic morphology versus 15/26 (57.7%) of patients in stage II and 3/7 (42.9%) of patients in stage I ($p=0.426$), while 9/11 (81.8%) of patients with stage III had diffuse pattern of infiltration versus 18/26 (69.2%) of stage II patients and 4/6 (57.1%) of stage I patients; the differences are statistically insignificant ($p=0.846$).

Fig. (1) Bone marrow infiltration by plasma cells highlighted by CD138 stain showing membranous pattern.

COX-2 and PCNA expressions by immunohistochemistry was significantly higher in patients as compared to controls (Table 2). Thirty four percent (15/44) of the cases were strongly positive for COX-2, 36.4% (16/44) showed moderate positivity whereas none of the controls had strong positivity and only 13.3% (2/15) were moderately positive.

COX-2 results were correlated with different prognostic parameters. Thirteen bone marrow biopsy specimens were negative, while 16 and 15 specimens were moderately positive and strongly positive for COX-2 immunostaining respectively. Fig. (2) shows bone marrow biopsy section from a patient with multiple myeloma immunostained for Cox-2 that demonstrates membranous and cytoplasmic staining.

Strong COX-2 expression was associated with higher bone marrow plasma cell burden and diffuse pattern of infiltration but the results were statistically insignificant, whereas advanced stage and non-responders were associated with strong Cox-2 expression with p-value of <0.001 for both. B2M levels were higher while albumin levels were lower in those with strong COX-2 expression with p-value of 0.016 and 0.007 respectively (Table 3). Kaplan-Meier survival estimate of those patients with negative or moderate COX-2 immunoreactivity in myeloma cells was significantly better than that of patients with strong COX-2 immunoreactivity ($p=0.001$) (Fig. 4).

PCNA positivity ranged from 8% to 80% in myeloma cells. Fig. (3) illustrates bone marrow biopsy section from a patient with multiple myeloma immunostained with PCNA antibody that show nuclear staining. PCNA expression was highly correlated to COX-2 expression ($p<0.001$). Similarly PCNA expression correlated significantly with elevated ß2M and reduced albumin ($r=0.385$, $p=0.010$; $r=0.350$, $p=0.020$, respectively). PCNA expression was associated with advanced stage ($p<0.001$). Higher PCNA expression was associated with plasmablastic morphology ($p=0.006$). Also higher PCNA expression was detected in non- responders ($p<0.001$) and in patients with short overall survival ($p<0.001$) (Table 4).

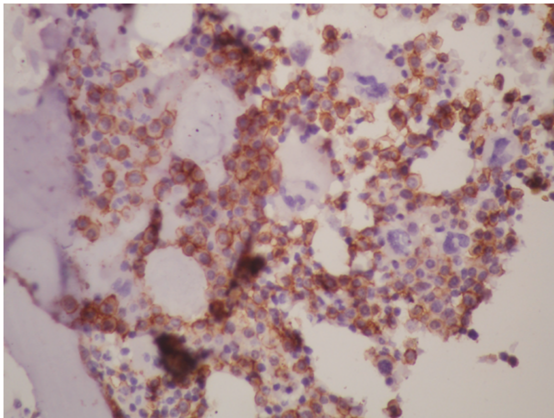

Fig. (1): Bone marrow biopsy section showing infiltration by plasma cells highlighted by CD138 stain that show the membranous pattern of CD138.

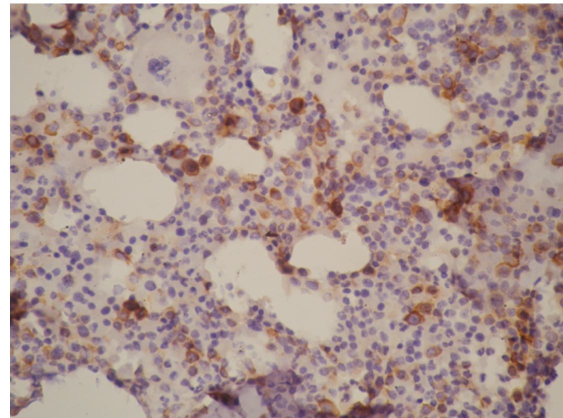

Fig. (2): Bone marrow biopsy section from a patient with multiple myeloma showing immunostaining with antibody with Cox-2 that demonstrates membranous and cytoplasmic staining.

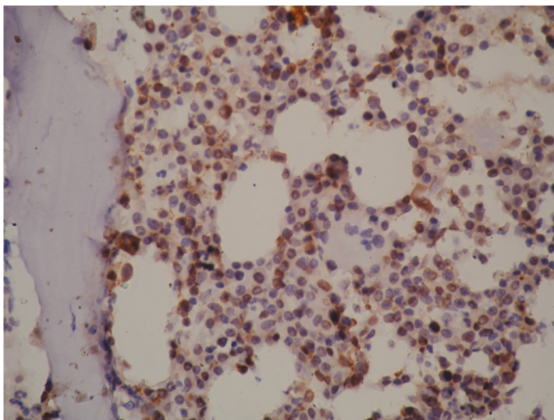

Fig. (3): Bone marrow biopsy section from a patient with multiple myeloma immunostained with PCNA antibody shows nuclear staining.

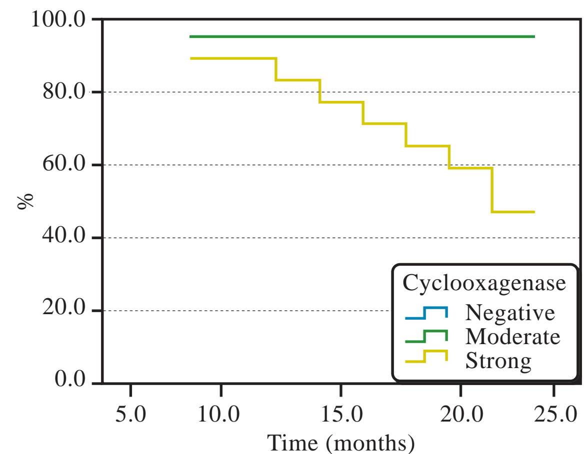

Fig. (4): Kaplan Meyer curve of survival in patients with negative, weak-moderate and strong COX-2 immunoreactivity in myeloma cells.

Table (1): Comparison between plasma cell percent in bone biopsy and aspirate.

	<20		20- 50		>50		MCp
	No.	%	No.	%	No.	%	
Bone marrow biopsy	0	0.0	24	40.7	35	59.3	<0.001*
Bone marrow aspirate	2	3.4	36	61.0	21	35.6	

MCp: *p*-value for Monte Carlo test. *: Statistically significant at $p \leq 0.05$.

Table (2): Comparison between patients and controls according to Cyclooxagenase-2 expression and mean of PCNA expressions.

	Cases (n = 44)		Controls (n = 15)		MCp
	No.	%	No.	%	
Cyclooxagenase-2:					
Negative	13	29.5	13	86.7	0.001*
Moderate	16	36.4	2	13.3	
Strong	15	34.1	0	0.0	
PCNA %:					
Range	8.0-80.0		0.0-3.0		<0.001*
Mean ± SD	32.80±16.81		1.60±0.99		
Median	31.0		2.0		

MCp: *p*-value for Monte Carlo test. *p*: *p*-value for Mann Whitney test. *: Statistically significant at $p \leq 0.05$.

Table (3): Cyclooxagenase 2 immunostaining in relation to the different studied parameters.

		Age (mean±SD)	Serum LDH (median)	Serum B2M (median)	Serum Albumin (mean±SD)	Plasma cell percentage in trephine biopsy			Pattern of infiltration in trephine biopsy			Stage			PCNA immune-staining (median)	Response to treatment	
						<20% Number (%)	20-50% Number (%)	>50% Number (%)	Diffuse Number (%)	Interstitial Number (%)	Mixed Number (%)	I Number (%)	II Number (%)	III Number (%)		Responder	Non-responder
Cyclo-oxygenase 2 Immune-staining	Negative	53±9.8	218	2.3	3.31±0.31	0 (0)	9 (69.2)	4 (30.8)	8 (61.5)	4 (30.8)	1 (7.7)	6 (46.2)	7 (53.8)	0 (0)	20	13 (100)	0 (0)
	Moderately-positive	58.1±9.2	267.5	3.2	2.95±0.37	0 (0)	8 (50)	8 (50)	10 (62.5)	3 (18.8)	3 (18.8)	1 (6.3)	14 (86.5)	1 (6.3)	30.5	13 (81.3)	3 (18.8)
	Positive	57.4±10.4	250	2.9	2.88±0.38	0 (0)	7 (46.7)	8 (53.3)	13 (86.7)	1 (6.7)	1 (6.7)	0 (0)	5 (33.3)	10 (66.7)	41	2 (13.3)	13 (86.7)
p		0.341#	0.079‡	0.016‡*	0.007#*	0.525#			0.402#			<0.001#*			<0.001‡*	<0.001#*	

*: Significant.
#: Monte Carlo test p-value.
‡: Kruskal wallis test *p*-value.
B2M: beta 2-microglobulin.

