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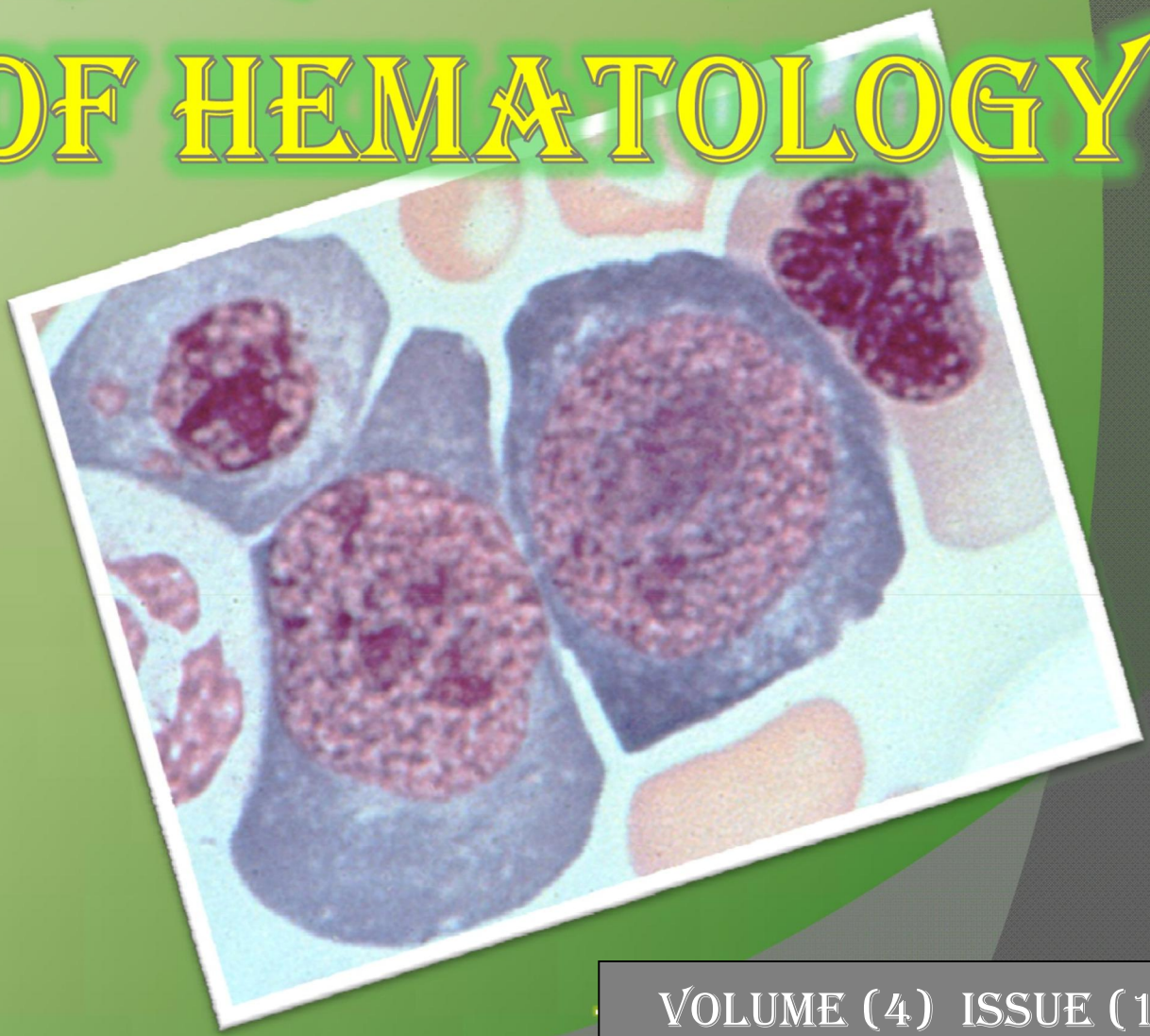

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Efficacy of combination of rituximab therapy with chlorambucil plus prednisolone (R-LP) Protocol as treatment line in chronic lymphocytic leukemia patients

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ABSTRACT

Background: Chronic lymphocytic leukemia (CLL) remains an incurable disease with variable course. It is typically responsive to several courses of chemotherapy.

Objectives: Evaluation of efficacy of combination of rituximab with chlorambucil plus prednisolone (R-LP) as first line of treatment of CLL patients whom are not fit for treatment with fludarabine combination therapy.

Patients & Method: Over 17 months duration, thirty patients with CLL were included in this a cohort prospective study. All of them were received 8 cycles combinations of rituximab (day 1) plus chlorambucil plus predinsolon (day1 to day 5). It had been used for those couldn't receive or those having intolerance to fludarabin based therapy. At end of courses , re-assessment had performed & included clinical examination ,blood count in addition to BM examination for evaluation of response in term of overall response rate(ORR) ,duration of response(DOR), and treatment free interval(TFI).

Results: Overall response rate (ORR) reported in 86.67% of patients. Both anemia &bone marrow lymphocyte percentage has significant relation to the treatment response (p=0.051, p=0.036 respectively) as well as positive direct antiglobulin test(DAT) & diffuse BM infiltration (p=0.033,p=0.04 respectively). Mean duration of response (DOR) is 9.23$\pm$0.50 months while mean treatment free interval (TFI) is 11.74$\pm$0.64 months. Bone marrow lymphocyte proportion is consistently predictive factor in long term remission durability in negative correlation(r = – 0.47, p= 0.04).

Conclusion: Rituximab plus chlorambucil and predinsolon (R-LP) combination therapy might be as good alternative regimen with equivalent response for CLL patients whom are nonsuitable for treatment with fludarabine combination therapy

Key words: chronic lymphocytic leukemia, rituximab, chlorambucil, treatment guidelines.

Introduction:

Indolent B cell lymphoid malignancies including Chronic lymphocytic leukemia (CLL) have many common characteristics like slow growth, a high initial response rate and a variable disease course besides the common cell of origin (mature B cell) [1,2] .

The Rai and the Binet staging system [3,4] are simple yet accurate predictors of survival and are widely used by clinicians and researchers.

The natural history of CLL is extremely variable and survival from initial diagnosis ranges from 2 to 20 years. The watchful waiting strategy is acceptable in most indolent B cell lymphoid malignancies including CLL .[2] ,however; it remains an incurable disease with an extremely variable course. [5]. As a result, therapy must be flexible and individualized for different patient groups [6]

Advances in the treatment of chronic lymphocytic leukemia (CLL) have improved initial overall response rates (ORR), complete response (CR) rates and progression free survival (PFS). [6]

Rituximab is a chimeric mouse anti-human CD20 monoclonal antibody. It is included in the treatment of chronic lymphocytic leukemia (CLL), low-grade or follicular lymphoma, and diffuse large B-cell lymphoma [7]

Combination of rituximab with fludarabine plus cyclophosphamide significantly improved ORR, CR and PFS both in untreated patients with CLL and in those with relapsed or refractory CLL, according to the results of two randomized, open-label, multicenter trials. [7] This improvement is directly resulted from an improved ability to eliminate minimal residual disease (MRD) [6,8]

For many years chlorambucil (LEUKRAN®) has been the standard treatment for CLL for 40 years, but it has not changed the natural history of the disease. [9]. It yields a small proportion of complete responses (5 – 10%) and improves symptoms, but the survival is only slightly, if at all, affected.

Because of this, chlorambucil is usually given to patients not able to tolerate more effective therapies. It should be noted that the doses of chlorambucil have varied widely in different studies and there is a clear indication of a dose – response curve. [10] Chlorambucil is still used as the treatment line in many current trials. Because of its relative safety it is still recommended for patients over the age of 70 years or those with comorbidities [6,9] .

The aim of this study is to evaluate the efficacy of combination of rituximab with chlorambucil plus predinsolon (R-LP) as first line of treatment of CLL patients whom are not fit for other standard first line therapy containing purine analogous.

Patients & Method

It is a cohort prospective study performed at hematology unit, Emammain kadhumain medical city throughout the period from Dec 2011 to May 2013 over 17 months duration.

It included 30 patients diagnosed to have CLL. The diagnosis based on clinical features & the combination of lymphocyte morphology on peripheral blood film as, there is >5 x 10^9/l of circulating mature looking lymphocyte cells persisting for >3 months with either characteristically more than 30% of the nucleated cells in the bone marrow aspirate are mature lymphoid cells [8] in addition to characteristic immunophenotyping markers that confirmed via either immune-histochemistry on bone marrow biopsy or flowcytometry on peripheral blood whenever available (which is defined according to the recommended scoring system allocates one point each for the expression of weak surface membrane immunoglobulins, CD5, CD23, and absent or low expression of CD 79b and FMC7) [11]

A marrow biopsy and aspirate were helpful in evaluation the factors contributing to cytopenias (anemia, thrombocytopenia) that may or may not be directly related to leukemia-cell infiltration of the marrow as well as for the type of marrow infiltration (diffuse vs. non diffuse) which reflects the tumor burden and provides some prognostic information.[8]

The following conditions had been excluded hairy cell leukemia, or leukemic manifestations of mantle cell lymphoma, marginal zone lymphoma, splenic marginal zone, lymphoma with circulating villous lymphocytes, or follicular lymphoma.

Patients data were reported and included age, genders, clinical manifestation, hemoglobin (Hb), white blood cells (WBC), lymphocyte proportion, and platelet count as well as BM aspirate lymphocyte percentage and pattern of distribution at biopsy (diffuse vs. non diffuse). Direct Coombs test (direct antiglobulin test-DAT-) had requested for each.

This study had been approved by local ethical committee for medical researchers at college of medicine Al-Nahrain University, and all patients were informed about therapeutic course &written consent had taken.

All patient were received rituximab (375mg/m2 at first cycle & 500mg/m2 at

subsequent cycles for day 1 only) every 28 day as intravenous infusion over 4-6 hours preceded by premedication (8) in addition to simultaneous use of combination chlorambucil (10 mg/m^2- per oral) plus prednisolone (60 mg/m^2- per oral) from day1 to day 5 as outpatient treatment [12] for 8 cycles.

This course was advised for all patients who couldn't commit to stay inside hospital to receive the classical approved course (due to personal or extraordinary circumstances) or those having intolerance to fludarabine based therapy, like positive DAT or allergy.

During the cycles of chemotherapy, all patients were monitored for any complication of treatments & managed accordingly.

At end of courses, re-assessment had performed and included clinical examination, blood count in addition to re-BM examination for evaluation of response in term of overall response rate (ORR), duration of response (DOR), and treatment free interval (TFI). The definitions of response used in the UK for CLL3 and CLL4 trials were broadly similar to the National Cancer Institute (NCI) 1996 guidelines. These definitions have been updated and clarified in the 2008 IWCLL guidelines [8].

CR requires normalization of blood counts and the bone marrow, whereas a PR requires the regression of at least 50% of organomegaly and lymphocyte counts. A bone marrow aspirate & biopsy are required to define the category of nodular PR by the presence of discrete or moderately large nodules of residual CLL [8]

Follow up monitoring every 2 months had advised & registered for all patient during the time of observation post chemotherapy

Those with failure of treatment had been shifted to another line of treatment.

SPSS version 14 program had been used for statistical analysis &included student t test , Fisher Exact test , Mann Whitney U test and correlation analysis considering $P< 0.05$ as significant.

Results

Demographic characteristics at initial presentation

The total number of patients was thirty. Nineteen (19/30) of them were female (Male: Female = 1:1.73). The mean age is 62.03$\pm$1.73 years (mean $\pm$SE). Its range between 45-76 years. Male patients reported higher mean age which is 64.4$\pm$2.1years.

The most common initial compliant was listed to be abdominal pain & heaviness (26.67%) as shown in frequency of clinical presentation description (table I).

Those eight patients (26.67%) presented with abdominal pain proved to have

massive splenomegaly despite presence of other 11(36.67%) patients who discovered to have mild to moderate clinically palpable splenomegaly. A radiological enlarged splenomegaly found in 6 (20.0%), while only 5 (16.67) patients found to have not enlarged spleen.

Investigations of the studied patients demonstrated that white blood cells (WBC) count varied between 19.8x 10^9/l - 230.0x10^9/l with mean of 78.42x10^9/l$\pm$11.38x10^9/l (the mean absolute lymphocyte count was72.0x10^9/l$\pm$ 2.2x10^9/l with range between 60.0x109/l -115.0x10^9/l)

Hemoglobin (Hb) mean level was 102g/l$\pm$4.1g/l in range between 60-142 g/l while platelet count mean was 141.93x10^9/l $\pm$8.53x10^9/l (65.0x10^9/l - 224.0x10^9/l)

At bone marrow (BM) aspirate, the mean lymphocyte percentage estimated as 64.13%$\pm$2.83% while BM biopsy formed mostly diffuse pattern infiltration in 53.3%(16/30) of studied group.

Direct Coombs (DAT) test was screened for all patients but only five showed positive results (16.67%) who were having severe anemia.

Treatment outcome at end of 8 cycles of R-LP courses

All patients had completed their 8 cycles of chemotherapy every 28 days. No serious adverse effects were demonstrated throughout follow up period. Response was assessed depending on clinical examination, laboratory evaluation and sonographic assessment before each cycle while BM study performed at the end of 8 cycles.

Overall response rate (ORR) reported in 86.67% of patients& included complete response in (CR) 50 %(15/30) with partial response (PR) in 36.67% (11/30). Four patients failed to show any primary response that necessitate changing to another protocol.

Concerning their clinical parameters, the age of patients showed no relationship to treatment outcome (p= 0.94) &similarly their gender (p=1.00) as well as the presenting symptoms whether accidentally or not (p=0.39) or the presence of clinically palpable splenomegaly (p=0.45).

Association between different laboratory parameters &treatment outcome revealed that both anemia &bone marrow lymphocyte percentage has significant relation to the outcome (p=0.051, p=0.036 respectively). Those with positive DAT showed worse outcome than their counterparts in statistical significant (p=0.033) as in (table II) while bone marrow diffuse pattern infiltration showed lower response rate to this regimen as most of them got either PR or Failure(68.75%)(11/16) (p=0.04).

Outcome at end of follow up period

Assessment of the long term outcome in terms of survival rate at the end 17 months of follow up in relation to their initial presentation & laboratory parameters demonstrates that both WBC, hemoglobin &positive DAT are associated significantly with long term outcome (p=0.05,p=0.03,p=0.04 respectively) in contrast to other parameters like platelet count or bone marrow lymphocyte percentage or even absolute lymphocyte number. Although the bone marrow pattern persists to be worse marker for long term outcome as 6/16(37.5%) reported death (p=0.051) during follow up (due to different reasons) (table III, table IV)

All other clinical factors don't showed any relationship with long term-outcome apart from age (p=0.03) unlike the gender (p=0.4), the clinical presentation (p=0.3) or the presence of massive splenomegaly (p=1.00)

In terms of correlation, it found that the bone marrow lymphocyte proportion is consistently predictive factor in long term remission durability in negative correlation {the higher the proportion ,the lower remission duration persistence & the earlier chance of relapse}(r = – 0.47, p= 0.04) unlike the rest of parameters (table V)

Discussion

Chronic lymphocytic leukemia (CLL), the most common form of adult leukemia [13,14]. During the last decade, the treatment approach to CLL has dramatically changed from palliative to potentially curative. The goal of treatment has shifted from controlling leukocytosis and disease-related symptoms to achieving eradication of minimal residual disease (MRD) [15]

In this study, the disease reported at mean age of 62.03±1.73 years which is slightly lower than what is published as the median age at diagnosis is between 65 and 70 years [11,13,16] or just similar to Stefano Molica report [17], while the females shown to be predominant affected gender here & this is unlike the reported gender ratio by others who identified that CLL more likely to affect male patients in average ratio of 2 [11,13],this variation may be due to different populations ratio.

Most of studied patient were complained of abdominal heaviness while asymptomatic patient presented second in order unlike Rozman et al report that define that 70% of CLL patients were asymptomatic at time of diagnosis [13]and this may be understood due to sample size as well as inclusion criteria in this study. Positive Direct anti globulin test(DAT) is similar to other reports (16.6% vs. 7.7%-35%) [13]

Therapeutic approaches to CLL should take into account patient classification, when to treat, and the potential role of new drugs. In this study ,the age of patients showed no relationship to treatment outcome(p=0.94) in contrast to the presence of anemia &bone marrow lymphocyte percentage with bone marrow diffuse pattern infiltration that indicate advanced disease stage (p=0.051,p= 0.036, p=0.04 respectively)in agreement of Jaksic et al postulation that tumor mass has a role in diseases outcome [18] and similarly the significance of positive DAT demonstrated worse outcome in statistical point of view (p=0.033) with lower response rate to this regimen later on .