Table (4): Relation between PCNA and different prognostic parameters.

	Range of PCNA	Mean of PCNA± SD	Median	Test of sig.
Plasma cells % in biopsy:				
<20	0.0-0.0	0.0±0.0	0.0	^{KW}p=0.272
20-50	10.0-80.0	30.79±16.75	28.0	
>50	8.0-77.0	35.20±17.0	34.50	
Pattern of infiltration in biopsy:				
Diffuse	8.0-80.0	36.06±18.13	34.0	^{KW}p=0.225
Interstitial	11.0-36.0	24.25±9.29	25.0	
Mixed	12.0-40.0	26.20±11.76	31.0	
Plasma cell morphology:				
Plasmablastic	8.0-80.0	38.62±18.32	35.0	^{MW}p=0.006*
Plasmacytic	10.0-40.0	24.39±9.72	24.50	
Stage:				
I	8.0-20.0	11.86±3.85	11.0	^{KW}p<0.001*
II	16.0-60.0	32.48±9.51	32.0	
III	30.0-80.0	58.83±21.02	65.0	
Response:				
Responder (28 cases)	8.0-50.0	25.04±10.34	25.0	^{MW}p<0.001*
Non responder (16 cases)	25.0-80.0	46.38±17.56	38.50	
Outcome:				
Alive (36 cases)	8.0-64.0	28.17±12.29	29.50	^{MW}p<0.001*
Dead (8 cases)	35.0-80.0	53.63±19.37	50.0	

^{KW}p: *p*-value for Kruskal Wallis test. ^{MW}p: *p*-value for Mann Whitney test. *: Statistically significant at $p \leq 0.05$.

DISCUSSION

This study analyzed 44 cases of myeloma, with respect to COX-2 and PCNA expressions by immunohistochemistry on bone marrow biopsies. The correlation of COX-2 and PCNA expressions with other myeloma parameters (clinical stage, laboratory parameters, bone marrow plasma cell infiltration, clinical outcome and survival) were investigated.

In the studied cohort, bone pain and easy fatigability were the most common symptoms seen in 84% and 75% respectively as was reported by others [22]. Most of our patients were in stage II (59.1%). When the morphology of the plasma cells and pattern of infiltration were compared with the clinical stage of the disease it was seen that although 72.7% of patients in stage III had plasmablastic morphology and 81.8% had diffuse pattern of infiltration, the results were statistically insignificant (p=0.426 and p=0.846 respectively). This may be due to the small number of cases in stage III (11 patients).

In our study, we observed that 59.3% of the patients had >50% plasma cell infiltrate in the biopsy, compared to only 35.6% in the aspirate (p<0.001), this is in agreement with Subramanian et al [24] who found that 71% of the patients had >50% plasma cell infiltrate in the biopsy, compared to only 40% in the aspirate (p<0.001). Also Pich et al [23] reported a higher mean percentage of plasma cell infiltrate in the biopsy (50.3%) as compared to the aspirate (32.89%).

In our study, positive COX-2 immunostaining was found in 70.5% of our patients; 36.4% were weak to moderate and 34.1% were strongly positive. Poor prognostic factors as clinical stage, B2M and albumin were correlated with COX-2 expression. Kaplan-Meier overall survival estimate of those patients with negative or weak-moderate COX-2 immunoreactivity in myeloma cells was significantly better than that of patients with strong COX-2 immunoreactivity. Cetin et al [24] found that COX 2 overexpression was associated with reduced estimated survival. Poor prognostic factors such as LDH, age and b2-microglobulin were also correlated with COX-2 expression.

We also found that PCNA expression increased with advancing disease stage and correlated significantly with prognostic factors such as B2M and albumin. Also there was strong correlation with COX-2 over expression, response to treatment and survival. Tsirakis et al [25]. found that PCNA value increased with advancing disease stage and correlated significantly with prognostic factors, such as IL-6, β2 microglobulin and LDH. Pretreatment PCNA expression correlated significantly with bone marrow MVD (p<0.05) plasma cell infiltration (p<0.01) and IL-6 (p<0.01) as reported by Alexandrakis et al [26].

In agreement with our results Usnarska-Zubkiewicz et al [27] found that PCNA positive cells ranged from 2% to 100%, higher PCNA expression was observed in patients with immature type of MM (mean 29.5%, SD=5.5), the highest expression was seen in plasmablastic type MM (mean 60.5%, SD=22.6) and correlated positively with the cytomorphology of plasma cells and clinical outcome.

In conclusion, Bone marrow biopsy is better than aspirate in estimating the plasma cell burden in the marrow. Also COX-2 over-expression by immunohistochemistry is associated with reduced survival and poor prognostic factors such as clinical stage, B2M and albumin. PCNA expression in myeloma indicates the myeloma's proliferative activity and correlates positively with the different parameters as advanced stage, plasmablastic cell morphology, elevated B2M, reduced albumin, poor response to treatment and shorter survival.

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Glucose-6-Phosphate Dehydrogenase Deficiency Among Newborns with Indirect Hyperbilirubinaemia in Bani Sueif Governate

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ABSTRACT

Background: Jaundice is the most common condition requiring attention in newborn. G6PD deficiency is among the causes of severe neonatal hyper-bilirubinemia with the potential of kernicturs.

Objectives: The aim of this work is to detect cases of G6PD deficiency among newborns with indirect Hyperbilirubinaemia in our country before recommending it as an obligatory screening test for all newborns using the quantitative method and for early diagnosis of G6PD deficiency.

Patients and Methods: This study was conducted on 200 full term neonates with neonatal jaundice including 158 males and 42 females. They were selected from the NICU in Beni-Suef University teaching hospital. Cases were recruited during the study period from May 2010 to January 2011.

Results: Out of the 200 neonates, 8 patients (4%) were G6PD deficient using the screening test.

Conclusion: Despite low incidence of G6PD deficiency in our study, we recommend screening for G6PD deficiency in any neonate presenting with jaundice or any neonate with positive family history for G6PD deficiency not only to detect the etiology of jaundice, but also to prevent kernicterus and future hemolytic episodes.

Key Words: *G6PD deficiency – Neonatal jaundice – Neonatal screening – Quantitative enzyme assay.*

INTRODUCTION

Hyperbilirubinemia is one of the most common problems in the neonatal period, and is a benign condition in most cases [1].

Nonetheless, untreated severe indirect hyperbilirubinemia is potentially neurotoxic, and conjugated direct hyperbilirubinemia often signifies a serious illness [2].

Kernicterus is a neurologic syndrome resulting from the deposition of unconjugated bilirubin in the brain cells. The risk in infants with erythroblstosis is directly related to serum bilirubin levels. The relationship between serum bilirubin level and kernicterus among healthy term infants is uncertain [3].

The mechanisms of neonatal hyperbilirubinaemia are variable including: Bilirubin overproduction which occurs in hemolytic diseases with either positive Coombs test (ABO incompatibility, Rhesus incompatibility, and minor blood group antigens) or negative Coombs test (red blood cell membrane defects, e.g., spherocytosis, elliptocytosis, and/or red blood cell enzyme defects, such as glucose-6-phosphate dehydrogenase (G6PD) and pyruvate kinase deficiencies) Nonhemolytic causes as cephalohematoma, bruising, polycythemia or decreased Bilirubin conjugation as Crigler-Najjar syndrome types 1 and 2, Gilbert syndrome, Hypothyroidism, Breast milk jaundice and Impaired bilirubin excretion as infection, metabolic disorder, chromosomal abnormality, drugs [4].

The prevalence of G-6-PD deficiency among Caucasian populations ranges from less than 1 in 1000 among northern European populations to 50 percent of the males among Kurdish Jews. G-6-PD deficiency is also found among certain Chinese populations and in Southeast Asia but it is rare in Japan. G-6-PD deficiency of the A- type is very common in West Africa, and the prevalence among African American males is approximately 11 percent. Some 16 percent of African American males carry the non-deficient G-6-PD A+ gene [5].