The bone marrow in CLL has traditionally been considered as important prognostic marker [17,19], especially if it is diffusely infiltrated by mature-appearing lymphocytes. The pattern of bone marrow infiltration separates CLL patients into two different prognostic groups [19] Patients with diffuse infiltration have a median survival ranging between 2 and 4 years, while this value is between 8 and 10 years for those with a nondiffuse pattern.

Clinically, The age of patients showed no relationship to treatment outcome (p=0.94) &similarly their gender (p=1.00)as well as the presenting symptoms whether accidentally or not (p=0.39) or the presence of clinically palpable splenomeglay (p=0.45) unlike Lee JS et al who report that advanced age & presence of organomegaly are reliable markers of early prognosis [20]

Chlorambucil, an aromatic derivate of nitrogen mustard, is the old most commonly used drug in CLL, but complete remissions are rarely reported .The combination of chlorambucil and prednisone does not appear to be superior to chlorambucil alone [21], however; it remains widely used in the UK for patients considered unfit for intensive therapy on fludarabine combination regimens but with no international consensus as to the optimal dose or duration of chlorambucil therapy [11] . Therefore David Oscier had encouraged a recommendation for the combination of ant CD 20 antibodies into chlorambucil or benadmustine based regimen in these conditions.[11].

Chlorambucil plus prednisolone, remains the best treatment for patients over 60, because of low side effects, oral administration and relatively acceptable response rates [22], but the responses were heterogeneous.

While it is well known that corticosteroids possess lymphocytolytic activity, prednisone by itself has a limited antileukemic effect in CLL. Nevertheless, it is useful when dealing with autoimmune hematological complications. The limits of corticosteroid treatment in B-CLL are related to the metabolic and/or cardiovascular complications that sometimes appear in long term therapy, especially in the elderly. [23]

CLL is typically responsive to several courses of chemotherapy, although the depth of response tends to decrease with each subsequent line of therapy [24] Response rate in fludarabine based therapy 81% to 100% (25% to 37% CRs) in untreated patients according to O'Brien S. et al (25) while in R-LP regimen used in this study, it showed that (ORR) overall response rate is 86.67% (CR :50%,PR: 36.67%).

A group of leading hematologists have suggested that the toxicity profile of fludarabine (particularly immunosuppression due to long-term T-cell toxicity) makes it unsuitable for around 50% of patients (generally those aged older than 65 years with comorbidities and poor performance status). These patients are treated with chlorambucil, which is generally well tolerated but has relatively poor efficacy compared with fludarabine combination chemotherapy regimens in terms of the depth of remission (22). Chlorambucil therefore tends to be used when clinicians decide to take a palliative approach. (24) (12)

Therefore; FCR(fludarabin-cyclophosphamide- rituximab) is recommended as initial therapy for previously untreated patients (11) (14) but in case of being unsuitable, chlorambucil was the preferred first-line treatment option for patients with CLL who would be considered unsuitable for fludarabine combination chemotherapy regimens (24) and the R-LP might be as good alternative regimen with equivalent ORR although There are currently no definitive criteria for determining which patients would be unsuitable for treatment with fludarabine combination therapy [24]

Conclusion

Rituximab plus chlorambucil and predinsolon (R-LP) combination therapy might be as good alternative regimen with equivalent response for CLL patients whom are nonsuitable for treatment with fludarabine combination therapy

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Table I: Frequency of Clinical Presentation

Clinical Presentation	No.	%
Upper abdominal pain &heaviness	8	26.67
Accidental (asymptomatic)	7	23.33
Lymph nodes swelling	5	16.67
Constitutional Symptoms	5	16.67
Anemia	3	10.00
Any Combination	2	6.67
Total	30	100.00

Table II:

Relationship between initial laboratory parameters with treatment outcome at end of 8 cycles of R-LP

Parameter†	CR (n = 15)	PR (n = 11)	Failure (n = 4)	P-value
	Mean±SE	Mean±SE	Mean±SE	
WBC(x 10^9/l)	89.74 ±14.86	88.40±22.09	93.5±29.15	0.757
Hemoglobin(g/l)*	103.4±5.5	98.73±7.3	72.75±4.7	**0.051***
Platelet (x 10^9/l)	117.62±52.58	104.27±57.49	112.50±33.56	0.521
Bone Marrow lymphocyte %*	59.80±3.30	69.30±5.50	91.00±7.65	**0.036***
Absolute Lymphocyte count (x 10^9/l)	72.40±2.98	76.36±4.05	63.75±2.39	0.194
DAT* Negative	14(46.67%)	11(36.67%)		**0.033***
Positive	1(3.33%)	4(13.33%)		

†Fisher Exact test * statistical significance

Table III: Final Outcome Summary at end of follow up period

Response duration range (mean ±SE)			
	Duration of Response-DOR (months)	4.00-15.00	(9.23±0.50)
	Treatment free interval-TFI (months)	6.00-18.00	(11.74±0.64)
Patients outcome frequency (percentage)	**Survival**	21/30	(70.00%)
	Death	9/30	(30.00%)
	Total	30/30	(100.00%)

Table IV:

Relationship between final outcomes with their initial presentation in term of survival

Parameter	Out come	N	Mean±SE	P-value*
WBC(x 10^9/l)*	Survival	21	77.53±14.42	**0.05***
	Death	9	105.33± 18.30	
Hemoglobin (g/l)*	Survival	21	112.29± 4.15	**0.03***
	Death	9	94.11±7.3	
Platelet (x 10^9/l)	Survival	21	136.86±52.5	**0.3**
	Death	9	104.40±18.08	
Bone Marrow lymphocyte (%)	Survival	21	73.72±3.59	**0.8**
	Death	9	65.00±4.61	
Absolute peripheral Lymphocyte count (x 10^9/l)	Survival	21	68.38±2.78	**0.6**
	Death	9	87.11±3.61	
Positive DAT**(frequency-percentage)	Survival	4/30	(13.33%)	**0. 04****
	Death	1/30	(3.33%)	

*Mann Whitney U test **Fisher Exact test

Table V:

Correlation between initial presentation and remission durability & treatment free interval

Parameter	Correlation parameter	Age	WBC count	Hemoglobin level	Platelet count	Bone Marrow lymphocyte percentage	Absolute peripheral Lymphocyte
DOR	R	-0.081	0.127	0.193	0.196	**-0.472***	0.297
	P	0.672	0.503	0.308	0.300	**0.043***	0.111
TFI	R	-0.152	0.022	0.169	0.170	-0.260	0.023
	P	0.448	0.912	0.400	0.397	0.190	0.909

DOR: duration of response

TFI: treatment free interval

Original article

Flowcytometric Measurement of CD5, CD23, and CD38 expression as a diagnostic and prognostic markers in CLL patients

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ABSTRACT

Background: B-Chronic lymphocytic leukemia (B-CLL) is a monoclonal malignancy characterized by an accumulation of terminally differentiated small and anergic B lymphocytes in the blood, bone marrow and other tissues. CLL is typically characterized by CD5+, CD23+, CD22 -, CD79b-, with weak expression of surface Ig. CD5 also is expressed in B1 subset of human B –lymphocytes. Mature B cell malignancies, such as B-cell chronic lymphocytic leukemia, are mostly CD5 +. CD23 promotes the activation and proliferation of normal B lymphocytes and has an important role in the process of malignant transformation in B-CLL.CD38 is expressed on the surface of leukemic cells in a significant percentage of patients with B-cell chronic lymphocytic leukemia (B-CLL).Its expression has prognostic value in CLL. The current immunophynotype antigens is used to diagnosed as CLL cases, and by using the modern multicolor Flow Cytometry, which made it possible to determine the expression of several such antigens on specific cell populations of the CLL cases.

Objectives: To measure the expression of CD5, CD23, and CD38 antigens on the B-cells of morphologically diagnosed CLL cases, and showing their correlation with the hematological parameters, and with each other.

Material and methods: A prospective cohort study including 20 patients including 11 females and 9 males morphologically diagnosed with CLL. The patients were attending the National Center of Hematology, Al-Yarmouk teaching hospital for the period from November 2012 to March 2013. A total of 2 ml of venous blood were collected from all patients who were selected randomly with respect to age, sex, duration and stage of the disease. The diagnosis was done by

measuring and calculating the total number of blood cells and lymphocytes and hemoglobin and other by using autoanalyzer blood counter, then flowcytometry was used to measure the appearance of antigens surface CD5 and CD23 CD38.

Results: The mean of age of all patients included was 61.95+8.88 SD, and a range of (45-75) years old. There were 11 (55%) males patients, the most common symptoms of patients is an enlarged spleen (45%). 85% of patients who were in Binet stage C, the most advanced stage of the disease. Within Binet stage C there was 94.1% percent of patients showed moderate intensity expression of CD5 and CD23, while 64.7% of them for CD38. There was no statistical significance of CD5, CD23 in relationship to age, hemoglobin or platelet, while CD5 showed a significant relationship with lymphocytes count and the total number of white blood cells ($P < 0.05$).CD38 showed significant relationship with hemoglobin ($P < 0.05$). There is a significant correlation between the CD5 and CD23 P) <0.05), while the CD38 show positive correlation with CD23) $P < 0.05$.

Conclusions

1-There is a significant correlation between CD5 expression and absolute lymphocyte count, so higher peripheral blood lymphocyte associated with greater CD5 antigen expression.

2-There is a significant correlation between CD5, and CD23 expression, so high CD5 expression associated with high CD23 expression.

3-There is a significant negative correlation between CD38 expression and Hb level that reflects a prognostic significance.

4-There is a correlation between CD38 expression and CD23 expression.

5-No correlation between the intensity of expression of CD5, CD23, and CD38 and stage of the disease.

6-No correlation between CD38 expression and age, WBC count, and lymphocyte count.

Keywords: Chronic Lymphocytic Leukemia, Flowcytometry, CD5, CD2, CD38

Introduction

B-Chronic lymphocytic leukemia (B-CLL) is a monoclonal malignancy characterized by an accumulation of terminally differentiated small and anergic B lymphocytes in the blood, bone marrow and other tissues. These malignant cells can be identified by their varying surface membrane molecules, many of which are quite different to those expressed by normal cells and other lymphoproliferative disorders [(1)]. CLL is typically characterized by CD5+, CD23+, CD22 -, CD79b-, with weak expression of surface Ig [(2)]. CD5 is a T-cell marker of 65000-67000 Dalton that also is expressed in B1 subset of human B – lymphocytes. Mature B cell malignancies, such as B-cell chronic lymphocytic leukemia, are mostly CD5 +(3). CD23 antigen, a trans-membrane glycoprotein, promotes the activation and proliferation of normal B lymphocytes and has an important role in the process of malignant transformation in B-CLL [(1)]

CD38 is a transmembrane glycoprotein was initially characterized in 1980 as a T-cell differentiation antigen. In the following years, several studies showed that CD38 expression is not limited to T cells but is widely expressed on different hematopoietic and non-hematopoietic tissues. The strength of expression of CD38 on hematopoietic cells varies with the stage of maturation, the type of activation, and the milieu in which activation takes place. It expressed on the surface of leukemic cells in a significant percentage of patients with B-cell chronic lymphocytic leukemia (B-CLL).Its expression has prognostic value in CLL [(4)].

The current study involved measuring the expression of these markers on lymphocytes in twenty patients, who were morphologically diagnosed as CLL cases, by using the modern multicolor Flow Cytometry, which made it possible to determine the co expression of several such antigens on specific cell populations of the CLL cases [(5)].

Symptoms and signs of CLL

It is not unusual for a patient to feel entirely healthy with no symptoms whatsoever when a routine blood count reveals an absolute lymphocytosis requiring additional follow-up investigations that establish a diagnosis of CLL. On the other end of the spectrum is a patient who presents with all of the typical “B” symptoms of lymphoma (i.e., marked weakness, profuse night sweats, unintended weight loss, and fever without infection). Each of these extremes accounts for approximately 20% of cases at presentation. The remaining 60% have varying symptomatology with milder constitutional symptoms. Most patients consult a physician because they have noted painless swelling of

lymph nodes, often in the cervical area (but also at times in any other lymph node-bearing site), that spontaneously waxes and wanes but does not altogether disappear [6,7].

Diagnosis of CLL: To achieve this, it is essential to evaluate the blood count, blood smear, and the immune phenotype of the circulating lymphoid cells [8].

1. Blood: The diagnosis of CLL requires the presence of at least 5×10^9 B lymphocytes/L (5000/μL) in the peripheral blood. The clonality of the circulating B lymphocytes needs to be confirmed by flowcytometry. The leukemia cells found in the blood smear are characteristically small, mature looking lymphocytes with a narrow border of cytoplasm and a dense nucleus lacking discernible nucleoli and having partially aggregated chromatin. Smudge cells, found as cell debris, are other characteristic morphologic features found in CLL [8].

2. Immunophynotype: it is achieved by means of labelled antibodies that recognize specific epitopes of cellular antigens. In general, the most useful antibodies are monoclonal antibodies (McAb) produced by hybridoma technology but, for some antigens, antisera containing polyclonal antibodies (PcAb) are better. The technique employed for immunophenotyping is usually flowcytometry [9]. Table 1 shows the markers used for phenotyping CLL and table 2 shows the score in various B cell neoplasms.

FLOW CYTOMETRY

Immunofluorescence is the basis of flowcytometry immunophenotyping. Flow cytometry has the advantage over immunocytochemistry that it is rapid and quantification of the percentage of positive cells is more precise because many more cells are evaluated. On the one sample it is possible to determine forward light scatter (FSC) and sideways light scatter (SSC), examine the co expression of multiple antigens and quantitate the strength of antigen expression. The antibody is bound to a fluorochrome that absorbs light then emits light of a longer wave length, detectable at a specific relevant wave length[9].A stream of cells, labelled with an antibody conjugated to a fluorescent dye, flows past a detector so that cells can be counted and their FSC, SSC and fluorescence intensity can be characterized[9]. Co expression of antigens on single cells or populations of cells can be detected by using two or more antibodies conjugated to different fluorochromes with specific emission spectra[9]. Fluorescence intensity is determined by the fluorochrome used, the strength of binding and the number of epitopes carried on a cell. Immunophenotyping laboratories often use ‘dim’ and ‘bright’ to refer to fluorescence

intensity. As a broad approximation, signals between 0 and 101 can be regarded as negative, between 101 and 102 as weak(+), between 102 and 103 as moderate (++) and between 103 and 104 as strong (+++) [9]

Table(1) IMMUNOPHENOTYPE USED IN SCORING CLL(10).

Marker(result)	Score
SmIg(weak)	**1**
CD5(+)	**1**
CD23(+)	**1**
FMC7(- or weak)	**1**
CD79b(- or weak)	**1**
Total	**5**

Table (2)CLL Score in B-cell disorders[10].

Disease	Score
CLL	
Typical	**4-5**
Atypical;CLL/PL	**3-5**
B-prolymphocytic leukemia	**0-1**
Hairy cell leukemia	**0-1**
NHL with leukemia*	**0-2**

***Follicular lymphoma, MCL, SMZL**

as informative as the core biopsy regarding overall cellularity and degree of infiltration[10].Bone marrow aspirate smears reveal a lymphocytosis of ≥30% of all nucleated cells in the bone marrow differential count. Bone marrow biopsy reveals that the marrow invariably is infiltrated with leukemic lymphocytes. There are four patterns of marrow involvement. In approximately one-third of patients, the marrow has an interstitial, or lacy, pattern, which is associated with a better prognosis and/or early stage disease. Approximately 10 percent of patients present with a nodular pattern of marrow involvement, and approximately 25 percent have a mixed nodular-interstitial pattern. These patterns also are associated with a better prognosis. A quarter of the patients present with extensive marrow replacement, producing a diffuse pattern that is associated with advanced clinical stage and/or more aggressive disease[11]

Staging of CLL

1.The two widely accepted systems are those of Rai (1975) and Binet (1981)[10,12].