Icterus neonatorum in G-6-PD deficiency probably is due principally to inadequate processing of bilirubin by the immature liver of G-6-PD-deficient infants, although shortening of red cell life span may play a role. Severe jaundice due to G-6-PD deficiency seems to be limited to infants who have also inherited a mutation of the (UDPGT-1) gene promoter [6].

Evaluation of G6PD deficiency should be considered, especially for infants who are older than four days, have a positive family history, or are of East Asian, Greek, Mediterranean, or African descent.

SUBJECTS AND METHODS

This study was conducted on 200 full term neonates with neonatal jaundice including 158 males and 42 females. They were selected from the NICU in Beni-Suef University teaching hospital. Cases were recruited during the study period from May 2010 to January 2011. The protocol was approved by the IRB of the Faculty of Medicine, Beni-Suef University and informed consent was obtained from children's guardians.

Inclusion criteria:

All full term neonates with indirect hyperbilirubinaemia.

Exclusion criteria:

Cholestasis and Preterm infants.

Methods:

All cases were subjected to: Careful history taking focusing on factors that might lead to neonatal jaundice as gestational age, mode of delivery and instrument use (forceps, or ventouse); factors that might lead to sepsis and urinary tract infections as premature rupture of membranes, maternal fever, vaginal discharge; pattern of breast feeding adequacy; passage of meconium; family history of jaundice in a previous sibling, RH or ABO incompatibility, family history of chronic haemolyticanaemia presenting in the neonatal period (spherocytosis & elliptocytosis); history of G6PD in a family member.

Thorough clinical examination: Vital data: Heart rate, respiratory rate, temperature. Complexion: Jaundice or pallor. Anthropometric measurements: Weight, length, head circumference and abdominal circumference; Modified Ballard Scoring to include full term only (above 37 weeks); decreased perfusion & lethargy; organomegaly and palpable flank masses. Complete systemic examination: GIT, chest, cardiac and neurological examination.

Investigations:

1- Serum bilirubin (total and direct) using automated (Cobas C 111) (Roche) [7].
2- Complete blood picture using Electronic counter Sysmexkx– 21N.
3- Reticulocytic count by Brilliant Cresyl blue stain (supravital stain).
4- Coomb's test (direct) [8].
5- Maternal & neonatal blood group & RH.
6- Serum C- reactive protein (CRP) using Latex serology test.
7- G6PD enzyme assay: Quantitative method using spectrophotometer Bayer RA-50 chemistry analyzer Biosystem according to manufacturer instructions.

Statistical analysis:

Analysis of data was done by using SPSS (statistical program for social science version 15) as follows Description of quantitative variables as mean, SD and range Description of qualitative variables as number and percentage Chi-square test was used to compare qualitative variables between groups Unpaired *t*-test was used to compare two groups as regard quantitative variable in parametric data (SD <50% mean). Mann Whitney test used instead of unpaired *t*-test in non parametric data (SD >50% mean) Spearman correlation test was used to rank different variables against each other either positively or inversely.

- p-value >0.05 insignificant.
- $p<0.05$ significant.
- $p<0.01$ highly significant [9].

RESULTS

The present study included 200 full term neonates admitted for neonatal jaundice. (Table 1) presents the characteristics of the study group. The majority of the cases were males (79%) and the majority was in 4-7 days old at the time of testing.

As regards the maternal history of the studied neonates, the majority (60%) had irrelevant history, urinary tract infection, Premature rupture

of membrane were each encountered in 15%, 2% had placenta previa while 8% had other conditions as maternal diabetes, instrumental delivery and maternal medication.

The laboratory findings of the studied neonates are presented in (Table 2).

The causes of hyperbilirubinemia in the studied cohort are presented in (Table 3). In the majority of cases (69%) no cause could be detected. Sepsis was the most common cause followed by ABO incompatibility. G6PD deficiency was detected in 8 cases (4%).

No statistically significant difference was encountered in any of the laboratory parameters between neonates with normal and those with deficient G6PD. As regards maternal history, 4/8 cases had irrelevant history while UTI and PROM were detected in 2/8 cases each.

No statistically significant correlation could be detected between G6PD level on one side and either age, birth weight, bilirubin level, Hb, TLC, Platelets or reticulocyte count on the other side.

Table (1): Characteristics of 200 neonates with hyperbilirubinemia.

Variable	No.	%
Age (days):		
≤3	78	39
4-7	84	42
≥8	38	19
Mean±SD	4.5±2	(1-15)
Gestational age:		
37 weeks	60	30
>37	140	70
Gender:		
Male	158	79
Female	42	21

Table (2): Description of laboratory data of 200 neonates with hyperbilirubinemia.

Parameter	Mean	±SD	Range
HB g/dl	13.1	1.9	11-16
HCT %	38.8±5	5	35-42
MCV fl	97	9	95-99
MCH pg	33.5	2	29-35
WBCs/cmm	9.9	2	4-11
Lymphocytes %	28	11	0-30
Neutrophils %	48	20	4-58
RDW %	14	2	11-16
Reticlocytes %	2.7	2	0.9-3
Platelets/cmm	280	100	150-350
Total bilirubin (mg/dl)	16	3	8-18
Direct bilirubin (mg/dl)	0.78	0.07	0.04-1.01

Table (3): Causes of indirect hyperbilirubinemia in 200 neonates.

Cause	Number	%
ABO incompatibility	18	9
Rheusus incompatibility	4	2
Cephalhematoma	2	1
Sepsis	30	15
G6PD deficiency	8	4
Undetermined (exaggerated physiological jaundice, breast feeding or breast milk jaundice, unknown)	138	69
Total	200	100

DISCUSSION

Glucose-6-phosphate dehydrogenase deficiency, the most common enzyme deficiency worldwide, causes a spectrum of disease manifestations including neonatal hyperbilirubinemia, acute hemolysis, and chronic hemolysis. Persons with this condition also may be asymptomatic. This X-linked inherited disorder most commonly affects persons of African, Asian, Mediterranean, or Middle-Eastern descent. Approximately 400 million people are affected worldwide [10].

The relationship between G6PD deficiency and hyperbilirubinemia in the newborn period is well recognized. Severe neonatal hyperbilirubinemia resulting in kernicterus is the most serious complication of this enzyme deficiency in the newborn period. Thus early neonatal screening programmes should be instituted especially in countries where the prevalance of enzyme deficiency is high [11].

Hyperbilirubinemia in G6PD-deficient neonates is thought to be secondary to reduced hepatic conjugation and excretion of bilirubin, rather than increased bilirubin production resulting from hemolysis. Thus no difference was encountered in reticulocyte count or hematocrit level between G6PD-deficient and normal groups [12].

In the present study, the G6PD deficiency was found in 4% of the studied population. Other studies reported slightly lower incidence of 1.57% in Spain [11] 2.1% in Zanjan province of Iran, 2.5% in Singapore [13] and 3.5% in Turkey [11]. On the contrary a much higher

incidence of 30%, 40% and 14% were reported in Al-Houfuf area in Saudi Arabia [14] in Nigerean neonates [15] and in Black Americans [16] respectively.

Our results showed that the G6PD deficient newborns are all males thus there is no G6PD deficient female neonates, this result comes in agreement with those obtained by Huang et al. [17] and Yu et al. [18]; they reported that G6PD deficient females are not at increased risk for the development of neonatal hyperbilirubinemia in Taiwan. Similarly another study in Taiwan reported that the prevalence of G6PD deficiency was 3.54% in males and 1.57% in females [19]. Thus the prevalence of G6PD deficiency in males was significantly higher than females in this study. The percentage of boys was higher than girls in other studies as well, such as the study by Koosha & Rafizadeh [20] which reported that 3.6% of males and 0.6% of females were G6PD deficient. Similarly the ratio between male: Female G6PD deficient neonate was 3: 1 in the study by Atay et al. [11].

However, such results were not matched with the reports obtained by Tan [21] in Singapore and by Kaplan and Abramov [22] in Israel that showed higher incidence of neonatal hyperbilirubinemia in G6PD-deficient females. Another study by Omran et al. [15] in Saudi Arabia showed higher incidence of G6PD deficiency in females may be in part due to the high rate of consanguinity among the Saudi population, leading to increased numbers of female homozygotes.

In the present study, no significant relationship was noted between the severity of jaundice and morphological changes in the neonates' RBCs, their reticulocyte count or their hemoglobin concentration. Such findings were also reported by Kaplan et al. [23] who suggested that jaundice is thought to be secondary to reduced hepatic conjugation and excretion of bilirubin, rather than increased bilirubin production resulting from hemolysis. This is in agreement with Abolghasemi et al. [24] who reported that jaundice may not necessarily be related to hemolysis, but probably to transferase activity in liver cells. This is supported by the fact that in jaundiced G6PD deficient neonates there are lower levels of bilirubin diglucuronide, normal packed cell volumes (PCV), normal reticulocyte counts and insignificant rise of carboxyhemoglobin [25].

Statistical analysis of our results showed that there were no differences in the highest total bilirubin concentration, reticulocyte count, or the lowest haemoglobin level between normal G6PD and G6PD-deficient newborns. This comes in agreement with the results obtained by Koosha and Rafizadeh [20] and Al-Omran et al. [15]. Also Atay et al. [11] in Turkey reported that no statistical difference was detected between G6PD deficient and normal groups in relation to reticulocyte count. These findings do not suggest significant hemolysis as a cause of jaundice in these infants, which is a common observation in G6PD deficient neonates. On the contrary, a Nigerian study done by Kaplan et al. [23] documented that the difference in the mean total Hb value of the G6PD deficient group compared with controls was extremely significant. These findings suggest accompanying hemolysis as a cause of this difference.

In the current study, we demonstrated that G6PD deficiency by itself is a risk factor for the development of neonatal hyperbilirubinemia even without exposure to chemicals that might cause hemolysis. As the Apgar scores of these neonates in our study showed that the stress from birth process was not likely the major cause to induce neonatal hyperbilirubinemia in G6PD-deficient neonates in the nursery, our findings implied that the possible cause of neonatal hyperbilirubinemia was not directly related to hemolysis, but was secondary to reduced hepatic conjugation and excretion of bilirubin. Our results came in concordance with another study by Weng et al. [20] in Taiwan.

However, the mechanism of the relationship between G6PD activity and neonatal hyperbilirubinemia is not clear. The presence of other genetic factors has been postulated in the pathogenesis of neonatal hyperbilirubinemia in G6PD deficiency. Kaplan et al. [23] reported that UGT1A1 gene mutation, diminishing activity of the conjugating enzyme UGT, was associated with neonatal hyperbilirubinemia in G6PD deficiency. However, this was not confirmed by Galanello et al. [26]. Thus the interaction between UGT1A1 and G6PD genes remains to be verified.

In conclusion, G6PD deficiency is encountered in 4% of neonatal hyperbilirubinema in our study. Despite its low incidence, we recommend screening for G6PD deficiency in any neonate presenting with jaundice or any neonate with positive family history for G6PD deficiency not only to detect the etiology of jaundice, but also to prevent kernicterus and future haemolytic episodes.

Genetic counseling is recommended; parents should know that the condition is an X linked disease, transmitted by the asymptomatic mother to affect 50% of her sons. So, the recurrence rate in subsequent pregnancies is 50% in male offsprings. In male siblings (brothers) of patient, the enzymatic activity of G6PD should be studied for early diagnosis of G6PD deficiency. In addition, we recommend that measurement of the enzyme UGT be made available for the clinical use in the evaluation of neonatal hyperbilirubinaemia.

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Routine Use of Mini-Pool Nucleic Acid Testing (MP-NAT) Multiplex Assay for Sero-Negative Blood Donors

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ABSTRACT

Background: Until December 2010, HBV-DNA NAT assay was not required by the FDA or recommended by the AABB. The FDA approved automated multiple assays that detect HIV-RNA, HCV-RNA, and HBV-DNA in one reaction chamber. These systems are approved for testing of individual donations and pool of 6-16 donor samples.

Material and Methods: With retrospective study, for blood donors, in Almana General Hospitals, Saudi Arabia, from January 2008 to April 2011 donor's blood was examined by serological assays for HBV, anti-HCV, and HIV. Sero-negative donor blood was then examined by mini-pool nucleic acid test (MP-NAT) multiplex assay. Individual donor NAT reactive samples were discriminated by PCR.

Results: The total number of blood donors was 13,435. Serologically non reactive cases but MP-NAT reactive was eight cases. Seven cases were discriminated by PCR as four HBV cases, two HCV cases and one HIV case. These seven cases became sero-positive when reevaluated after three to six months. So they were considered to be diagnosed in the window period. One case was sero-negative but NAT reactive, non reactive by PCR and persisted sero-negative when followed-up after three, six and twelve months. This case was considered as false positive.

Conclusion: The routine use of MP-NAT multiplex assay for detection of HBV-DNA, HCV-RNA, and HIV-RNA should be mandatory for all sero-negative donor blood to reduce the serologic window phase and increase the safety for the patients. In spite of NAT false positive and cost effective drawbacks should be considered.

Key Words: *Nucleic acid test – Multiplex NAT – HIV – HBV – HCV – Blood donor's safety – Transfusion Transmitted Diseases.*

INTRODUCTION

In October 2004, the implementation of HIV-RNA and HCV-RNA NAT examination of donor blood was added to the FDA guidance [1]. It was found to detect infection earlier than antibody or antigen assays. Also the FDA permitted discontinuation of HIV-1 p24 antigen testing with implementation of a licensed HIV-1 NAT assay [2]. Until December 2010, HBV-DNA NAT assay was not required by the FDA or recommended by the AABB. Some blood banks have implemented automated multiplex assays that screen for HIV-RNA, HCV-RNA, and HBV-DNA simultaneously [3]. In recent years, fully automated NAT systems have been developed. The FDA approved two manufacturers' automated triplex (HIV-1/HCV/HBV) nucleic acid testing (NAT) assays, used either in mini-pools (MP-NAT) of 6 or 16 donations, or for testing individual donations (ID-NAT) that detect HIV-RNA, HCV-RNA, and HBV-DNA in one reaction chamber.

Objective:

This study aimed for the detection of the advantages and limitations of the routine use of mini-pool Nucleic Acid Testing (MP-NAT) for the detection of HBV-DNA, HCV-RNA, and HIV-RNA in one chamber for sero-negative donor blood.

MATERIAL AND METHODS

With retrospective study, for all blood donors, in Almana General Hospitals, Saudi Arabia (SA), between January 2008 to April 2011. Blood donors were examined by serological assays for HBV (HBsAg, and HBc-Antibody), anti-HCV, and HIV Ag/Ab Combo (anti HIV-1, anti HIV-2 and HIV p24) by ARCHITECT system (i-1000, Abbott). Sero-negative donor blood for HBV, HCV, and HIV were then examined by mini-pool Nucleic acid test (MP-NAT) multiplex assay (Cobas, S-201 system, TaqScreen Multiplex (MPX) Test, Roche).

TaqScreen Test is a qualitative multiplex test that enables the screening and simultaneous detection of HIV-1 Groups M and O RNA, HCV RNA and HBV DNA in infected pooled and individual plasma specimen donations. The Cobas TaqScreen MPX Test uses a generic nucleic acid pre paration technique on the Cobas AmpliPrep Instrument. HIV-1 Groups M and O RNA, HCV RNA and HBV DNA are amplified and detected using automated, real time PCR on the Cobas Taqman Analyser. The test incorporates an Internal Control for monitoring test performance in each individual test as well as the AmpErase (Uracil-N-glycosyl) enzyme to reduce potential contamination by previously amplified material (amplicon). The Cobas TaqScreen Test does not discriminate which virus is detected in a specimen. COBAS Ampliscreen HIV Test v1.5, COBAS Ampliscreen HCV Test v 2.0 and Cobas AmpliScreen HBV Test were used for discrimination of HIV, HCV and HBV respectively. Minipool of 5 samples were prepared and examined for HBV-DNA, HCV-RNA, and HIV-RNA in one chamber according to the manufacture instructions. NAT reactive pools were then resolved to the single donation. Individual donor NAT reactive samples were discriminated by PCR- based diagnostic assay (COBAS Ampliscreen, Roch). Also the NAT reactive donors were followed-up after 3-6 months by serological testing for HBV, HCV and HIV.