Rai staging system: This divides CLL into 5 stages: Rai stage 0: The blood lymphocyte count is too high, usually defined as over

10,000 lymphocytes/mm3 of blood. The lymph nodes, spleen, and liver are not enlarged and the red blood cell and platelet counts are near normal. Rai stage I: Lymphocytosis plus enlarged lymph nodes. The spleen and liver are not enlarged and the red blood cell and platelet counts are near normal. Rai stage II: Lymphocytosis plus an enlarged spleen (and possibly an enlarged liver), with or without enlarged lymph nodes. The red blood cell and platelet counts are near normal. Rai stage III: Lymphocytosis plus anemia (Hb less than 11g/dl), with or without enlarged lymph nodes, spleen, or liver. Platelet counts are near normal. Rai stage IV: Lymphocytosis plus thrombocytopenia (platelets count less than 100×10^9/L, with or without anemia, enlarged lymph nodes, spleen, or liver.

Binet staging system: The Binet classification integrates the number of nodal groups involved with the disease with bone marrow failure.

Binet stage A: Fewer than 3 areas of lymphoid tissue are enlarged, with no anemia (HB<10g/dl) or thrombocytopenia (platelets<$100x10^9$) [12].

Binet stage B: 3 or more areas of lymphoid tissue are enlarged, with no anemia or thrombocytopenia(12).

Binet stage C: Anemia and/or thrombocytopenia are present(12).

Materials and Methods

This study was conducted on twenty patients, including 11 males and 9 females, all with morphologically diagnosed CLL cases, 5 received treatment and 15 of them were newly diagnosed and no treatment was given. The patients were attending the National Center of Hematology, Al-Yarmouk teaching hospital, and some from private clinics and labs, through the period from November 2012 to March 2013.

Criteria for selection of patients:

1- All patients were diagnosed as CLL depending on morphology of their peripheral blood and bone marrow examination.

2-Randomly selected regarding the age, sex, duration and the stage of the disease.

3-Two cases were excluded as they showed negative expression for CD5, and CD23.

-Immunophenotyping by flowcytometry analysis to measure the expression of CD5,CD23, and CD38 surface markers was done for each patient at private lab.

Blood samples: A total of 2 ml of venous blood was collected by clear venipuncture into an EDTA tube, CBC was done for each sample by automated Abbot Ruby autoanalyzer at Al-Yarmouk teaching lab, blood film slides were revised for some of

the patients, and then the samples were sent within six hours to private lab for immunophenotyping.

Immunophenotyping for CD5, CD23 and CD38 expression were investigated by using four –color Cyflow® Cube 6 flow cytometry device (Partec Cyflow®, German), which is a fully equipped desktop Flow Cytometer (FCM). CyFlow Cube features a modular optical concept. This allows using different lasers as light sources (up to 2 light sources simultaneously: blue solid state laser: 488 nm and red diode laser: 638 nm and the detection of up to 6 optical Parameters (4 Colors + FSC + SS C) (parameters which denotes a measured property of the particles.) with selected PMTs with integrated electronic preamplifier for FSC, SSC, FL1-FL4(13). The CyFlow Cube allows easy optimization of the optics for any application by simple exchange of optical filters and (n) of cellsin a given volume (v), c = n/v. In the CyFlow® Cube 6, the volume is precisely measured directly by mechanical means, rather than indirectly with expensive and sometimes problematic beads, thus eliminating any errors related to varying bead concentrations or bead aggregations. The CyFlow® Cube 6allows the analysis of a fixed volume as defined by the distance between two platinum electrodes. The desired volume can also be freely selected, based on digital sample mirrors. Data acquisition, instrument control, and data analysis are controlled and performed by the CyView software. Compact flow cytometer for automated sequential analysis of single cells and microscopic particles (scatter particle size range: 50 nm -200µm), or cell subpopulation using True Volumetric Absolute Counting (TVAC). This advanced technology is solely based on the fundamental definition of absolute counting i.e.: the particle concentration (c) is equal to the counted number speed control by software [13]

Reagents: Product name: CyLyse Erythrocyte lysing reagent kit for wash and no wash procedures.

Contents: Reagent A for leukocyte fixation, 25 ml, Reagent B for erythrocyte lysing, 500 ml, Monoclonal Abs kits, Control beads

Description: CyLyse stands for an erythrocyte lysing reagent kit with a complete preservation of the surface proteins and practically no loss of cells.CyLyse is particularly suitable for absolute cell counting and for assays, demanding a minimum loss of leukocytes. Residual debris does not need to be removed by centrifugation due to the properties of the lysing reagent buffer. Fixative reagent A fixes and stabilizes the leukocytes. The fixed

samples can be stored for up to 24 hours at 2C-8C before analysis.

Method and Procedure [14] :1-Antibody labeling Antibody labelling was done by mixing 100 microliter of whole blood with conjugated antibodies (10 microliter) in a test tube, mixed thoroughly. Incubated for 15 minutes in the dark at room temperature.2-Leukocyte fixation For leukocyte fixation, 100 microliter of reagent A was added and mixed thoroughly and incubated for 10 min in the dark.3-Erythrocyte lysis For erythrocyte lysis 2.5ml of reagent B was added, shaken gently and incubated for 20min in the dark. Then the sample was analyzed on the flowcytometry. Some samples after fixation were stored at 2-8°C, protected from light, up to 24hr until analysis.

Flow cytometry data was analyzed in bivariate plots of two- or three-color analyses with the application of electronic gates based on the scatter characteristic of cells. The measurement of the intensity of staining of cells by flowcytometry to provide an absolute value for the light intensity it measures is performed by comparing cell fluorescence with an external standard by using different commercially available beads in kits, which usually comprise two tubes. One tube contains four types of beads with four different levels of fluorescence uptake: one very dim, one very bright and two intermediate; the other tube contains blank (non-fluorescent beads) [15].

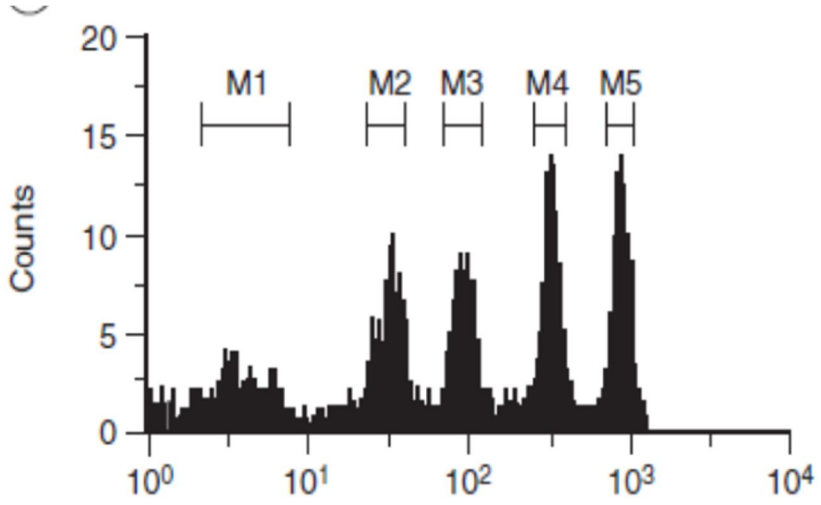


Fig (1) Beads fluorescence intensity showing five peaks: blank (M1); dim (M2); bright (M5); and intermediate (M3 and M4).

The instrument set up was so that the fluorescence signal of the tube with the blank (unlabeled) beads isolated in the region between 0 and 101 and four other peaks of fluorescence are seen along the axis of the relevant fluorochrome. The fluorescence voltage is established, and these settings maintained throughout the rest of the analysis of the unknown samples.(15).The samples for a particular McAb run with the fluorescence settings obtained from beads stained with the corresponding McAb, so that one fluorescence standard curve obtained for each McAb. The data obtained from the flow cytometer and, a standard curve is automatically produced. Identification of cells was performed using forward scatter (FSC) versus side scatter (SSC) parameters. Antigen expression was considered to be positive when the percentage of positive cells was equal or greater than 20%.[9]

Statistical analysis: Analysis of data was carried out using the available statistical package of SPSS-20 (Statistical Packages for Social Sciences- version 20). Data were presented in simple measures of frequency, percentage, mean, standard deviation, and range (minimum-maximum values). Pearson correlation was calculated for the correlation between two quantitative variables with its t-test for testing the significance of correlation. Statistical significance was considered whenever the P value was equal or less than 0.05.

Results

This study includes 20 adult patients with chronic lymphocytic leukemia, who were diagnosed morphologically by Lieshman stain on peripheral blood and bone marrow aspirates, biopsies stained by H&E.Immunophenotyping was done by Flow Cytometry to detect CD5,CD23 markers as diagnostic ,and CD38 as prognostic markers.

Clinical parameters

Age Groups: The mean of age of all patients included in this study was 61.95+8.88 SD, and a range of (45-75) years old. Table 3 shows that the highest percent of patients is within the age group of (60-69) years old.

Table (3) Age group distribution of the patients.

		No	%
Age (years)	<50	2	10.0
	50—59	4	20.0
	60—69	9	45.0
	=>70years	5	25.0
	Mean±SD(Range)	61.95±8.88	45-75

Gender: The patients included in this study were males (55%) and in females (45%). And within the most common age group of (60-69 yr), the percent of males (25%) was more than that of females (20%). As shown in table 4.

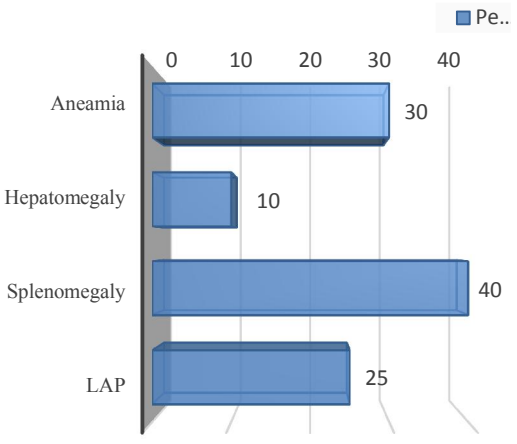
Table (4)The frequency of gender in relation to age group

Age group	Male	Female	Total
< 50	2 (10%)	0	2 (10%)
50-59	2 (10%)	2 (10%)	4 (20%)
60-69	5 (25%)	4 (20%)	9 (45%)
=>70	2 (10%)	3 (15%)	5 (25%)
Total	11 (55%)	9 (45%)	20(100%)

Clinical presentations

The clinical features of the patients are shown in the figure (2). The most common presenting feature of the patients is splenomegaly (45%) including (splenomegaly alone, splenomegaly with anemia or with hepatomegaly), followed by anemia (30%) as alone or with splenomegaly or with hepatosplenomegaly.

Fig. (2) presentation of the CLL patients.



Distribution of the patients according to Binet staging

The highest percent of the patients in this study (85%) fell within Binet stage C which is considered the most advanced stage in CLL patients, while (10%) stage B and (5%) stage A.

Hematological parameters

The mean Hb concentration was 10.98+ 2.84g/dl (mean+ SD) with a range between (5.6-16.5 g/dl)(16.5g/dl who was male 60 years old) and the Hb concentration was <10g/dl in 12 patients. The mean of lymphocyte count was 46.72+30.22x109/l (mean+SD), while the mean of platelets count was 162+71.28x109/l, and six presented with platelets count less than 100x109/l. The hematological parameters are depicted in table 5 below.

Table (5) Mean and Range of Hematological parameters of CLL cases

	Mean±SD	Range
WBC($x10^9$)	59.10±31.19	13.0-153.0
Lymphocyte($x10^9$/l)	46.72±30.22	7.5-141.0
Hemoglobin(g/dl)	10.98±2.84	5.6-16.5
Platelets($x10^9$/l)	162.0±71.28	30.0-280.0

Markers expression

The range of the percent of expression of the CD5 was between 20% which is the lowest positive value and 95% with a mean 65.65+22.6 (mean+SD), with all the morphologically diagnosed CLL cases show positive expression for CD5.

The percent of expression of CD23 range between 21%-96% (all show positive expression) with a mean of 67.08+21.41. While the CD38 range between 12- 86% with a mean of 38.79+22.58. Five patients out of twenty were negative (<20%) for CD38. Table 6 shows the expression of the three markers.

Table (6) Surface Markers CD5, CD23 & CD38 expression in CLL cases

Marker	Mean+SD	Range
CD5	65.65±22.60	20.0-95.0%
CD23	67.08±21.41	21.0-96.0%
CD38	38.79±22.58	12.0-86.0%

Hematological and clinical parameters in relation to Binet Staging: within Binet C stage, 12 (70.6%) out of 17 patients are with Hb less than 10g/dl .37.5% of patients are within (60-69) years old followed by 29.4% above70 years old. Within Stage C 52.9% are males while equal sex incidence within Binet stage B. 6% of patients had platelets less than $100x10^9$/l, six patients (35.3%) with splenomegaly, three (17.6 %) with lymphadenopathy and (5.9%) with hepatomegaly. As shown in table 7

Table (7) Hematological and clinical parameters in relation to Binet Staging

		A		B		C	
		No	%	No	%	No	%
Age (years)	<50	1	100	-	-	1	5.9
	50—59	-	-	-	-	4	23.5
	60—69	-	-	2	100	7	41.0
	=>70years	-	-	-	-	5	29.4
Gender	Male	1	100	1	50	9	52.9
	Female	-	-	1	50	8	47.1
Aneamia	Hb<10g/dl	-	-	-	-	12	70.6
	Hb≥10g/dl	1	100	2	100	5	29.4
Hepatomegaly	Yes	-	-	1	50	1	5.9
	No	1	100	1	50	16	94.1
Platelets	<$100x10^9$/l	-	-	-	-	6	35.3
	≥$100x10^9$/l	1	100	2	100	11	64.7
Splenomegaly	Yes	-	-	2	100	6	35.3
	No	1	100	-	-	11	64.7
LAP	Yes	1	100	-	-	3	17.6
	No	-	-	2	100	14	82.4

Markers intensity in relation to Binet Staging: Within Binet Stage C;16(94.1%) of patients expressed moderate intensity for both CD5 and CD23, and one (5.9%) patient expressed mild intensity for those markers, while (11) 64.7% of them expressed moderate intensity for CD38, , and 5(29.4%) of patients showed negative expression for CD38 (<20%).Within Binet B: all patients expressed moderate intensity for CD5, CD38, while 50% showed mild intensity for CD23 and 50% moderate. This is illustrated in figure3.

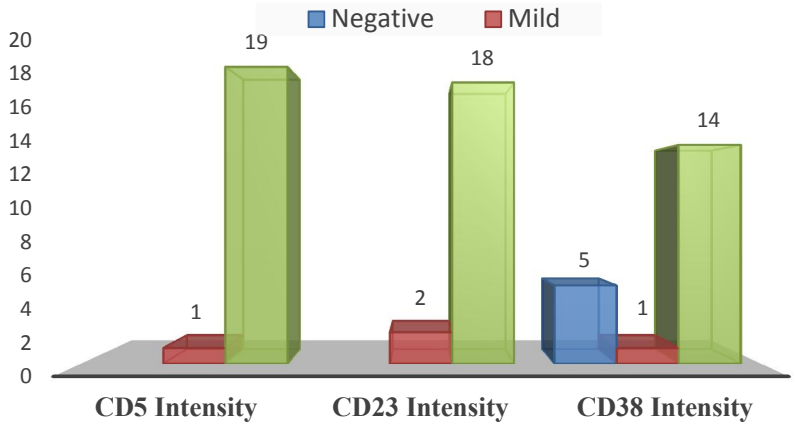


Figure (3) Intensity of markers expression in CLL patients

Markers expression in relation to Clinical and Hematological parameters

Within Binet group C patients , CD5,CD23 show no significant relation to each age, Hb or platelets, While CD5 show significant relation to lymphocyte counts (P <0.05) , and CD38 show relation with Hb level (P <0.05). As shown in table 8.

Table (8) Correlation between Clinical, hematological parameters and markers expression

C group patients (n=17)		CD5	CD23	CD38
Age (years)	r	0.995	0.641	0.361
	P	0.061	0.557	0.765
WBC ($X10^9$)	r	-0.996	-0.634	-0.353
	P	0.055	0.563	0.770
Lymphocyte	r	0.999	0.595	0.307
	P	0.024*	0.594	0.802
Haemoglobin (g/dl)	r	0.453	-0.480	-0.668
	P	0.701	0.681	0.047*
Platelets ($X10^9$)	r	0.727	-0.156	-0.464
	P	0.482	0.900	0.693

Markers Relation to each others

Within all Binets groups, there is a significant correlation between CD5, and CD23 ($P < 0.05$) , While CD38 show significant relation to CD23 ($P<0.05$) . This is expressed in table 9 below.