RESULTS

From January 2008 to April 2011, blood donors were 13,435. Their age range was between 20 to 59 years old with median age of 35 years. Male: Female ratio was 10:1. Serological assays revealed reactive cases of HBV in 71 (0.53%), HCV in 112 (0.83%), and HIV in 39 (0.29%) donors. Serologically non reactive cases (for HBV, HCV and HIV) but MP-NAT reactive were 8 cases. Seven cases were discriminated by PCR as four HBV cases, two HCV cases and one HIV case. These 7 cases became seropositive when re-evaluated after 3 to 6 months. So these 7 cases were considered to be diagnosed in the window period. One case was sero-negative (for HBV, HCV, and HIV) but NAT reactive and non reactive by PCR for HBV, HCV or HIV and persisted sero-negative when followed-up after 3,6 and 12 months. This case was considered as false positive.

DISCUSSION

Since 1990, the national strategy to eliminate hepatitis B virus (HBV) infection in Saudi Arabia has included obligatory administration of HBV vaccine to all infants. The prevalence of hepatitis B surface antigen (HBsAg) among children before this program was reported to be 6.7% [4]. Nowadays, the prevalence of HBV is 0.22%. The prevalence varied by region, ranging from 0.03% to 0.72% with a mean prevalence of 0.15% [5]. In a recent study, in the Eastern Province-Saudi Arabia (SA), the incidence of HBV infection was found to be 0.5%. Similarly, HCV prevalence in Saudi Arabia varies in different provinces being highest in the Western and Southern provinces [7]. It was reported to be 0.6% in Eastern Province [6]. In this current study the prevalence of HCV infection was 0.83%. The latest statistics of the Ministry of Health (MOH) on the numbers of AIDS patients in Saudi Arabia revealed cumulative number of all AIDS cases detected since 1984 and until the end of 2009,15,213 cases including 4,019 Saudis, and 11,194 non-Saudi (i.e. The proportion of non-Saudis represent almost three times the Saudis). A woman to men ratio is 1:4 [7]. Human immunodeficiency virus infection prevalence in Saudi Arabia was shown to be 0.02% in a previous study [8]. In a current study it showed to be 0.29% which is much higher than general Saudi population study. This may be attributed to regional variation or due to that Eastern Province being too close to Bahrain which is a free country.

On October 2004, the implementation of HIV-RNA and HCV-RNA NAT examination of donor blood was added to the FDA guidance [1]. These were found to detect infection earlier than antibody or antigen assays. Also the FDA permitted discontinuation of HIV-1 p24 antigen testing with implementation of a licensed HIV-1 NAT assay [2]. Until December 2010, HBV-DNA NAT assay was not required by the FDA or recommended by the AABB. Some blood banks have implemented automated multiplex assays that screen for HIV-RNA, HCV-RNA, and HBV-DNA simultaneously [3]. In recent years, fully automated NAT systems have been developed. The FDA approved two manufacturers' automated triplex (HIV-1/HCV/HBV) nucleic acid testing (NAT) assays, used either in mini-pools (MP-NAT) of 6 or 16 donations, or

for testing individual donations (ID-NAT) that detect HIV-RNA, HCV-RNA, and HBV- DNA in one reaction chamber. These recent FDA licensures may offer an opportunity to further reduce the risk of transfusion-transmitted infection. Furthermore, it has been estimated that ID-NAT screening would minimally increase detection of infected donors, whereas the associated testing cost would be significantly increased [9]. An additional important concern is that donors would be deferred for false-positive results much more frequently with ID-NAT screening than MP-NAT screening [2]. Both licensed assay systems appeared to perform adequately in terms of analytical sensitivity and specificity, and when applied to contemporary US donors they generate incremental yields of 1:300,000 to 1:600,000 HBV DNA-positive donations not detected by current serological tests (HBsAg and anti-HBc). This rate is similar to the yield rate of HCV MP-NAT, and substantially higher than that for HIV MP-NAT. The HBV yield donations tend to contain low copy numbers of HBV genome that are not detected by currently available ultrasensitive HBsAg assays [10].

Transfusion- transmission of HIV, HCV, and HBV is now so rare that the rate of transmission cannot be measured by prospective clinical studies. The primary cause of residual transmissions however is thought to be related to donations made by individuals in the window period of early infection, before serological test results are positive [2].

The FDA requires donor screening for HBsAg and for anti-HBC (IgM and IgG antibody). It is difficult to estimate the HBV residual risk of transmission from donor because neither the window period duration nor the incidence for donor HBV infection is precisely known. The duration of the infectious window period before HBsAg has recently been estimated at 30 to 38 days. A recent publication estimated US HBV transfusion-transmission risk to be between 1/280.000 [11,12] and 1/357.000 [13] units. Also current donor screening for HIV includes serologic testing for antibodies to HIV-1 and HIV-2 (both IgG and IgM) and NAT testing for HIV-RNA. This gives risk of HIV transmission of 1 of 1.5 million for a unit of blood obtained from general donor population, but the risk is too much higher in high risk donor (1 of 4100) which could be missed by current screening methods despite the short window period due to inclusion of donors with high risk of acquiring HIV [14,15]. Current FDA donor screening for HCV includes NAT testing for HCV RNA and serologic testing for antibodies to HCV. The average window period between exposure and detection of infection by MP-NAT is estimated to be 7.4 days [9]. The serologic test detects only IgG antibody, a relatively late marker of infection, and therefore they may be a significant lag (1.5 to 2 months) between detection of RNA and detection of antibody [16]. The current estimated US risk of HCV transmission by transfusion after application of NAT testing is extremely low approximately 1 in 1.1 million. Accordingly, questioning of donors for risk to minimize window-period donations continue to be critical for preserving blood safety [2].

Overtime, the window periods have been shortened by implantation of donor screening tests that detect earlier infection. However, because there are no tests that will give a positive result instantly after an individual acquires an infection, the window period remains. With mini-pool NAT (MP-NAT), the average duration of the window period for HIV and HCV infections is estimated to be 9.4 and 7.4 days, respectively [9]. The window period for HBV is longer. The use of NAT assay allows the differentiation between new infections and established infections and it has been found that new HIV and HCV infections are two to four folds more common among first-time donors compared to repeat donors [13,14,17]. Current study showed 7 cases out of 13,435 blood donor diagnosed during the window period (negative serological tests with positive NAT assay). Discrimination of these 7 cases showed HBV, HCV, and HIV infected donor in 4,2,1 cases respectively. Those 7 cases became sero-positive when followed-up after 3 to 6 months so this confirms their diagnosis early after few days of exposure to infection and during the window phase.

Blood donations collected at the National Blood Center, the Thai Red Cross Society, Bangkok, in 2007 were tested by nucleic acid amplification technology (NAT) using the Chiron TIGRIS/Procleix Ultrio test and the Roche Cobas s 201/cobas TaqScreen multiplex (MPX) test. The sensitivity, specificity, and robustness were determined by testing 486,676 sero-

negative blood donations. Samples from each day of collection were divided into two sets; the odd-numbered samples were tested individually on the TIGRIS and the even-numbered samples were tested in pools of 6 on the Cobas s 201. The status of reactive samples was confirmed by duplicate testing of samples from the plasma bag to calculate the test specificity. Reactive samples were tested on the alternate system and followed-up. The analytical sensitivity of both systems met the 95% limits of detection claimed by the respective package inserts. No cross contamination was seen with either system. Test specificity was 99.93 and 99.90% for the Procleix Ultrio and Cobas TaqScreen tests, respectively. The NAT yield rates for human immunodeficiency virus Type 1 (HIV-1), hepatitis C virus (HCV), and hepatitis B virus (HBV) were 1:97,000, 1:490,000, and 1:2800, respectively. Several occult HBV donors, the majority of whom were detected by both tests, were also identified. The HIV-1 and HCV window cases were detected with both tests. The performances of the systems and tests indicated that both were acceptable for routine NAT by the National Blood Center, the Thai Red Cross Society. However, the Procleix Ultrio test appeared to be less sensitive than the cobas TaqScreen test for HBV [18].