Table (9) Markers Relation of CLL patients

CD5		CD5	CD23	CD38
	R		0.544*	-0.037
	P		0.013*	0.878
CD23	R	0.544*		0.654
	P	0.013*		0.018*
CD38	R	-0.037	0.654	
	P	0.878	0.018*	
Pearson correlation with its t-test*Correlation is significant at the 0.05 level.				

Discussion:

B-Chronic lymphocytic leukemia (B-CLL) is a monoclonal disorder characterized by a progressive accumulation of functionally incompetent lymphocytes. It is the most common form of leukemia found in adults in Western countries[6]. These malignant cells can be identified by their varying surface membrane molecules, many of which are quite different to those expressed by normal cells and other lymphoproliferative diseases[1].

Clinical and Hematological parameters of the patients

In this study the mean age of the patients included was 61.95+8.88 SD, and the range of the age was between 45-75 years old, which are close to the results obtained by other Iraqi studies as shown in table (10)(16,17,18). These results also were comparable to the results reported by other studies in Asian countries [19]. While the results obtained from western countries show higher median age of presentation which was reported to be 70 years old [7].Other studies reported 68 year as median age at presentation [20]. This difference can be attributed to the difference in population structure, environmental difference, genetic predisposition between Iraq and Western countries, and difference in life expectancy.

The male to female ratio in this study was 1.2:1 which was lower than that reported by other Iraq studies [16-18], as shown in the table below. This difference may be due to the difference in sample size. But it was comparable to that of Western countries and other world studies [1,7,21]. But in all studies obvious finding of male predominance was fixed. Which might be related to genetic bases as shown by results reported by Cantu ES, McGill JR et al [22]. These results provided a genetic basis for the notion that the FISH abnormalities found underlie the phenotypic M/F sex ratio and also that they may be sex chromosomes (X and/or Y) influenced [22].

The most common presenting clinical feature of CLL in this study was splenomegaly followed by anemia, then lymphadenopathy, these results were comparable with other Iraqi workers [16-18].

Similar to the current study, results obtained by a study in Thailand [19].While other Western studies showed that the incidence of lymphadenopathy is more common than splenomegaly, anemia and hepatomegaly [3]. This might be attributed to earlier diagnosis, and sample size, in addition 5 patients in the current study received treatment. Regarding staging of the CLL cases in this study, by applying Binet staging, 85% of the patients fell within Binet stage C, which is considered as a high-risk

stage. This may be attributed to lack of regular checkup and general follow up of the people's health, so most patients are not presented until signs of advanced disease begin to appear. This percentage appears higher than other Iraqi studies. The cause may be due to the smaller sample size of the current study. While Western studies showed decreasing percent of cases in stage C at diagnosis, and increasing percent of stage A cases at time of diagnosis, which is attributed to the regular checkup and the facilities available for early detection and diagnosis of the disease [20].

The mean Hb level was 10.98+2.84g/dl, with a range (5.6-16.5g/dl), which was close to the results obtained by other Iraqi workers(16,17,18), as shown in table 4.1. As most patients in the current study presented in advanced stage of the disease with infiltration of the bone marrow by leukemic cells , in addition to the fact that nearly all the patients in the current study were elderly and many of them suffered from chronic illnesses. These results were also comparable to Western studies [3]

The mean of platelets count was 162±71.28X109/l, and six patients (35.3%) presented with platelets count less than 100X109/l, and a range between 30.0-280.0X109/l. As 85% of the patients in the current study were within Binet stage C, the most advanced stage of the disease, which involves infiltration of the bone marrow by leukemic cells and suppression of platelets production. These results agreed with Iraqi and Western studies [3,16-18].

Table (10) The results of the current study and other Iraqi workers

Parameters	Current study	Huda,et al M.Sc thesis 2010 [93]	Shaimaa,et al M.Sc thesis 2010[94]	Abdulkareem ,et al PhD thesis2008[95]
Patients number	20	50	68	60
Age range	45-75	39-75	40-88	48-72
Mean age (year)	61.95±8.88 SD	59.2 ± 1.34	61.70 ± 11	61.4 ± 9.1
Male : female ratio	1.2:1	3:1	2.4:1	3:1
Mean Hb (g/dl)	10.98±2.84g/dl	10.52 ± 0.26	10.16 ± 6.6	10.0 ± 1.5
Mean platelet count ±SE ($x10^9$\L)	162.00±71.28	142.82±10.11	162.45 ± 96.96	149 ± 73.8
Mean lymphocyte count±SE($x10^9$\L)	46.72±30.22	110.97±14.40	104.56±113.64	96.9 ± 99.8
Most common presenting sign	splenomegaly	lymphadenopathy	Lymphadenopathy	splenomegaly
Percentage of high risk patients Binet stage C	85%	54%	64.7%	63.3%

Regarding the intensity of marker expression, the majority of patients show moderate intensity of expression for CD23, (this agreed with results of Gong JZ and coworkers who demonstrated that the majority of CLL cases showed either moderate or bright expression of CD23 [23], and moderate CD5 expression [24], regardless the stage of the disease , as evidenced no relation between the stage of the disease and the intensity of expression P>0.05. This was in contrast to Geisler, et al study, which demonstrated that low intensity CD23 was associated with shorter survival in CLL[25] Dadmarz and Cawley demonstrated an association of low intensity CD23 with more advanced stage of disease in CLL [26]. Study of additional cases is necessary to confirm this association. The current study is somewhat limited, however, by relatively short follow up periods, small sample size. This issue will be readdressed after long

follow-up. Regarding the relation of the marker expression to the clinical and hematological parameters, there was a significant relation between CD5 and absolute lymphocyte count ($P<0.05$), this passed in agreement with other Western studies , which showed that greater CD5 antigen expression ,with higher peripheral blood lymphocyte count [(1)].

While in the current study CD23 percent of expression showed no correlation with peripheral blood lymphocyte count, other Western studies like Jurisic et al [(1)], showed that no correlation was found between CD23 expression in the patients with peripheral blood lymphocytes less than $100x10^9$/L, as the patients were still in early stages of the disease with low lymphocyte count and no cell membrane changes regarding expression of molecules had been occurred yet, while the patients with peripheral blood lymphocytes $>100X10^9$/L exhibited negative correlation with very low percentages of CD23 expression, as an increase in the lymphocyte count and an accumulation of anergic B cells have been associated with marked membrane molecule alterations [(27,28)]. These molecules are often functionally altered and diversely expressed in comparison to those of normal cells, show different cell membrane densities, differing receptor avidity and receptor saturation, and with the expression of activation molecules [(26)].This is contrary to the suggestion that advanced disease is associated with higher levels of CD23 expression, based on the measurement of elevated serum levels of soluble cleaved CD23 molecules using ELISA techniques [(29,30)], these soluble CD23 molecules (sCD23) resulting from the spontaneous proteolysis, cleavage and release of one of the two isoforms of the trans-membrane CD23 molecule [(31)].

So based on the findings of the current study , and the different findings of the different studies, we conclude that CD23 is not uniformly expressed by lymphocytes in CLL patients, and its expression may depend on number of clinical parameters like stage of the disease, absolute lymphocyte count, high lymphocyte count $>100x10^9$/l .

The current study showed that were no correlation between CD38 expression and the age of the patients , WBC count, lymphocyte count and platelets count , whereas there was a relation between CD38 expression and the Hb level($P<0.05$) as low level of Hb was seen with higher CD38 expression. This may be explained as low Hb levels are usually associated with advanced stages of the disease, and this pass with the positive expression of CD38, which is a poor prognostic factor. This was in agreement with the studies of other workers, like Ibrahim S, Keating M, et al[(4)].

The current study showed that there is a significant correlation between CD5, and CD23 expression as ($P<0.05$), indicating a predominance of the B cell subpopulation within the pool of the circulating lymphocytes. This was in agreement with other workers studies, like, Jurisic V, et al. Who showed that with high expression of CD23, there was also a high percentage of CD5 expression ($P<0.05$), which was positively correlated [(1)].

Also there was a correlation between the percent of expression of CD38+ cells and the expression of CD23 ($P<0.05$). This was in agreement with the results of other workers, like Poeta GD, Maurillo L, et al [(32)]. Who showed that high CD38 expression associated with higher CD23 expression emphasizes that CD38+ CLL cases are authentic B-CLL showing a greater disease activity as CD38 had been reported to play a complex role in lymphocyte proliferation [(32)].

Conclusions

1-There is a significant correlation between CD5 expression and absolute lymphocyte count, so higher peripheral blood lymphocyte associated with greater CD5 antigen expression.

2-Thereis a significant correlation between CD5, and CD23 expression, so high CD5 expression associated with high CD23 expression.

3-There is a significant negative correlation between CD38 expression and Hb level that reflects a prognostic significance.

4-There is a correlation between CD38 expression and CD23 expression.

5-No correlation between the intensity of expression of CD5, CD23, and CD38 and stage of the disease.

6-No correlation between CD38 expression and age, WBC count, and lymphocyte count.

Recommendations

1-Application of flow cytometry on larger sample size, and on other B-cell markers, including CD79b, FMC7, sIg, to show the complete scoring of CLL.

2-Depending on CD19 measuring as a gating step for B-lymphocytes in stead of the forward and side scatter characteristics, as it is more specific for B-lymphocytes.

3-Comparing the expression of CD38 before and after treatment of the patients with chemotherapy.

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Bronchial wash miR-21 as a potential biomarker for non-small cell lung cancer

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ABSTRACT

Background: Lung cancer is one of the major health problems all over the world. Most of cases are discovered at advanced stages because of late appearance of symptoms and the lack of efficient and effective methods for early diagnosis and screening of high risk groups. The microRNA-21(miR-21) was stably present and reliably measurable in all samples of bronchial wash whether positive or negative (control) for lung cancer.

Objectives: to evaluate the expressions of the miRNA-21 as a minimally invasive diagnostic biomarker for non-small cell lung cancer (NSCLC).

Results: Relative quantification of miR-21 gene showed overexpression in samples positive for NSCLC (non-small cell lung cancer) and ROC study yielding 85% sensitivity and 98% specificity in distinguishing NSCLC patients from controls with p-value < 0.05.

Conclusion: altered expressions of the miR-21 in samples of bronchial wash may provide a potential biomarker for detection of non-small cell lung cancer.

Keywords: bronchial wash, miRNA-21, non-small cell lung cancer

Introduction

Lung cancer is one of the leading cause of death from cancer, if not the first among various cancers, all over the world [1,2,3,4,5,6,7,8]. There is increase in the incidence of lung cancer starting from the fifties of the last century as a result of widespread tobacco smoking [9,10,11,12]. There are two types of lung cancer; the non-small cell (NSCLC) and the small cell lung cancer (SCLC) [5,9,10]. NSCLC is the commonest type of lung cancer worldwide, including Iraq, comprising about 85% of whole lung cancer [3,4,5,7,8]. NSCLC consists of three major histological types: squamous cell carcinoma (SqCC), adenocarcinoma (AC), and large cell undifferentiated carcinoma (LCC). The disease is frequently diagnosed at advanced and terminal inoperable stage in more than 75 % of cases [9,10]. Finding of

NSCLC at earlier stage reduces the mortality and improves the outcome [9,10]. Nowadays, the diagnosis of lung cancer depends on the development of symptoms, which are late in the course of disease, especially worrying symptoms such as repeated cough with or without hemoptysis and/or chest pain [9,10]. Therefore, developing a minimally invasive technique by taking advantages of recent developments in molecular genetics for diagnosis of NSCLC at an early stage is important clinically. MicroRNAs (miRNAs) are one of these studied biomarkers and still under investigation [13,14,15,16]. The miRNAs are small non-coding single stranded RNA consisting of 21-23 nts (nucleotides) bases. MicroRNAs exert their effect by post-transcription translation inhibition of target messenger RNAs (mRNAs) by complementary binding to 3'-untranslated regions (3'UTRs) of the target mRNA gene leading to its degradation or translation inhibition, with the resultant decrease in expression of target gene [9,10]. Due to its effect on target mRNAs, miRNAs participate in the physiological processes of proliferation, differentiation, apoptosis, and cell death as well as in pathological processes especially in carcinogenesis [9,10]. They are acting as oncogenes or tumor suppressor genes according to nature of their targeted mRNA genes [9,10]. MiR-21 is now considered as an oncomiR (oncogene) due to its ability of suppressing the actions of several tumor suppressor genes, promoting tumor cell growth, invasion and metastasis [9,10]. It was found to post-transcription down regulate PTEN which is a tumor suppressor gene [9,17]. MiR-21 also promotes growth, invasion, chemo and radio-resistance of NSCLC [9,10,17]. The high expression of miR-21 especially in bronchogenic squamous cell carcinoma has been found to be associated with poor prognosis [9,10,17]. MiR-21 also targets programmed cell death 4 (PDCD4), decreasing apoptosis in lung and breast cancer [18,19].

Materials and Methods

This is a prospective case-control study in which 54 patients were enrolled. Patients were recruited at the Thoracic Surgical Unit in the Specialized Surgery Hospital / Medical City during the period from March 2012 to April 2014.Twenty-four selected patients with NSCLC lung cancer proved by cytopathology on bronchial wash (15 were males and 9 were females).Thirty selected patients with benign pulmonary lesions (control) proved by cytopathology of bronchial wash (20 were males and 10 were females).

Inclusion Criteria

1. Patients were presented for the first time complaining from chest problem (cough, sputum, hemoptysis, tightness, chest pain ...), proved to be due to

pulmonary diseases malignant or benign.

2. Patients underwent Fiberoptic bronchoscopy with bronchial wash.

Exclusion Criteria

1. All cases that have received any form of specific cancer treatment (radical surgery, chemotherapy and/or radiation therapy) prior to sample collection were excluded from the study.
2. All cases known to have a second primary tumor other than lung cancer were excluded from the study.
3. All cases with uncertain diagnosis, whether benign or malignant were also excluded from the study.

Ethical approval for this work was obtained from Baghdad Medical College Ethics Committee. Every patient participated in the study have received a written information sheet explaining to them the aim of the study, and a signed consent form was taken from each one before participating in the study and the right was given to them to withdraw from this study at any time.

Samples collection

In a labeled nuclease free tube (2 – 3) milliliters of bronchial wash was taken directly from bronchoscope and kept on ice until transferred to the laboratory for separation. The separation is performed by centrifugation at a speed of 1500 g for 15 minutes at a temperature of 4 degree centigrade. Then, the supernatant fluid was discarded and the sediment was re-suspended in a 5 times volume of RNA Later solution is kept in a deep freeze at -80 degree centigrade (Ć) until RNA extraction. The rest of the bronchial wash specimen was taken for cytopathological study.

Cytopathological diagnosis

The smears that were taken from deposit of bronchial wash samples are stained by Papanicolaou's stain and/or Hematoxlin and eosin stains. The following criteria were used for cytopathological diagnosis of the main types of NSCLC lung cancer.

1. Squamous cell bronchogenic carcinoma

The cells are enlarged with a raised nucleo-cytoplasmic ratio (N/C ratio), the nucleus of malignant cell exhibits hyperchromatism, abnormal chromatin pattern, and irregular nuclear membrane. The cytoplasm of malignant cells is abundant and dense, in the well differentiated type it is cyanophilic, while basophilic in less mature type. Other features of malignant cells are also seen such pleomorphism, bizarre shaped cells and, giant tumor cells [9,10,20,21].

2. Bronchogenic adenocarcinoma

There is moderate hyperchromasia of their nuclei with fine granular chromatin pattern, prominent nucleoli with occasional mitotic figures. The cytoplasm is

amphophilic with fine or coarse vacuoles which are due to degeneration rather than mucus secretion, and cells are never ciliated. The presence of papillary clustering or three dimensional appearances may be seen as well [9,10,20,21,22,23].