In a pilot study in Taiwan among 10,727 sero-negative donations, 12 HBV NAT yield cases (0.11%) and one HCV NAT yield case (0.01%) were detected. Follow-up results for 1 to 8 months showed that the HCV yield case was a window case and all HBV NAT yield cases were occult carriers. The use of NAT detected occult HBV and reduced HCV window period. The yield rate, especially occult HBV, was 10 to 100 fold higher than that in developed, HBV non-endemic countries. Therefore, NAT implementation for routine donor screening in a more cost-effective manner would contribute to safer blood transfusion in Taiwan [19]. A one year pilot study conducted by the University of North Carolina in collaboration with the North Carolina Department of Health and Human Services (NC Study) showed that testing HIV serologically negative individuals using NAT can help in the early identification of primary HIV infection. The NC Study performed NAT in pools of 90 samples on more than 100,000 HIV serologically negative samples tested by the state, with a positivity rate of approximately one in 5,000 screened samples [9]. South African Blood service screening 732,250 donations by Individual donor-NAT (ID-NAT) showed 16 HIV, 20HBV, and one HCV window phase donations [20].

In current study, one other case was sero-negative for HBV, HCV and HIV but showed MP-NAT reactive. This case was non reactive for HBV, HCV or HIV infection when tested by the discrimination PCR-based assay and also when followed-up after 3,6,12 months where no sero-conversion happened. This case was considered as false positive result which is the main drawback of the use of NAT assay. In a retrospective survey done in China, NAT was used to analyze 28,800 HBsAg-negative samples by ELISA from blood donors in Dongguan city from August, 2006 to August, 2007 with Roche Cobas AmpliScreen systems; and follow-up research including NAT for HBV-DNA, ELISA for HBsAg and multiple factors analysis for HBV infection was carried out on HBV NAT screening-positive crowd. Ten positive pooling were screened from 28,800 samples; after further detection, 2 of these positive pooling were HBV-DNA negative and 8 HBV-DNA positive samples were found. They concluded that NAT is more sensitive than ELISA in screening HBV, but the probability of being false positive of NAT cannot be ignored at the same time. On the other hand, only screening HBsAg for HBV is a relative limitation in high infection region of China [21].

Steven Kleinman [10] states that it is reasonable for FDA licensed blood establishments to implement HBV MP NAT on a voluntary basis until the FDA mandates such testing. This mandate should be consistent with FDA-approved labeling of the two manufacturers' tests that allows NAT in MPs of up to 6 or 16 donations. There is no benefit to smaller MP sizes from either modeling studies or clinical trials. We recognized the potential benefit of MP NAT for HBV, and therefore believe that this test should be adopted. As a final comment, the absence of effective reimbursement mechanisms by which hospitals can recover the increased costs of blood safety initiatives, implemented voluntarily or after an FDA recommendation, remains a serious flaw in the regulatory process. HBV NAT is an example of such an initiative that will come as an unfunded mandate if FDA recommends its use.

In conclusion, the routine use of MP-NAT multiplex assay for detection of HBV-DNA, HCV-RNA, and HIV-RNA should be mandatory for all sero-negative donor blood to reduce the serologic window phase and hence reduce the incidence of transfusion transmission of viral infection and increase the safety for the patients. It should be considered in spite of NAT false positive and cost effective drawback.

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GSTM1 Genotype as a risk Modifier of MDR1 C3435T-Induced Risk Susceptibility to Pediatric Acute Lymphoblastic Leukemia

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ABSTRACT

Background: GSTM1 and GSTT1 appear to be associated with a modest increase in the risk of acute lymphoblastic leukemia (ALL). MDR1 C3435T polymorphism was also suggested as a risk factor for childhood ALL; individuals with TT allele have lower expression of P-gp than those with CC genotypes and hence less capable of extruding toxic substances and carcinogens.

Objectives: To investigate the impact of MDR1gene C3435T polymorphism and both GSTM1 and GSTT1 polymorphisms, separately and in combination, on risk susceptibility to childhood ALL.

Patients and methods: The study included 94 children with ALL and 314 apparently health control subjects. Multiplex-Polymerase chain reaction (PCR) was used to evaluate GSTM1 and GSTT1 status while PCR-restriction fragment length polymorphism (PCR-RFLP) was used for the detection of MDR1 C3435T single nucleotide polymorphism.

Results: There was no significant effect of either GSTM1 null or GSTT1 null variant allele or both of them combined on susceptibility to ALL. On the other hand, MDR1 CC, CT and TT genotype frequencies in childhood ALL patients were found to be 78.0%, 17.1% and 4.9%, respectively vs. 91.2%, 8.8% and 0 % in the control group (*p*=0.016). MDR1 gene C3435T homozygote and heterozygote have a 2.9 fold increased risk to develop ALL (OR=2.918, 95% CI:1.193-7.137,). There is a significant synergistic association between GSTM1-null allele and mutant MDR genotype homozygous TT or heterozygous allele CT on susceptibility to ALL with a 3.672 fold increased risk (*p*-value. 0.032 OR=3.672, 95% CI:1.059-12.733), however the presence of GSTM1 abolished the effect of mutant MDR1 allele on risk susceptibility to ALL (*p*-value. 0.193). In conclusion, the increased risk to develop pediatric ALL associated with MDR1 gene C3435T homozygote and heterozygote is further potentiated by the presence of GSTM1 null and abolished by the presence of GSTM1wild. Molecular genetic analysis is still required to understand genotype-genotype interaction and to clarify genotype-phenotype relation and their reflection on disease risk.

Key Words: *Glutathione s-transferase – GSTM1 – GSTT – MDR1 – Combined genotype polymorphisms – Acute lymphoblastic leukemia.*

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is a frequent malignancy affecting both children and adults. Despite much investigation, the causes are not yet fully understood. Like many other cancers, acute leukemia is considered to be a complex disease, which is determined by a combination of genetic and environmental factors [1,2]. There is increasing evidence that predisposition to acute leukemia is associated with exposure to chemicals such as benzene and chemotherapeutic agents [3,4]. Glutathione S-transferases (GSTs) are a family of cytosolic enzymes involved in the detoxification of various exogenous as well as endogenous reactive species [5,6]. GSTs function as dimers by catalyzing the conjugation of mutagenic electrophilic substrates to glutathione. In humans, 4 major subfamilies of GSTs can be distinguished and are designated as GSTα, GSTμ, GST0, and GSTπ. Within the GSTμ subfamily, the gene coding for GSTM1 exhibits a deletion polymorphism which in case of homozygozity (GSTM1 null) leads to absence of phenotypic enzyme activity [7]. Similar mechanism is described for GSTT1 within the GST0 subfamily [8]. Variant GST alleles have been identified within the general population. The most extensively studied variant GSTs include two GST deletion alleles namely GSTM1*0/*0 and GSTT1*0/*0 [9]. The functional consequences of the GSTM1 and GSTT1 (*0/*0) genotypes are obvious in terms of enzyme activity; gene deletion results in loss of conjugation potential. Molecular epidemio-

logical studies indicate that individuals lacking the genes GSTM1 and GSTT1 are more likely to develop cancer than those having these genes [6]. Based upon this meta-analysis of 30 case-control studies, GSTM1 and GSTT1 appear to be associated with a modest increase in the risk of acute lymphoblastic leukemia (ALL). It is conceivable that GSTM1 and GSTT1 null genotypes may thus play a role in leukemogenesis. The pooled analysis of both GSTM1 and GSTT1 null genotypes produced a similar risk estimate [10]. Thus, some studies indicate that GST polymorphisms are associated with ALL; however, this association is not accepted across all observational studies. Discrepancies may be partially attributed to failure to consider gene combinations or interactions with environmental factors. Greater understanding of the numerous factors affecting GST expression and activity, accompanied by more incisive genetic analysis, may reveal further connections between GST genotypes and ALL risk [11].

The multi-drug resistance 1 gene (MDR1) which belongs to the family of ABC transporter proteins, encodes P-glycoprotein (P-gp). P-gp is a membrane-associated protein that acts as an ATP-dependent pump involved in the membrane transport of various substrates including toxic xenobiotics [12-15] and it thus has a protective function in various cells and tissues/organs [16]. Several polymorphisms of this gene have been characterized. Several studies suggested that MDR1 C3435T polymorphism was a risk factor for childhood ALL. Carriers of the TT genotype are more at risk of developing ALL than other individuals, whereas CC genotype carriers are supposed to have worse prognosis [17-20].

To the best of our knowledge, there is no study addressing the potential synergistic effect of GSTM1 and/or GSTT1 Null and MDR1 c>T polymorphism risk susceptibility to pediatric ALL. In this study we examined GSTM1 and GSTT1status and C3435T polymorphism in 94 pediatric ALL cases and 314 apparently healthy controls to verify if any of their impact on risk susceptibility to ALL separately and in combination.

PATIENTS AND METHODS

The study included 94 newly diagnosed Pediatric ALL patients who presented to the Pediatric Oncology department, NCI, Cairo University and 314 apparently healthy controls blood bank donors. We chose adults to avoid ethical concerns of using children as control taking in consideration that genotype is not affected by age. Diagnosis was performed according to clinical, morphological, cytochemical and immunophenotypic examination. The IRB of the NCI, Cairo University approved the study and written informed consent was obtained from all participants or their guardians.

The criteria for inclusion in patient's group were:

1- Egyptians origin residing in Egypt area as judged by their names, languages and places of birth.

2- Availability of biological material.

The recruited patients comprised 58 males and 36 females between the ages of 1.5 and 18 years with a median of 6 years.

The criteria for inclusion in the control group were:

1- Anonymous, healthy, and unrelated individuals.

2- Egyptians origin residing in Egypt area as judged by their language and place of birth.

Genotyping:

DNA isolation: DNA was isolated from peripheral blood at diagnosis using Qiagen column Kit (USA) according to the manufacturer’s recommendations. DNA concentration was determined by measuring the optical density at 260 nm and the purity of the nucleic acid (the absence of proteins contamination) was determined by the ratio of absorbance at 260 nm to the absorbance at 280 nm. The ratio of 260/280 should be 1.7-2.0 [21].

Genotyping for MDR1 C2334T was performed by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method and GSTM1 and GSTT1 were performed by multiplex PCR with the housekeeping gene B-globin as internal control.

GSTM1 polymorphism:

The polymorphic deletion of the GSTM1 gene was genotyped using the multiplex PCR of GSTM1 Primers with ß-globin housekeeping gene used as internal control as described by

[22]. PCR was performed in 20 µL reaction mix containing 20 ng of genomic DNA, 0.5 µmol/L of each primer, 200 µmol/L of each dNTPs, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, and 0.5 U of ampliTaq DNA polymerase (Hoffman-LaRoche, Branchburg, NJ). After initial denaturation for 10 minutes at 95°C, the PCR was performed for 35 cycles of 1 minute at 94°C, 1 minute at 59°C, and 2 minute at 72°C. The last elongation step was extended to 7 minutes. Negative and positive control samples were included in each amplification series. The amplicon was analyzed by electrophoresis on a 3-4% agarose gel, at 100 volt for 30 min. The presence of GSTM1 allele, identified by a 219-bp fragment indicates wild type while its absence indicates complete deletion (null genotype) [23].

GSTT1 Polymorphism:

The polymorphic deletion of the GSTT1 gene was genotyped using the multiplex PCR of GSTT1 Primers and ß-globin housekeeping gene as internal control [22]. The amplification reaction was performed in 20 µL reaction mix, containing 20 ng of genomic DNA, 0.5 µmol/L of each primer, 200 µmol/L of each dNTPs, 2.0 mmol/L $MgCl_2$, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, and 0.5 U ampliTaq DNA polymerase (Hoffman-LaRoche). After initial denaturation for 10 minutes at 95°C, the PCR was performed for 35 cycles of 1 minute at 95°C, 1 minute at 59°C, and 2 minute at 72°C. The last elongation step was extended to 7 minutes. Negative and positive control samples were included in each amplification series. The amplicon was analyzed by electrophoresis on a 1.5% agarose gel, at 100 volt for 30 min. The presences of GSTT1 alleles, identified by a 480-bp fragment indicate wild type while its complete deletion indicates null genotype [23].

MDR1:

MDR1 C3435T mutation was determined using PCR-RFLP assay [24]. The PCR products were digested at 37 C° using 4 units Sau3AI restriction endonuclease (New England BioLabs, UK). The restriction fragments obtained were separated by electrophoresis on a 3% agarose gel for 45 min at 140V and analyzed after staining with ethidium bromide under ultraviolet light. The 3435-C (wild type) allele can be detected by the presence of two fragments, which are 158 bp and 39 bp long. The presence of 3435-T (Mutant type) allele results in uncut amplified segment of 197 bp; the presence of a heterozygous genotype results in the presence of all three bands. The primer design was based on published sequences (Table 1).

Table (1): The oligonucleotide primer pairs used for amplification of the candidate genes.

Gene/ primer	Primer sequence	Reference
GSTM1 forward	GAA CTC CCT GAA AAG CTA AAG C	23
GSTM1 reverse	GTT GGG CTC AAA TAT ACG GTG G	
GSTT1 forward	TTC CTT ACT GGT CCT CAC ATC TC	23
GSTT1 reverse	TCA CCG GAT CAT GGC CAG CA	
ß-Globin forward	ACA CAA CTG TGT TCA CTA GC	23
ß-Globin reverse	CAA CTT CAT CCA CGT TCA CC	
MDR1 Forward	TGTTTTCAGCTGCTTGATGG	24
MDR1 reverse	AAGGCATGTATGTTGGCCTC	

Statistical analysis:

Statistical analysis was performed using SPSS 15.0 (Statistical Package for the Social Science). The level of significance was calculated by Fisher's exact test. Odds Ratio (OR) was used to measure the strength of association between the tested genotypes and ALL risk. Crude ORs are given with 95% Confidence interval (CI). All of the statistical tests were based on two-tailed probability.

RESULTS

Glutathione S transferase:

The frequencies of the GSTM1 wild allele was 46/94 (49%) among patients compared to 141/314 (44.9%) among controls. The frequency of the GSTM1 deleted (Null) allele was 47 (51%) among patients compared to 173 (55.1%) among controls; the difference was found to be statistically insignificant (p-value=0.553). No significant association was observed between GSTM1 genotype and ALL development.

The frequency of the GSTT1 present allele was 76 (82.6%) among patients compared to

257 (86%) among controls. No significant association was observed between GSTT1 genotype and ALL development. The frequency of the GSTT1 deleted (null) allele was 16 (17.4%) among patients compared to 42 (14%) among controls; the difference was found to be statistically insignificant (*p*-value=0.430).

The GSTM1 and T1 genotypes were double deleted in 4 ALL patients (4.4%) cases compared to 25 normal controls (8.4%); the difference was found to be statistically insignificant, *p*-value=0.069.

The MDR C3435T genotypes distribution among the study samples of 80 childhoods ALL patients in comparison to 91 normal healthy controls. The frequency of the MDR C/C, C/T and T/T alleles were 64 (78 %), 14 (17.1%) and 4 (4.9%) respectively among patients compared to 83 (91.2%), 8 (8.8%) and 0 (0%) among controls as shown in (Table 2). MDR 3435 CT Heterozygous and TT Homozygous genotype polymorphism is significantly associated with childhood ALL (*p*-value=0.016) which result in increased risk of ALL by 2.91 folds. Also, T allele was significantly high in Children All (13.4%) as compared to control (4.4%), *p*=0.035. The risk to ALL increased in ALL patients (OR= 3.37).

When we studied the frequency of both MDR 3436 CT/TT genotypes and null GSTM1 genotype we found a significantly higher frequency in ALL patients than controls (*p*=0.032) (Table 3). These combined genotypes showed an increased risk of ALL by 3.67-folds.

Table (2): MDR1 C3435T genotyping and allele frequency among ALL cases and Controls.

MDR1 C3435T	C/C		C/T		T/T		C/T+T/T		C allele	T allele
	No	%	No	%	No	%	No	%	%	%
Control: n=91	83	91.2	8	8.8	0	0	8	8.8	95.6	4.4
ALL: n=82	64	78	14	17.1	4	4.9	18	22.0	86.6	13.4
p			0.078				0.016*		0.035	
OR			2.270				2.91		3.37	
95% CI			0.897-5.740				1.193-7.137		1.029-11.038	

Table (3): MDR1 C3435T combined to GSTM1 or GSTT1deleted genotypes among ALL and control groups.