3. Large cell bronchogenic carcinoma

LCC is characterized by syncytial clusters of cell and dispersed cells, the cells have irregular nuclei with striking chromatin clearing, prominent, often multiple nucleoli and ill-defined, feathery cytoplasm [9,10,20,21,22].

qRT-Realtime PCR

The extractions was performed by the use of extraction kit (mirVana™ miRNA Isolation Kit, with phenol) according to the manufacturer's instructions [24]. The extracted total RNA then, was treated by DNA-free kit to get rid of any contaminant DNA using DNase and according to manufacturer's instructions [25]. The purity and concentration of RNA was measured by Nano-drop spectrophotometer and a samples with a ratio of A260/A280 (1.9 – 2.0) were taken for gene quantification [25]. The extracted total RNAs were stored at a temperature of (– 80 C) for reverse transcription into corresponding cDNAs.

Reverse Transcription of total RNAs to cDNAs

Two samples from DNA free total RNA (each containing 1 µg of total RNA) were taken for reverse transcription of miRNAs into cDNA using (TaqMan® MicroRNA reverse Transcription Kit and primers) according to manufacturer's instructions [26]. A Primer pool for reverse transcription of miRNAs into cDNAs were created from miRNA specific primers and a mix of reagent was also used to reduce pipetting errors. The concentration and purity of cDNA was also checked with Nano-drop spectrophotometer. The quality of cDNA as a template for real time PCR amplification of miRNA was assessed by qRT-PCR amplification of (miR-RNU-48 housekeeping gene) with a non-template sample as a control (NTC). The optimum concentration of primers and the optimum annealing temperature was assessed by serial dilutions of primers and the temperature also investigated by test changing annealing temperature and performing PCR runs. The optimum primer concentrations and annealing temperature in table 1

Table1: Properties and amounts of primers used in real-time qRT-PC assays of microRNAs.

Property	microRNA-21	microRNA-RNU-48
Forward primer	5'GCCCGCTAGCTTATCAG ACTGATG-3'	5'TCTGAGTGTCTTCGCTGA CG-3'
amount of use	10 pmol	15 pmol
Reverse Primer	5'GTGCAGGGTCCGAGGT-3'	5'GAGGTATTCGCACCAGA GGA-3'
amount of use	10 pmol	15 pmol
Optimized annealing temp.	55 C	56.5C

Realtime qRT-PCR amplification of cDNA

The realtime PCR amplification of miRNAs (21, & housekeeping gene RNU48) in the samples taken from bronchial wash was performed in duplicate using TaqMan® MicroRNA Master Mix II, no UNG Kit with primers and probes from Applied Biosystems [27]. The assays were performed according to the manufacturer's instructions with the use of master mix pool to reduced pipetting errors with a non-template control (NTC). The thermal prolife was designed including hold at 95Ć for 10 minutes followed by forty cycles of 15 seconds at 95 C followed by annealing and extension at 60Ć for forty cycles. The mean of Ct value for each specimen was taken for gene expression study.

Statistical analysis

Analysis of the present results were carried out using IBM SPSS-22 statistical software .Frequency of positive results were studies as number and percentage. Differences between frequencies were calculated by applying Chi square. Results were considered significant when p value < 0.05.Mean and standard deviation (SD) were calculated for total RNA concentration and purity by SPSS-21 software. Mean and SD of calculated Ct values were subjected to student *t*-test, ANOVA, and LSD statistical tests. Results of differences were considered significant when p value is < 0.05.The ΔCt and ΔΔCt

for each gene were calculated according to the equations:

a. The ΔCt of target gene = [Ct of target gene – Ct of housekeeping gene].

b. The ΔCt of control gene = [Ct of control gene – Ct of housekeeping gene].

c. Expression = ($2^{-\Delta\Delta Ct}$), the result of expression = $2^{-\Delta\Delta C}$ {[Ct of target gene – Ct of housekeeping gene]-[Ct of control – Ct of housekeeping gene]} [28].

ROC area (Receiver Operator Characteristic) for sensitivity and specificity of expression of each gene were calculated with significant being *p*-value <0.05.

Results

The cytopathological findings of bronchial wash samples that were positive for NSCLC summarized in figure1. Bronchogenic squamous cell carcinoma was the most frequent type of lung cancer accounting 19/24 (79.17 %) of whole NSCLC cases, subdivided according to sex into 13/24(54.17%) in men and 6/24 (25 %) in women from whole non-small cell lung cancer cases. Bronchogenic adenocarcinoma subtype of NSCLC were 4/24 (16.67 %) also subdivided into 1/24 (4.17 %) in males and 3/24 (12 %) in females, while large cell bronchogenic carcinoma accounting for 1/24 (4.16 %) of whole lung cancer. The results showed cancer was more common in the age group (60 – 69 years), and followed by the age group (70 – 79 years) and in most age groups the males were more common than females or equal to them figure 2.

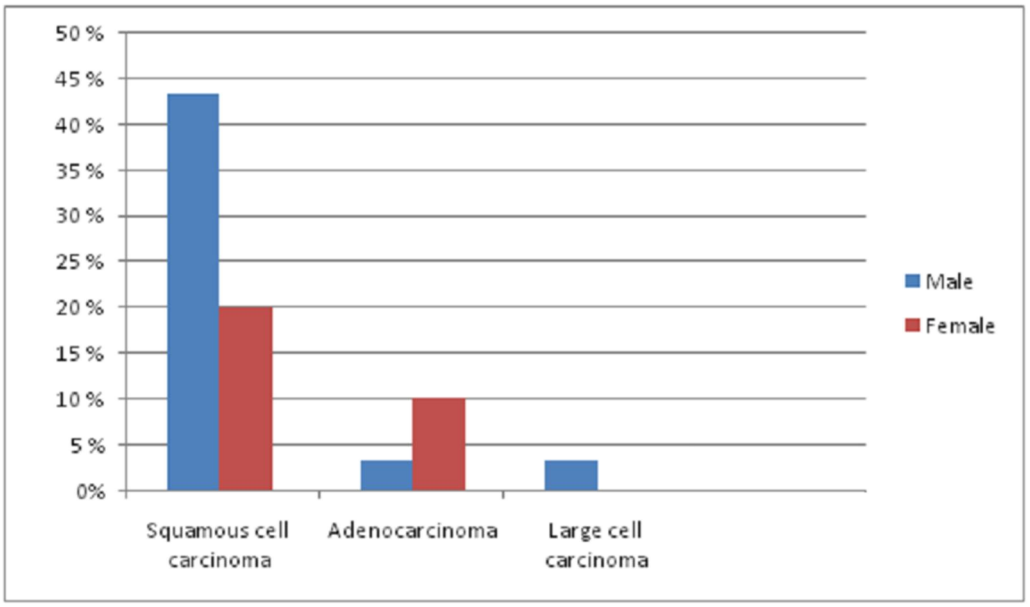

Figure 1: Cytopathological results of bronchial wash positive for malignant cells, frequency of the cytopathological type according to sex.

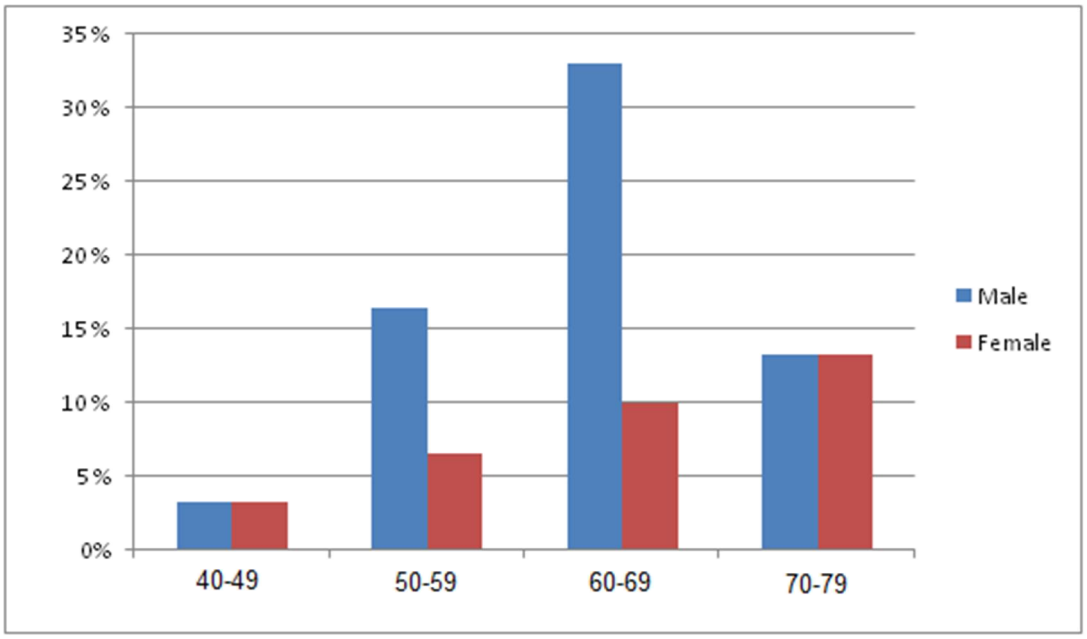

Figure 2: Cytopathological results of bronchial wash positive for malignant cells, the frequency according to the age groups and sex.

The total RNA concentration and purity of each sample, after total RNA extraction by mirVana Isolation kit and treatment with DNase kit, was measured by nano-drop and the ratio of A260 / A280 and samples results 1.9 – 2.0 were considered as good samples and taken for further steps. The mean ± SD for concentration of whole samples of bronchial wash positive for lung cancer cells was 4346 ± 53.147µg/ml and for males the mean concentration was 4785.88±65.174 µg/ml and for females it was 3466.96±23.201µg/ml. The mean ± SD of concentration of whole samples of bronchial wash that were negative for lung cancer cells was 3230.45±22.404 µg/ml and for males 3205.995±23.908 µg/ml and for females it was 3279.36±19.078 µg/ml.

The mean of purity ± SD of whole samples of bronchial wash that were positive for lung cancer cells was 1.9602±0.03616 and for males it was 1.9553±0.0386606 and for females it was 1.970±0.0299629. The mean of purity ± SD of whole samples of bronchial wash that were negative for lung cancer cells was 1.95510±0.0372479 and for males it was 1.95035±0.395731 and for females it was 1.96460±0.0318441.

Results for raw Ct values of all studied microRNA genes compared between NSCLC cases and control by *t*- test as studied from bronchial wash specimens

Table 2: Recorded results for raw Ct values of all studied mRNA and microRNA genes compared between lung cancer cases and control as studied from bronchial wash specimens.

	Study group		
	Control group (C)	**Cases (lung Ca)**	***p*- (*t*-test)**
Ct value - miR-RNU-48			0.02
Range	(22.19 to 25.96)	(23.2 to 26.6)	
Mean	24.35	24.99	
SD	1.10	0.90	
SE	0.20	0.16	
N	30	24	
Ct value - miR-21			<0.001
Range	(25.96 to 30.16)	(22.68 to 29.97)	
Mean	28.87	26.14	
SD	0.90	1.79	
SE	0.16	0.33	
N	30	24	

Comparison between different histological types and control cases in mean raw Ct value of markers studied in bronchial wash. The mean raw Ct value was compared between different histological types of lung cancer and control cases for each of the studied markers in bronchial wash using ANOVA test. The *p*–LSD (Least significant difference) for difference in mean raw Ct values was studied different types of lung cancer themselves. In miR-RNU-48, ANOVA testing for difference in mean raw Ct value between different histopathological types of lung cancer

and control was not significant; the *p*-value was > 0.05. The *p*-(LSD) for difference in mean raw Ct value was significant the *p*-value was < 0.05, on comparing between C (control) and SqCC (squamous cell carcinoma) only. However, it was not significant, *P*-value > 0.05, when comparing between C and AC (adenocarcinoma), and between SqCC and AC. In miR-21, ANOVA testing for difference in mean raw Ct value between different histopathological types of lung cancer and control was significant; the *p*-value was < 0.05. The *p*-(LSD) for difference in mean raw Ct value was significant; the *p*-value was < 0.05, on comparing between C and SqCC, and between C and AC. However, it was not significant, *p*-value was > 0.05, when comparing between SqCC and AC, table 3

Table 3: ANOVA and LSD for differences in means of raw Ct values of different markers studied in bronchial wash according to histological types.

	Final diagnosis			*p*-value (ANOVA)
	SqCC	**AC**	**C (Control)**	
Ct value miR-RNU-48			0.13[NS]	
Range	(23.2 to 26.6)	(24.4 to 25.65)	(22.19 to 25.96)	
Mean	25.03	25.07	24.35	
SD	1.03	0.52	1.10	
SE	0.24	0.26	0.20	
***p*-(LSD) for difference in mean between:**				
C X SqCC = 0.028				
C X AC = 0.2[NS]				
SqCC X AC = 0.95[NS]				
Ct value - miR-21			<0.001	
Range	(24.32 to 29.56)	(25.21 to 29.6)	(25.96 to 30.16)	
Mean	26.05	27.08	28.87	
SD	1.43	1.85	0.90	
SE	0.33	0.93	0.16	
***p*-(LSD) for difference in mean between:**				
Cont X SqCC <0.001				
Cont X AC = 0.022				
SqCC X AC = 0.19[NS]				

On studying the sensitivity and specificity of each marker, the P value for ROC curve was statistically significant, P value < 0.05, in all microRNAs.

Table 4: The ROC area occupied by each test and its *p*-value of markers studied from bronchial wash samples.

	ROC area	***P*-value**
Ct value - miR-21	0.871	<0.001
Ct value - miR-RNU-48	0.658	0.036

The differences in the mean normalized Ct values between malignant and control cases were statistically significant, *P*-value was < 0.05 with miR-RNU-48 & miR-21, table 5.

Table 5: Recorded results for normalized Ct values of studied markers (miR-RNU-48 & miR-21), compared between lung cancer cases and control, as obtained from bronchial wash specimens.

	Study group		
	Control group	**Cases (lung Ca)**	***P*- (*t*-test)**
Standardized Ct value mir-RNU-48			<0.001
Range	(0.78 to 0.93)	(0.85 to 0.98)	
Mean	0.87	0.92	
SD	0.04	0.03	
SE	0.01	0.01	
Standardized Ct value - miR-21			<0.001
Range	(0.94 to 1.07)	(0.85 to 1.11)	
Mean	1.03	0.96	
SD	0.03	0.06	
SE	0.01	0.01	

Comparison between different histopathological types of lung cancer and control cases in mean normalized Ct values of markers (miR-RNU-48 & miR-21) studied in bronchial wash samples. The mean of normalized Ct values were compared between different histopathological types of lung cancer and control cases for each of the studied markers in bronchial wash samples using ANOVA test. The *P*-(LSD) also studied for differences in mean between control and different histopathological types of non-small cell lung cancer and also between different histopathological types themselves. ANOVA testing for miR-RNU-48 between all histopathological types of lung cancer and control was statistically significant, *P*-value was <0.05. The *P*-(LSD) for difference in mean normalized Ct values of miR-RNU-48 was statistically significant; *P*-value was < 0.05, between C and SqCC, and between C and AC. The *P*-(LSD) for differences between the types of lung cancer was not significant, *P*–value was > 0.05, between SqCC and AC.ANOVA testing for miR-21 between all histopathological types of lung cancer and control was significant, *P*-value was <0.05. The *P*-(LSD) for difference in mean normalized Ct values of miR-21 was statistically significant; *P*-value was < 0.05, between C and SqCC, and between SqCC and AC. The *P*-(LSD) for differences between the types of lung cancer were not significant, *P* – value was > 0.05, between C and AC.

Table 6: ANOVA and LSD for differences in means of normalized Ct values of different markers as studied in bronchial wash samples, according to histopathological types of lung cancer.

	Final diagnosis			
	SqCC	**AC**	**Control**	***P*- (ANOVA)**
Standardized Ct value - miR-RNU-48				<0.001
Range	(0.85 to 0.97)	(0.93 to 0.96)	(0.78 to 0.93)	
Mean	0.91	0.94	0.87	
SD	0.04	0.01	0.04	
SE	0.01	0.01	0.01	
P (LSD) for difference in mean between:				
Control X SqCC <0.001				
Control X AC <0.001				
SqCC X AC = 0.12[NS]				
Standardized Ct value - miR-21				<0.001
Range	(0.88 to 1.07)	(0.96 to 1.11)	(0.94 to 1.07)	
Mean	0.95	1.02	1.03	
SD	0.05	0.07	0.03	
SE	0.01	0.03	0.01	
***P*-(LSD) for difference in mean between:**				
Control X SqCC <0.001				
Control X AC = 0.47[NS]				
SqCC X AC = 0.009				

The P-value for ROC curves was statistically significant, *P*-value < 0.05, in both studied microRNAs, table 7

Table 7: The ROC area occupied by each test and the p-value of markers studied from bronchial wash samples.