Gene	Genotype	ALL (n=56)		Controls (n=67)		*p*-value	OR	95% CI
		N	%	N	%			
MDR1C3435T and GSTM1 null	CT+TT+M1null	10	23.8%	4	.8	0.032*	.672	1.059-12.73
	CC+M1present	32	76.2%	47	92.2			
	Total	42	100	51	100			
MDR1C3435Tand GSTT1null	CT+TT+T1 null	6	42.9	2	12.5	0.101	5.250	0.850 - 32.430
	CC+T1present	8	57.1	14	87.5			
	Total	14	100	16	100			
MDR1C3435Tand GSTM1 present	CT+TT+M1present	8	20.5	4	10	0.193	2.323	0.638 - 8.461
	CC+M1 present	31	79.5	36	90			
	Total	39	100	40	100			
MDR1C3435Tand GSTT1 present	CT+TT+T1 present	12	17.6	6	8	0.082	2.46	0.870 - 6.982
	CC+T1 present	56	82.4	69	92			
	Total	68	100	75	100			

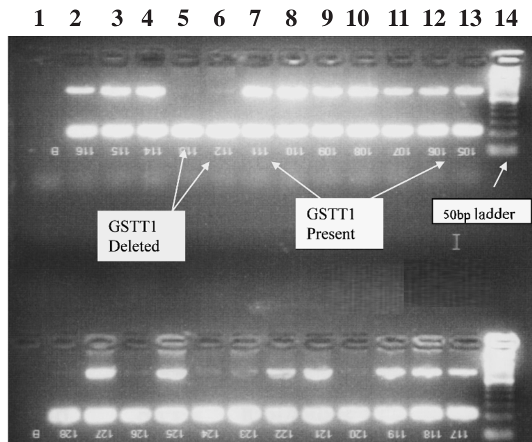

Fig. (1): GSTT1 and B-Globin in multiplex-PCR Lane 1: No amplification. Lane 2-4, 7-13: GSTT1Present (480 bp) + B-Globin (110 bp). Lane 5, 6: GSTT1 Deleted (B-Globin110 bp). Lane 14:50 bp marker.

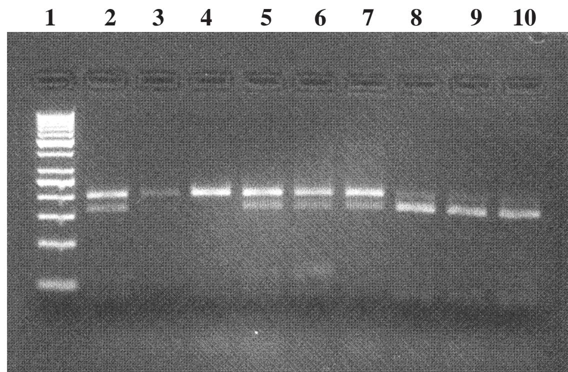

Fig. (2): MDR1 C3435T polymorphism after Sau3AI Digestion Lane: 50 bp DNA ladder. Lane 2: Heterozygous genotype. Lane 3 & 4: Homozygous. Lane 5-7: Heterozygous. Lane 8-10 Wilde type.

DISCUSSION

In our study, GSTM1 null and GSTT1 null genotype has no effect on the risk of ALL. Our findings are in agreement with Chen et al. [25]. In their study, the GSTM1 null genotype was detected in 55.2% of white colored children with ALL and 53.5% of normal controls, and the GSTT1 null genotype was found in 14.1% of white colored children with ALL and 15.0% of their controls. In our study, deletions in GSTM1 and GSTT1 genotypes either separately or in combination were not found to be significantly associated with an increased risk of acute leukemia. Thus, combined GSTM1 and GSTT1 genotypes did not show any synergistic effect on pediatric ALL susceptibility. This is contrary to many studies [9,10,26-28] which reported that individuals with null GSTM1/GSTT1 genotypes may have an impaired ability to detoxify carcinogens, thus, carrying an increased risk of developing cancer. Arruda et al. [2] observed a 4.7-fold (95% CI: 2.1-11.0) and 2.3-fold (95% CI: 1.0-5.2) increased risk of acute myeloid leukemia with the GSTM1 and GSTT1 null genotypes, respectively, and a 6.6-fold (95% CI: 2.4-7.9) increased risk with the combined null genotype. Although many studies have shown significant association of GST polymorphisms with acute leukemia, others have shown contradictory results [10]. This may be attributed to many factors like different ethnic group and exposure to different types of carcinogens in different environments which means different gene-environment interaction. Also sample size may affect the results of the study. Therefore, studies with large sample sizes will reflect much better the association of genotypes and cancer risk since they provide statistical power. Meta-analyses studies have indicated statistically significant but small increases in risk for specific genotypes, while many studies have been negative [10]. However, the genetic analysis used in most of these studies has been limited, especially by the failure to discriminate between heterozygous and homozygous deletion genotypes (gene dose). It is also well known that humans express a large number of different GSTs with overlapping substrate specificities, and the effects of polymorphisms (including gene deletions) affecting one GST may be masked by the activity of others. On the other hand, GST activity is highly variable among individuals, but genetic factors may account for only a fraction of this variability [29,30]. Factors such as diet [31], environmental chemical exposures [32], age [33], or gender [34]; that remain only poorly understood; may be more important determinants. Nevertheless, our understanding of human GST polymorphisms is still incomplete which hardens any interpretation of contradictions in literature relevant to clinical aspects.

In our work, MDR1 C3435T gene polymorphism showed significant impact on the risk susceptibility to pediatric ALL. In the present study, MDR1 C3435T heterozygous (CT) and homozygous (TT) genotypes was associated with 2.91-fold increased risk of childhood ALL while MDR1 3435T allele frequency was associated with 3.37 fold increased risk. The risk is in line with previous findings showing that patients with MDR1 C3435T TT-allele might

be at a higher risk of development of ALL than those with C3435T CC-allele [17-20].

The potential mechanism of the interaction of MDR1 polymorphisms with GSTM1 and GSTT1 in modifying risk susceptibility to childhood ALL has not yet been satisfactorily described. In the present study, we have compared the frequency of MDR1 C3435T combined with GSTM1 and/or T1 polymorphism in childhood ALL patients and healthy controls in an attempt to identify a possible impact of combined gene variation on the susceptibility to ALL. The MDR 3436TT genotype combined to GSTM1 null genotype showed a significantly higher frequency in ALL patients than controls (p=0.032). With the complex metabolic pathways of xenobiotics, synergistic or antagonistic interactions between genotypes are of great interest as they affect phenotypic features In our work, MDR T allele carriers when associated with deleted GSTM1 genotype, showed significantly higher risk than CC genotype individuals. We found a 3.67-fold increased risk of ALL when MDR C3436T (CT/TT) genotypes are combined with GSTM1 null allele compared to 2.91 fold increased risk of All in MDR genotype alone. On the other hand, the presence of both GSTM1 and GSTT1 abolish the risk effect of the variant allele of MDR1 C3435T gene polymorphism. This means that when the GST M1 and T1 enzyme activity are normal they can overcome the decreased efflux due to the presence of variant allele of MDR1 C3435T polymorphism and detoxify carcinogens.

In contrast to our findings, MDR1 polymorphisms have not been shown to be associated with overall risk of ALL in previous studies [35,36]. However, in one of these studies, the patient sample size was 44 [35]. The association of MDR1 C3435T gene polymorphism with incidence of different diseases is yet controversial. However, the genotype TT was shown to be associated with more than two-fold lower duodenal P-gp expression levels compared with CC genotype reducing the rate of efflux of carcinogens. There are several hypotheses regarding the influence of this polymorphism on phenotypically revealed features. One hypothesis assumes a change of the substrate's affinity to the P-gp transporter [37]. These authors showed that the polymorphism alters the substrate specificity in mammalian membrane transport protein affecting the timing of co-translational folding and may result in altered function. Other authors suggested that MDR1 C3435T is a silent polymorphism which leads to a more unstable mRNA and consequently, lowering overall activity of the variant allele [38].

The very large MDR1 gene includes 28 exons and is highly polymorphic which makes it difficult to identify causal polymorphisms. In addition, linkage patterns and allele frequencies in MDR1 are highly variable between different ethnic groups and thus between the studied populations. Hence, case-control studies with assessment of multiple polymorphisms in parallel with P-glycoprotein activity, mRNA and protein level measurements will be required to understand MDR1 genotype-phenotype relation.

In conclusion, the present study indicated that the studied GSTM1 and GSTT1 polymorphisms are not associated with risk of ALL, whereas MDR polymorphism was found to increase the risk of childhood ALL.

To our knowledge, this is the first study showing combination of GST polymorphisms affecting detoxification reaction and MDR genotypes controlling the efflux of carcinogens in association with childhood ALL. This gene interaction synergistically increases the susceptibility to childhood ALL in homozygote or heterozygote MDR1 C3435T variant type carriers associated with null GSTM1 polymorphism while the present GSTM1 and T abolish this risk effect. The present results stress the importance of molecular genetic analysis of combined genotypes with different role in carcinogenesis in stratifying individuals at increased risk of ALL.

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