	ROC area	***P*-value**
Standardized Ct value - miR-RNU-48	0.833	<0.001
Standardized Ct value - mir-21	0.821	<0.001

Studying the sensitivity of and specificity of each marker, the P-value for ROC curves was statistically significant, *p*-value < 0.05, all microRNAs, figure 3

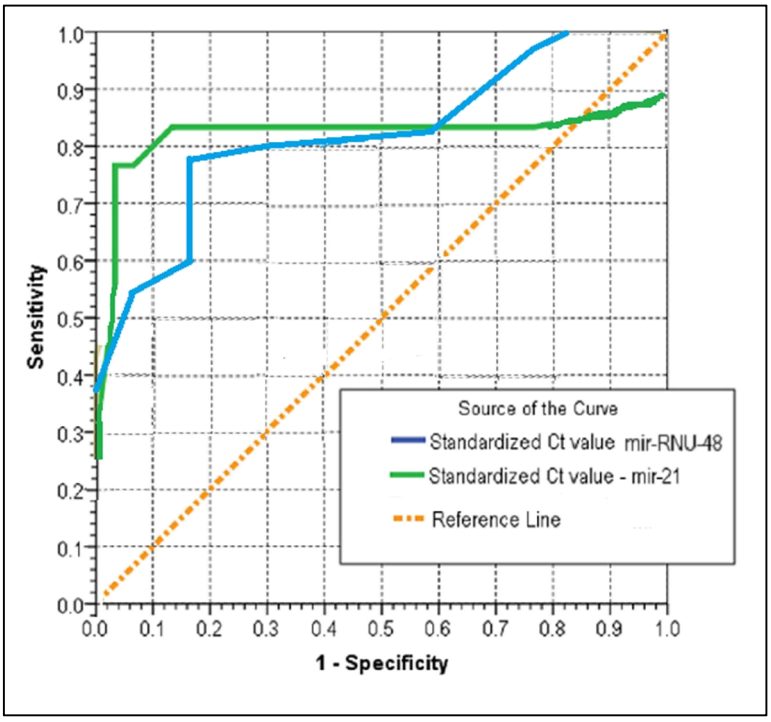

Figure 3: The ROC curves for sensitivity and specificity of studied markers (miR-RNU-48 and miR-21), in samples of bronchial wash.

Comparative CT Method

Calculation of expression by comparative Ct method using the formula, expression = ($2^{-\Delta\Delta C}$), the result of expression = $2^{-\Delta\Delta C}$ {[Ct of target gene – Ct of housekeeping gene]-[Ct of control – Ct of housekeeping gene]} [28].

The gene is overexpressed when its comparative Ct value is over (1), and under expressed when its comparative Ct value is less than. MiR-21 was (10.4) times more overexpressed in samples positive for NSCLC when compared to negative samples (control), table 8

Table 8: Mean Ct value with SD of cancer cases and control cases with comparative Ct values of studied markers and their P-values, in samples of bronchial wash.

	Ca cases		**Controls**	**Comparative Ct- value**		***P*- value**
	Mean	SD	Mean	SD		
Ct-miR-RNU-48	Control gene					
Ct-miR-21	0.591	0.992	0.057	0.094	10.4	<0.001

The expression of miR-21 gene in samples of bronchial wash positive for bronchogenic squamous cell (SqCC) compared to controls cases using comparative Ct method was (6.8) times more than control, table 9

Table 9: Comparative Ct values with mean and SD of mir-21 gene in samples of bronchial wash positive for lung squamous cell carcinoma by cytopathology.

	SqCC cases		**Controls**		
	Mean	SD	Mean	SD	Comparative Ct Value
Ct-miR-21	0.39	0.378	0.057	0.094	6.8

The expression of miR-21 gene in samples of bronchial wash positive for squamous cell lung (AC) compared to controls cases using comparative Ct method was (9.7) times more than controls, table 10.

Table10: Expression of mir-21gene in bronchial wash samples positive for lung adenocarcinoma.

	AC cases		**Controls**		
	Mean	SD	Mean	SD	Comparative Ct value
Ct-miR-21	0.551	0.703	0.057	0.094	9.7

When the standardized Ct value 0.935, the sensitivity is 30%, specificity is 100.0% and accuracy is 65%. When standardized Ct value 1.065, the sensitivity is 86.7%, the specificity is 6.7%, and the accuracy is 46.7%, table 11

Table 11: Specificity, sensitivity, accuracy, positive predictive values and negative predictive values.

					PPV at pretest probability =		
Positive if ≥ cut-off value	Sensitivity	Specificity	Accuracy	Optimal if minimum	50%	90%	NPV at pretest probability = 10%
Standardized Ct value - miR-21							
0.935	30.0	100.0	65.0	0.700	100.0	100.0	92.8
0.945	53.3	96.7	75.0	0.468	94.1	99.3	94.9
0.955	60.0	96.7	78.3	0.401	94.7	99.4	95.6
0.965	73.3	96.7	85.0	0.269	95.7	99.5	97.0
0.975	76.7	96.7	86.7	0.236	95.8	99.5	97.4
0.985	76.7	93.3	85.0	0.243	92.0	99.0	97.3
0.995	80.0	90.0	85.0	0.224	88.9	98.6	97.6
1.005	83.3	86.7	85.0	0.213	86.2	98.3	97.9
1.015	83.3	76.7	80.0	0.287	78.1	97.0	97.6
1.025	83.3	66.7	75.0	0.373	71.4	95.7	97.3
1.035	83.3	56.7	70.0	0.464	65.8	94.5	96.8
1.045	83.3	43.3	63.3	0.591	59.5	93.0	95.9
1.055	83.3	23.3	53.3	0.785	52.1	90.7	92.6
1.065	86.7	6.7	46.7	0.943	48.1	89.3	81.8

Discussion

In the present study, we found that the miRs (RNU-48 & 21) were stably present and readily measurable in the bronchial wash samples. Additionally, miR-21 was significantly over-expressed in specimens of cancer patients compared with those in normal controls. The data produced from our present study imply that miR-21 in bronchial wash could serve as a biomarker for diagnosis of lung cancer and miR-21 also could serve as a target for future therapy. The current study was in agreement with other studies. Shen et al. [29] have found that the expression of miR-21 may help in diagnosis of lung cancer in solitary lung nodule; Schwarzenbach et al. [30] suggested that miR-21 is a marker for early diagnosis of lung cancer. Cortez et al. [31] who reported the presence of miR-21 in body fluids as a marker for diagnosis and prognosis. Gao et al. [32] reported that over expression of miR-21 is associated with poor prognosis of lung cancer.

Inspite of our result appears to be promising, there are some limitations in this study. First, the sample sizes of the two study groups were too small, so further launching of miR-21 in large sample seize and in an independent studies is clearly required. Secondly, most of the studied cases were in advanced stages, and we were not sure of miR-21 expression in early stages of lung cancer, however, investigations in the future may give an answer.

In conclusion, we found that the expressions of the miRNA-21 in samples of bronchial wash could readily and specifically measured to be used as a minimally invasive diagnostic biomarker for NSCLC. Nonetheless, further independent cohort is required for validating the utility of this potential biomarker.

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Original article

Assessment of side effects of venesection (phlebotomy) procedure in Iraqi patients presenting with erythrocytosis: single center experience

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Background: Venesection or phlebotomy is generally considered to be a safe method, but occasionally adverse effects of varying severity may occur during or at the end of the procedure.

Objectives: The aim of the study was to estimate the frequency and type of adverse events occurring during venesection and to assess the practices which would help to minimize them.

Materials and methods: This is prospective single-center study was conducted from October 2011 to November 2012 at the emergency unit of the national center of hematology in Baghdad. All phlebotomies procedures made at the center were analyzed. All adverse events occurring during or at the end of procedure were registered by using a standardized questionnaire.

Results: Overall 3 adverse events were reported in relation to 960 venesections done, resulting in an overall adverse event rate of 0.3125%, that is, an incidence of 1 in every 320 venesections. One adverse effect was presyncopal symptoms of mild intensity, and the other two that observed were extravasation at site of puncture.

Conclusions: Only 0.3% of phlebotomies were complicated by adverse events which were very mild and easily managed. Our study confirms the fact that venesection procedure is a very safe method which could be made even more event-free by following certain friendly, reassuring and competent practices.

Key words: side effects, venesections, erythrocytosis

Introduction

Despite that great knowledge about venesection procedure and its safety in regards to patients, still there are rare cases in which serious adverse event happened as nerve injury or profound vasovagal shocks and soft tissue infections. Medical personnel's (doctors and nurses) who do venesection should be aware about the anatomical landmarks of target sites and have enough idea about the of pathophysiology of these adverse events to

avoiding them, and to be capable to treat them properly. Injury to peripheral nerves is one of the serious complications of which medical staff should take the utmost attention. It may lead permanent motor and/or sensory nerve damage. Selection of proper vein and careful procedure of venipuncture are essential. Vasovagal shock is quite common complication causing hypotension, pallor and infrequently syncope. Infections, particularly those by blood-borne pathogen, are rare but carry grave consequences. other side effects which occur less frequently include Hematoma at site of puncture, allergy, hyperventilation, air embolism, anemia and thrombosis. Finally, medical staff should know that close communication with patients undergoing venesection is crucial and efforts to advise them about risk of these side effects are becoming increasingly important in the current medical environment.[1]

Erythrocytosis, an increase of the red blood cell count above the threshold value of 6,000,000/μL, with a corresponding rise in the hematocrit (Hct) to over 50%, leads to a increase in the total red blood cell mass. Erythrocytosis is usually secondary to various conditions such as cardiac disorders (particularly congenital), lung diseases [in particular chronic obstructive pulmonary disease (COPD) and emphysema], tumors that produce erythropoietin, renal cysts, smoking, residence at high altitude, post-hematopoietic cell transplant, and endocrine disorders (with adrenocortical dysfunction). Patients with erythrocytosis have a high risk of severe thrombo-embolic events, which are directly related to the clear increase in blood viscosity [2]

From a therapeutic point of view, venesection (phlebotomy) is the most commonly used, cheapest and practical form of treatment for erythrocytosis and polycythemia, with the use of specific drugs such as busulphan and hydroxyurea [3]

The aim of this study was to estimate the frequency and type of adverse events of venesection occurring in persons presenting with erythrocytosis at the national center of hematology in Baghdad and to assess the practices which would help to minimize them.

Materials and methods

This is a prospective, single-center study of all adverse reactions related to all the consecutive venesections done between October 2011 and November 2012. All venesections were collected using a 16 gauge needle inserted into a vein in the antecubital area. Strict asepsis was maintained by cleaning the site of venipuncture sequentially using betadine and alcohol. The minimum hematocrit required for venesection was 45 and the lowest acceptable hemoglobin concentration was set at 15 g/dL. For venesection 350 to 450 mL of whole blood were collected from donors aged more than 18 years. As part of our study we assessed certain practices which could help to minimize the adverse incidents associated with venesection. The periods between two consecutive treatments were dictated by the hematocrit (Hct): patients underwent phlebotomy when the Hct rose to 50±1%. The aim was to bring the Hct to below 45% and maintain it at about this value for as long as possible. Full blood counts were performed 48 h after the

treatment and systematically repeated every 15-30 days in order to follow the trend in Hct over time. When the Hct reached about 50% or above, a new therapeutic procedure was performed. Besides being prescribed specific drugs, the patients were advised to increase their intake of fluids, stop smoking, limit food intake. It is always advisable to provide a friendly, warm and comfortable atmosphere for the donor and to engage particularly anxious donors in conversation during the donation, in order to distract their attention. It is also very important to react swiftly to initial complaints of giddiness, light headedness, pallor by the donor by stopping the procedure immediately and raising the legs of the donor (anti-shock position) as pallor, sweating, agitation are harbingers of a severe vasovagal reaction which could be prevented by taking corrective measures right at the onset of symptoms.

The classification scheme employed for recording the adverse events was suggested by the American Red Cross Hemovigilance Program that classifies complications into defined categories with severity ratings (minor/major) for certain types of reaction (4,5). Presyncopal symptoms include pallor, sweating or light-headedness without loss of consciousness. Syncopal types of complications are classified as minor if there is a transient loss of consciousness lasting less than one minute, while prolonged loss of consciousness for more than a minute or complicated by loss of bowel/bladder control, seizures or convulsions is said to be a major syncopal complication. Local adverse events include hematomas which can be small (<25.8 mm2) or large (>25.8 mm2), bruises, infiltration, allergic reactions, and a tingling/burning sensation.

Results

This study included a total of 960 venesections for whole blood (350 mL/450 mL) during the study period of which 943 were made by males and 17 by females aged between 18 and 65 years old. The age groups most represented were those between 30 and 40 years old (23 patients) and between 40 and 50 years old (31 patients).table 1

The venesections were made by 794 as first-time venesections and 166 repeated donors. Overall 3 adverse events were reported in relation to 960 venesections done, resulting in an overall adverse event rate of 0.3125%, that is, an incidence of 1 in every 320 venesections. One adverse effect was presyncopal symptoms of mild intensity, and the other two that observed were extravasation at site of puncture of all venesections; none necessitated hospitalization of the donor. The frequency distribution of the various types of adverse reactions that occurred in donors during the study period is presented in Table 2.

Table 1: distribution of age in the studied persons

Age(years)	Number	Present
15-25	84	8.75
26-35	204	21.25
36-45	387	40.31
46-55	175	18.22
56-65	67	6.97
>66	43	4.47

Table 2 - Frequency of various types of adverse reactions occurring in erythrocytosis persons

Type of adverse reaction	Number affected	Percentage
presyncopal complications	1	0.001
Syncopal complications		
Minor	0	0
Major	0	0
Hematoma	2	0.002
Numbness/tingling	0	0

Discussion

Hematology centers have a dual responsibility to offer sufficient supply of blood components to the community they serve and to ensure the safety and well-being of their donor's weather they are come to donation or to do venesection due to erythrocytosis. The most common systemic and venesection-related complications are presyncope and small hematomas. The importance of these minor complications, nonetheless, lies predominantly in the observation that any complication, even a minor one, decreases the likelihood of adherence to therapy [6]Although whole blood donation is considered to be safe, reports in the medical literature about the frequency of adverse events during donations show broad heterogeneity [7,8]

The aim of this study was to assess the frequency of various types of adverse reactions associated with venesection and to assess the measures that would help prevent or reduce the occurrence of these side effects. Donation-related adverse events were recorded according to standardized criteria as suggested by The American Red Cross Hemovigilance Program [(4)]. In our study, 0.3% percent of all venesections were complicated by an adverse event. This is similar to that found in various studies conducted all over the world in which the rate of adverse events associated with donations ranged from 0.3% to 3.8% [(5,6,7-11)]. Presyncopal symptoms, which include giddiness, sweating or light-headedness without loss of consciousness, accounted for approximately 33% of all adverse events. This is in contrast to the results of a study conducted by Crocco *et al*. in 2009, who found that vasovagal reactions of mild intensity constituted 71% of all adverse incidents reported.[(9)]

As regards local reactions, hematomas were found to be the most common adverse event 67%. Local reactions are mainly caused by blood donation-related neurological needle injuries which are commonly experienced by the donors after the donation in the form of hematomas, numbness/tingling, excessive or radiating pain, loss of arm/hand strength. The time to recover from these complications can range from less than 3 days to more than 6 months [(12)]. Since these complications are mostly experienced by the donor sometime after venesection and we recorded only adverse events occurring during the donation period and stay in the emergency unit, the rate of local adverse incidents observed in our study was not recorded. Like other studies, we found no incidence of severe reactions (major syncopal reactions with no episodes necessitating hospitalization of the donor or administration of intravenous fluids. It is worth noting that the maximum volume of blood withdrawn during venesection (350 to 450 mL) represents only about 10% of the total blood volume in a subject weighing 70 kg. Since at least 800-1,500 mL of blood, i.e. 15-20% of the total blood volume would have to be lost in order to be in at least class I risk of hypovolemia, erythrocytosis patients are unlikely to experience severe vasovagal reactions [(13)]. As part of our study we also assessed certain practices which could help to minimize the adverse incidents associated with venesections. It is always advisable to provide a friendly, warm and comfortable atmosphere for the persons who want to do venesection and to engage particularly anxious one in conversation during the procedure, in order to distract their attention. It is also very important to react positively to initial complaints of giddiness, lightheadedness, or pallor by the patients by stopping the procedure immediately and raising the legs of patient (anti-shock position) as pallor, sweating, agitation are early signs of a severe vasovagal reaction which could be prevented by taking correct measures right at the onset of symptoms.

In conclusion only 0.3% of whole blood venesections were complicated by adverse events and one of these events were presyncopal symptoms. Thus our study confirms the fact that venesection is a very safe procedure with no major complications.

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Original article

Hepatitis G virus infection and genotypes in Iraqi thalassemia patients

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ABSTRACT

Background: HGV infects patients at risk for parenteral exposure and chronic blood transfusion, such as those with β- thalassemia major.

Objectives: This study was designed to investigate the prevalence of HGV infection in thalassemia patient and furthermore to sequence and analyze phylogentic of HGV.

Materials and methods: A prospective study conducted between Feb. to May, 2014. A total of 154 Beta thalassemia patients (87 male; 67 female), from Alkarama teaching hospital and Ibn AL-baladi hospital maternity &children's hospital; aged 18-50 years , who received regular blood transfusions were included in the study. All patients were screened for Antibody against E2 glycoprotein of HGV (Anti-E2 Ab) by using ELISA to detect the presence of HGV infection. While Detection of HGV genomes was done by RT-PCR.

Results: One hundred fifty four thalassemia patients (87 male, 67 female) of 18-50 years were involved in this study. Using the ELISA method, Anti-HGV was detected in 16 patients out of 154 (10.4%) with peak prevalence age group was between 20-24 years. Reverse transcription - polymerase chain reaction (RT-PCR) showed HGV-cDNA was detected in 28 (18.2%) only. The peak age prevalence of HGV infection was under 20 years; however, there were no significant differences in prevalence among two sexes or number of blood transfusion. The results of genotyping in 12 randomly selected patients showed presence of genotype 2 and genotype 5 with percentage of 91.7% and 8.3% respectively.

Conclusion: the prevalence rate of HGV RNA in β-thalassemia major patients is 18.2%, while the prevalence rate of anti-HGV (past infection) is 10.4%. No one of thalassemia patients had HGV RNA and anti-HGV simultaneously. The Gene sequence analysis of PCR products identified HGV genotypes 2 and 5 with percentage of 91.7% and 8.3% respectively

Key words: hepatitis G virus (HGV), prevalence, genotype, thalassemia

Introduction

Regular blood transfusions for patients of thalassemia have improved the overall survival although these transfusions carry a definite risk of transmission of certain viruses. Due to repeated blood transfusions hepatitis B, C, G, and HIV infection can occur. All these are transmitted by blood or blood products in 10% cases [1]. Hepatitis G virus (HGV, or GB virus C) is an enveloped positive-sense, single-stranded virus [2] GB agent hepatitis originally was described by Dienhardt and colleagues, who inoculated tamarins (a type of New World monkey (Saguinuslabiatus) with the serum of a 34-year-old surgeon G. Barker (GB) who fell ill with acute hepatitis of moderate enzymatic activity and three-week icteric period [3].In 1995, a group of investigators from Abbott Laboratories identified two viruses in the serum and liver of tamarins. These viruses were called GB virus A and B (GBV-A and GBV-B) due to the pedigree of the infectious serum .GBV-A and GBV-B belonging to closely-related viruses of the Flaviviridae family. Later on, A PCR product from a West African specimen discovered a novel virus-like nucleotide sequence that was named GBV-C, which is most likely derived from a human virus [4]. Only HCV and HGV, assigned within genus Hepacivirus (type species Hepatitis C virus). The GBV-C genome is similar to hepatitis C virus (HCV) RNA in its organization, i.e. the structural genes are situated at the genomic 5' region and non-structural genes are at the 3' end [5].The 5’ terminal of the HGV, and HCV virus genomes represent untranslated regions (UTRs), while the 3’ ends of GB viruses and HCV represent the U’I’Rs. GB viruses and HCV are similar in size and structure. Each possesses single, large, open reading frames encoding putative poly-proteins of about 3000 amino acid residues. Infection with HGV is common in the world. Frequencies of GBV-C/HGV infection are hard to determine, but prevalence studies suggest that 1–4 % of healthy blood donors in most developed countries are viraemic at the time of blood donation [6].Based on analysis of the 5’ noncoding region (NCR) or E2 sequence, there are 5 major genotypes [7], and a recently identified sixth genotype [8], all distributed distinctly in different geographical regions. The mechanism responsible for the development of GBV-C-induced hepatitis is not clear so far. In spite of the described cases of acute and chronic hepatitis G, its hepatotropicity remains controversial [9].Viral hepatotropicity is supported by the detection of GBV-C RNA in hepatocytes and by the development of acute and fulminant hepatitis following the transfusion of infected blood and its products [10]. The outcome of acute hepatitis may be: (1) recovery with the disappearance of serum

GBV-C RNA and the emergence of anti-E2; (2) development of chronic hepatitis (CH) with serum GBV-C RNA being persistently detectable; (3) presence of GBV-C RNA without biochemical or histological signs of liver disease [11] Following clearance of GBV-C/HGV viraemia, most individuals develop conformation-dependent antibodies to the envelope glycoprotein E2, and thus E2antibody serves as a marker of past infection[11,12].

Patients, materials and methods

A prospective study including 154 Beta thalassemia patients (87 male; 67 female), from Alkarama teaching hospital and Ibn AL-baladi hospital maternity &children's hospital; their aged between 18-50 years , all patients received regular blood transfusions, the study had been conducted between February to May, 2014.

HGV serological analysis: All patients were screened for Antibody against E2 glycoprotein of HGV (Anti-E2 Ab) by using ELISA to detect the presence of HGV infection.

Detection of HGV genomes: Steps of RT-PCR for the detection of HGV infections

1. Extraction of viral RNA from specimens.
2. Estimation of RNA concentration
3. Reverse transcription of the RNA (cDNA synthesis)
4. Amplification of the cDNA: and this includes: Primers preparation:

Viral RNA in plasma sample was detected by RT-PCR using oligonucleotide primers deduced from highly conserved 5'UTR region of GBV-C genome/HGV. These primers are shown in table (1):

Table (1): Primers for PCR amplification of HGV

Primer	Primer sequence	Size of product (bp)
Forward - G1	**5′CGGCCAAAAGGTGGTGGATG -3′**	**185**
Reverse –G2	**5′- CGAGGAGCCTGAGGTGGGG- 3′**	

B. PCR Amplification Mixture and Condition.

Each 25 µl PCR mix composed of the ingredients listed in table (2). DNA amplification was carried in a thermal cycler according to caudai *et al.*, (1998).A negative control without template DNA was used in each reaction to reveal the hazardous cross contamination of the PCR components

.

Table(2): Master Mix components of HGV gene PCR

Component	Final Concentration	Amount
GoTaq Master Mix 2X (5X GoTaq flexi reaction buffer, Mgcl2, PCR nucleotide mix, and GoTaq Flexi DNA polymerase.	1X	12.5 µl
Primer Forward G1	40 Pmol	1µl
Primer Reverse G2	40 Pmol	1µl
cDNA	<250ng	5 µl
Nuclease free water	-	5.5µl
Total volume	-	25 µl

C. PCR program:

To detect the 5'UTR HGVgene amplification, cDNA amplification was carried in a thermal cycler, following the cycles listed in table (3).

Table (3): The PCR program for the 5'UTR HGV gene amplification

No.	Step	Temperature(C°)	Time	No.Of Cycles
1	Denaturation	94	1 min.	40
2	Annealing	55	45 sec.	
3	Extension	72	1 min.	
4	Final Extension	72	4 min.	1

The PCR products (10µg) with 100bp Ladder (promega) were subjected to electrophoresis on 2% agarose gel stained with ethidium bromide electrophoresis Then the gel was visualized on UV transilluminator at 320 nm. Gel was was performed at 80 V for 1 hr.30 min. photographed by Polaroid system (figure 1).

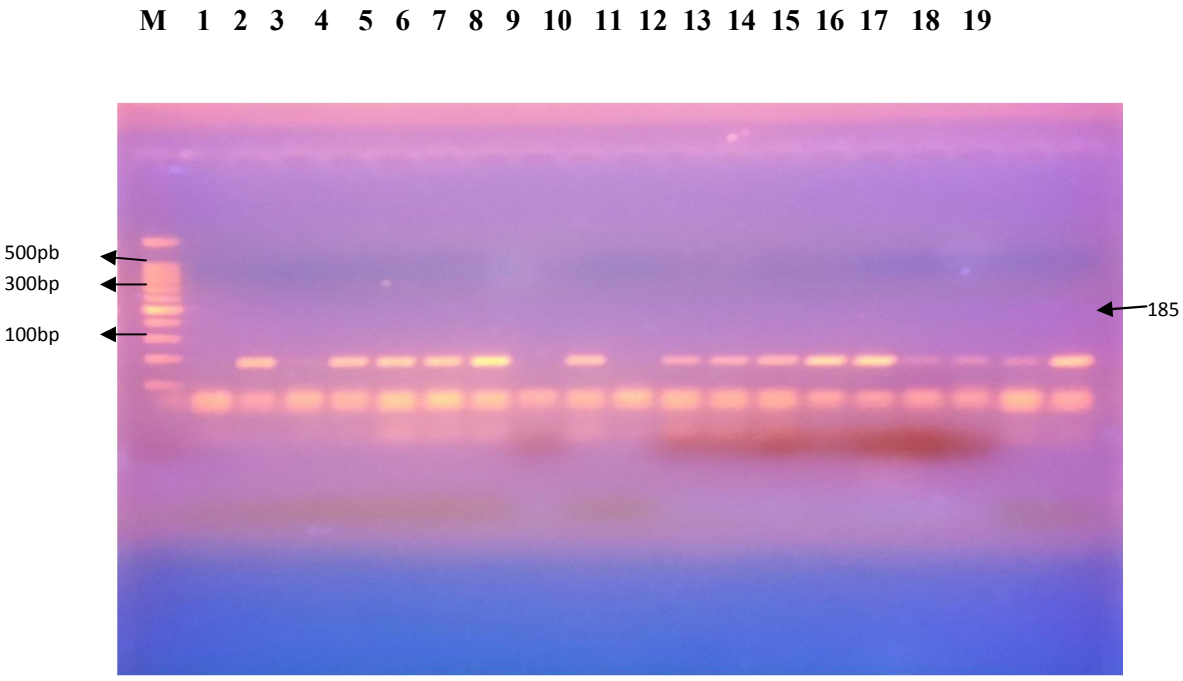


Figure 1. Agarose gel electrophoresis of 5'UTRPCR products of HGV-cDNA (M: 100 bp molecular weight marker, 2 % agarose, TAE buffer (1X), 80V, 1 hr.30 min; lane 1: Negative control, lane 2,4-7,9,11-19: amplicon of 185 bpPosetive HGV-cDNA , lane 3,8,10 Negative HGV-cDNA.

Gene sequencing:

Sequencing of PCR product was carried out by NICEM Company (South Korea) in reverse direction, and primer (G1&G2) was used in each sequencing reactions. Homology search was conducted between the sequence of standard gene BLAST program which is available at the National Center Biotechnology Information (NCBI) online at (http://www.ncbi.nlm.nih.gov) and using BioEdit program. Evolutionary analysis was conducted in MEGA 5 [15]

Statistical analysis: Analysis of data was carried out using the available statistical package of SPSS-22 (Statistical Packages for Social Sciences- version 22). Data were presented in simple measures of frequency and percentage .The significance of difference of different percentages (qualitative data) was tested using Pearson Chi-square test.

Results

ELISA was used for identification of hepatitis G virus antibody (IgG). Anti-HGV was detected in 16 patients (10.4%).Table (4) shows that the peak prevalence age group of thalassemia patients with anti-HGV was between 20-24 years, so There were a significant ($p<0.05$) decrease in prevalence of anti-HGV with increasing age, in addition male to female percentage was 37.5% to 62.5% respectively, however there were no significant differences in prevalence of anti-HGV in frequency of blood transfusion.

Table4: Prevalence of anti- HGV in thalassemia patients according to age, gender and frequency of blood transfusion

Parameter		Anti-HGV				P value
		Positive		Negative		
		No	%	No	%	
Age (years)	<20	1	6.3	56	40.6	0.041*
	20---24	10	62.5	45	32.6	
	25---29	3	18.8	23	16.7	
	=>30years	2	12.5	14	10.1	
Gender	Male	6	37.5	81	58.7	0.105
	Female	10	62.5	57	41.3	
No. of Blood transfusions (pints)	<30	1	6.3	17	12.3	0.120
	30---	3	18.8	58	42.0	
	60---	5	31.3	34	24.6	
	=>90	7	43.8	29	21.0	

*Significant difference between proportions using Pearson Chi-square test at 0.05 level.

All samples were subjected to PCR testing for detection of HGV nucleic acid**.** HGV-cDNA was detected in 28of 154 (18.2%) of patients with thalassemia. Statistical analysis of the data which was shown in the table (5) reveals that there were no significant differences in prevalence of HGV RNA among different age groups, gender and frequency of blood transfusion. The peak age prevalence of HGV infection was under 20 years; however, there were no significant differences in prevalence among two sexes.Nevertheless, no HGV one had RNA and anti-HGV simultaneously (table 6).

Table 5: Prevalence of HGV RNA in thalassemia patients according to age, gender and frequency of blood transfusion.

Parameter		HGV RNA				P value
		Positive		Negative		
		No	%	No	%	
Age (years)	<20	11	39.3	46	36.5	0.848
	20---24	9	32.1	46	36.5	
	25---29	4	14.3	22	17.5	
	=>30years	4	14.3	12	9.5	
Gender	Male	17	60.7	70	55.6	0.618
	Female	11	39.3	56	44.4	
No. of Blood transfusions(pints)	<30	-	-	18	14.3	0.190
	30---	12	42.9	49	38.9	
	60---	9	32.1	30	23.8	
	=>90	7	25.0	29	23.0	

*Significant difference between proportions using Pearson Chi-square test at 0.05 level.

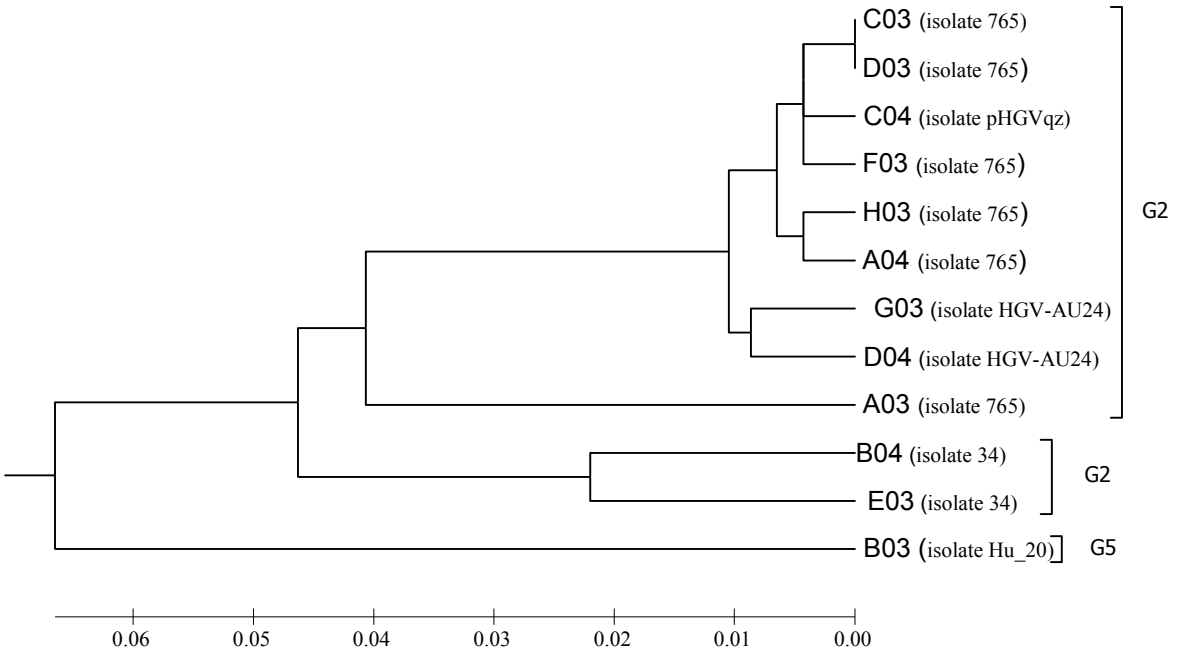
Table 6: Prevalence of HGV RNA in thalassemia patients with or without anti- HGV.

Parameter		Anti-HGV				P value
		Positive		Negative		
		No	%	No	%	
HGV RNA	Positive	-	-	28	20.3	-
	Negative	16	100.0	110	79.7	

*Significant difference between proportions using Pearson Chi-square test at 0.05 level.

Molecular typing and phylogenetic analysis of Hepatitis G Virus (HGV):

The amplified products from 12 / 28 HGV positive were subjected to sequencing procedure for the tree for each sample was obtained as seen in figure (2). region (5'UTR). Sequencing of PCR product was carried out by NICEM Company/ (South Korea) using an ABI 3730XL DNA Analyzer in reverse direction by primer G1&G2. Nucleotide sequence and phylogenetic analysis of 12/28 (%) HGV positive isolates belong to G2 (91.7%), G5(8.3%).This allowed us to construct a phylogenetic tree based on a partial polymerase 185bp fragment of 5'UTR in ORF1.Phylogenetic



G2 = **genotype 2** , G5 = **genotype 5**

Figure 2: Phylogenetic tree on the basis of the HG virus partial open reading frame 1 (ORF1) sequence as constructed by the neighbor-joining (N-J) method. Note that there are two major clusters, tentatively named G2, G5.

Genotyping results for the amplified region of each sample are shown in table (6). Nucleotide sequence analysis showed that the HGV genotypes in Iraqi thalassemia patients were closely related to the novel that isolated from the United States , Europe, Egypt, Thailand, , India, Turkey, Nepal, Saudi Arabia, UAE, Pakistan, Myanmar (genotype 2) and south Africa (genotype 5) that deposited in the Gene Bank with accession number as shows in table(6)

.

Table 6: The characteristics of genotyped HGV-positive clinical samples

	Name of gene	Accession N.	Homology	Genotype	Organism
1	Hepatitis G virus isolate 765	AY196904.1 GI:28543032	92%	2	GB virus C
2	GB virus C isolate Hu_20	KC618400.1 GI:558518560	96%	5	Uganda
3	Hepatitis G virus isolate 765	AY196904.1 GI:28543032	99%	2	GB virus C
4	Hepatitis G virus isolate 765	AY196904.1 GI:28543032	99%	2	GB virus C
5	Hepatitis G virus isolate 765	AY196904.1 GI:28543032	99%	2	GB virus C
6	Hepatitis G virus isolate HGV-AU24	AF177614.1 GI:9836767	98%	2	GB virus C
7	Hepatitis G virus isolate 765	AY196904.1 GI:28543032	99%	2	GB virus C
8	Hepatitis G virus isolate 765	AY196904.1 GI:28543032	98%	2	GB virus C
9	Hepatitis G virus isolate 34	AF489995.1 GI:20271200	94%	2	GB virus C
10	Hepatitis G virus isolate 34	AF489995.1 GI:20271200	94%	2	GB virus C
11	Hepatitis G virus strain HGV-Iw isolate pHGVqz	AF081782.1 GI:4093140	100%	2	GB virus C
12	Hepatitis G virus isolate HGV-AU24	AF177614.1 GI:9836767	99%	2	GB virus C

Discussion

Viral hepatitis infections, including HGV and HCV and their related hepatic and extra hepatic clinical manifestations are at the higher risk of interfering in the pathophysiology of thalassemia [16].Diagnosis of HGV depends on two methods; acute infection can be diagnosed by detection of HGV RNA by PCR, while past infection can be diagnosed by detection of antibody to E2 protein of the virus by ELISA. However, anti E2 antibodies to HGV and HGV RNA are almost mutually exclusive [17]. It had been reported that 60%-70% patients develop antibodies after infection [18]. As such, the detection of HGV RNA and anti-E2 is necessary to accurately define the prevalence of HGV infection in a population [19].

In our study the prevalence of anti-HGV was 10.4% in thalassemia patients. In Iraq Al-karkhy in his study demonstrate that 14.63 % of healthy volunteer blood donors have positive HGV-IgG [20], while the prevalence of HGV/GBV-C was reported in Iraq by Al-Obeidy et al. who revealed that HGV-IgG were detected in 25% of healthy blood donors [21]. The ongoing HGV infection can be diagnosed by demonstration of viremia in patient blood by reverse transcriptase-PCR [22]. The present study shows that 68.8 (62.5+6.3) of anti-HGV was found below age 24 years, so the prevalence was more in young age. In our result HGV RNA was detected by RT-PCR in 18.2% of thalassemia patients. In earlier studies, the significantly higher prevalence of HGV viremia was found in blood donors versus patients with multi-transfused anemia (1.7 vs. 18%) in Taiwanese children (23). However, HGV RNA was also detected in 12.9% of Iranian patients with thalassemia [24], which is similar to Italian patients (11%) with thalassemia [25].Moreover, a higher prevalence (19%) of serum HGV-RNA positive has been reported in Greek thalassemic patients [26]. Also Moatter et al. was detected the HGV RNA in 21% of thalassemia patients examined in Pakistan [27]. This may be explained by a greater number of transfused blood units per year in those thalassemia patients. Although a positive correlation between the prevalence of HGV infection and the history and/or numbers of blood transfusion have been reported in some studies from Japan [28] and [29] from France. Other reports indicated that infectiousness of HGV through blood products was low [22] or the clearance of the virus was rapid. Various factors have been present in patients who developed HGV infection with renal failure and those who were on dialysis despite they had multiple blood transfusions [30] or they had a history of surgeries and multiple injections.

In our study, we found that the HGV RNA was prevalent among individuals who were younger (39.3% in <20 year). In contrary to our findings, Shev et al. found no age relation in GBV-C/HGV positive patients with chronic hepatitis [31]. However, in many studies, HGV infection appeared to be acquired at younger ages than other known types of viral hepatitis, [32] which is similar to our finding. By contrast, the mean ages of anti-HGV were from 20-24 years. These findings might be explained by the fact that clearance of HGV may only occur after a long period of viremia [33] Hence, patients who were exposed to HGV may not yet have completely recovered. This theory is supported by the findings of Dille et al. who found that in patients with a post-transfusion hepatitis, the development of E2-specific HGV antibodies was associated with the loss of HGV viremia [34]. Gene sequencing, based on the nucleotide sequence of residue 134-395 of

the 5'-UTR region of HGV samples were studied and the results showed that genotype 2 was the most prevalent genotype and genotype 5 was only detected in one sample out of twelve. Reports from United States and Europe [35] and from other Middle Eastern countries (Iran, United Arab Emirates, Turkey, and Saudi Arabia) [36-39] showed that genotype 2 was the most prevalent genotype in these countries. In one case, genotype 5 was detected in thalassemia patient; this may reflect the African origin of this patient or donated blood. Finally, diagnosis of significant higher serological and molecular prevalence of HGV in patients with thalassemia in Iraq emphasized more on the importance of these viral hepatitis infections in introducing and complicating the clinical outcome of thalassemia patients.

Conclusion

In Iraq, the prevalence rate of HGV RNA in β-thalassemia major patients is 18.2%, while the prevalence rate of anti-HGV (past infection) is 10.4%. No one of thalassemia patients had HGV RNA and anti-HGV simultaneously. The Gene sequence analysis of PCR products identified HGV genotypes 2 and 5 with percentage of 91.7% and 8.3% respectively

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Elevated ceruloplasmin and leucocyte count in Type 2 diabetic nephropathy

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Abstract

Background : Type 2 diabetes mellitus is chronic disorder that requires proper medical care and education of patients to reduce long term complication like nephropathy .

Patients and methods: Forty-five Type 2 diabetic nephropathy patients in early stage were recruited for this study. Twenty-nine control subjects matched for age were also included.

Results : this study demonstrated a significant increase in glycosylated hemoglobin ,random blood glucose , blood urea , creatinine and total White blood cell count while a significant decrease in , estimated glomerular filtration rate in Type 2 diabetic nephropathy patients compared with healthy control . Urine markers including microalbumin and ceruloplasmin showed a significant increase in level in Type 2 diabetic nephropathy patients when compared with the healthy control.

Conclusion: leucocyte count can be considered as indicator for an inflammatory marker and a ceruloplasmin a good urinary marker.

Keywords: Diabetic nephropathy, ceruloplasmin, leucocyte count.

Introduction

Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia with disturbances in metabolism of carbohydrate, fat, and protein that results from the defect in insulin secretion, insulin action, or both [(1)] Chronic hyperglycemia is an associated with long-term dysfunction, and failure of various organs, especially the kidneys, eyes, heart, nerves, and blood vessels [(2)] Diabetic nephropathy (DN) is the most microvascular complication of diabetes including kidneys damage [(3)], it is the leading cause of kidney disease in patients starting renal transplant therapy

and affects about 40% of type 2 diabetic patients [4].

DN has many pathways for development such as glomerular hyperfiltration, advanced glycation end products, activation of the polyol pathway, upregulation of growth factors and increased oxidative stress [5]. Classical factors contributing to the pathology of diabetic nephropathy include hyperglycemia, hypertension, hypoinsulinemia, and hyperlipidemia [6].

It was noticed that leucocyte play an important role in progression of diabetes vascular complications. The leucocyte can be activated by glycation end product, oxidative stresses, angiotensin II resulting from hyperglycemia that produce factors like tumor necrosis factor α and interleukin β1that involves in pathogenesis of diabetic complication [7-11].

The ceruloplasmin /creatinine ratio is higher in diabetic nephropathy compared to nondiabetic nephropathy patients [12]. It has been reported that urine ceruloplasmin/ creatinine ratio has a sensitivity of 90-91%, specificity of 61–66% and 75% concordance, in diagnosing diabetic nephropathy [12,13].

The early medical treatment and lifestyle adjustments have been shown to reduce the progression from microalbuminuria to macroalbuminuria and end stage of renal failure. Therefore, the early detection of microalbuminuria as possible in the course of the disease is very important.

Subjects materials and method:

The study was conducted on 45 type 2 diabetic nephropathy patients, 23 females and 22 males, their ages ranged between 50-89 years. The patients were randomly selected from those attending the Al-fajer laboratory at Abdalmajid private hospital between December 2013 and July 2014.

The patients were having microalbuminuria or elevated serum creatinine was selected to this study. The information about the age and the presence of hypertension were documented from the patients.

For the purpose of comparisons, 29 healthy controls subjects comparable to diabetes mellitus patients in respect to age (50-89 year) and gender (12 females and 17 males), were included in the study. The controls were selected among subjects who were apparently healthy in terms of non-diabetic, no other endocrine disorders or metabolic kidney diseases and were free of acute illness or infection at time of sampling.

Five ml of blood were obtained from patients and control subjects by venipuncture, using a 5 ml disposable syringe, 3ml of blood and dispensed in a gel tubes and centrifuged at 3000 rpm for 10 minutes to collect serum. The serum was divided into aliquots in Eppendorf tubes for measuring the random blood glucose(RBG), blood urea , serum

creatinine, cholesterol and Triglyceride and stored in deep freezer at (-20◦C), other 2 ml were collected into EDTA tubes for measuring HbA1c , EDTA plasma for HDL cholesterol and total leucocyte count.

Urine was collected two times by spot urine collection. The urine was collected into sterile cups without any blood or urinary tract infection.

Serum creatinine and HDL cholesterol was measuring by Reflotron plus .

The microalbumin was determined by used microalbumin orgentic ELISA kit.

Human ceruloplasmin US Biological ELISA kit was used for determination the level of ceruloplasmin in urine.

Total leucocyte count was counted by using mindray BC-2800 hematology autoanalyzer. The following formula was used for calculated estimated GFR: [14]

$$eGFR = 186.3 \times [sCr]^{-1.154} \times age^{-0.203}$$

Age and s. Cr = serum creatinine concentration was measured in years. This formula is for white males. For females was multiplied by 0.742.

Statistical analysis:

Statistical analysis was carried out using statistical package (SPSS v.20). Students t-test was used and the differences were considered significant at the level of $P \leq 0.05$.

The values of the studied parameters were given in term of mean ± standard deviation.

Ethical issues :

All patients were informed a signed consent were obtained from patients and controls included in this study .the medical information were kept secret during analysis of the data for all participants in this research.

Results:

The result of Random blood glucose (RBG) was 154.91 mg/dl vs 94.90 mg/dl , Blood urea was 56.60 mg/dl vs 38.28mg/dl , Serum creatinine was 1.82 mg/dl vs 0.81mg/dl , eGFR was 49.52 ml/min/1.73m^2 vs 91.90 ml/min/1.73m^2, low density lipoprotein (LDL) was 115.66 mg/dl vs 102.48 mg/dl , glycosylated hemoglobin A1C was 7.90 vs 5.64 and total W.B.C count 10.8 x10^9 /L vs 7.69 x10^9 /L.

Table 1: The Biochemical and hematological parameter in Type 2 diabetic nephropathy and healthy control.

Parameters	Healthy Control (n=29)	T2 DN patients (n=45)	p-value
RBG (mg/dl)	94.90 ± 11.43	154.91 ± 61.65	**≤ 0.05**
HbA1c %	5.64 ± 0.35	7.90 ± 1.70	**≤ 0.05**
Blood .Urea (mg/dl)	38.28 ± 3.85	56.60 ± 24.53	**≤ 0.05**
S.Creatinine (mg/dl)	0.81 ± 0.20	1.82 ± 1.07	**≤ 0.05**
eGFR (ml/min/1.73m^2)	91.90 ± 20.48	49.52 ± 28.26	**≤ 0.05**
W.B.C count (x10^9/L)	6.69 ± 1.79	10.8 ± 2.70	**≤ 0.05**
S. Cholesterol (mg/dl)	177.52 ± 34.25	191.06 ± 54.50	N.S.
S.TG (mg/dl)	162.48 ± 98.66	181.60 ± 57.73	N.S.
HDL (mg/dl)	42.45 ± 5.55	39.66 ± 5.50	N.S.
LDL (mg/dl)	102.48 ± 29.98	115.66 ± 52.18	**≤0.05**

T2DN : Type 2 Diabetic nephropathy . s.TG : serum triglyceride . HDL : High density lipoprotein . LDL: Low density lipoprotein .RBG : Random blood glucose . HbA1c : glycosylated hemoglobin , W.B.C Count :White blood cell count, N.S. : Non-significant. Values are expressed as mean ± SD.

All the above - mentioned parameters demonstrated a significant ($P \leq 0.05$) increase in type 2 diabetic nephropathy patients as compared to healthy controls except eGFR shown a significant decrease (Table 1). The result of cholesterol 191.06 mg/dl vs 177.52 mg/dl , the triglyceride mg/dl 181.60 vs. 162.48 mg/dl and high density lipoprotein (HDL) 39.66 mg/dl vs 42.45mg/dl shows no significant ($P> 0.05$) difference between the Type 2 diabetic nephropathy patients and healthy controls, Also the patients age shows no significant ($P> 0.05$) difference between the patients and healthy controls (Table 1) .

The levels of microalbumin (200.7 µg/ml vs 32.54 µg/ml) increase significantly ($p\leq 0.05$) in Type 2 diabetic nephropathy patients compared with healthy control. Also, urine ceruloplasmin levels (60.72 ng/ml vs18.96 ng/ml) show a significant ($P\leq 0.05$) increase in Type 2 diabetic nephropathy compared healthy controls as shown in table (2).

Table 2 : the urinary parameters in Type 2 diabetic nephropathy and healthy control

Parameters	Healthy control (n=29)	T2 DN patient (n=45)	p-value
MALB strip (mg/L)	35.71 ± 19.87	99.78 ± 65.45	< **0.05**
MALB (µg/ml)	32.54 ± 25.22	200.70 ± 52.73	< **0.05**
Urine Ceruloplasmin (ng/ml)	18.96 ± 5.46	60.72 ± 10.93	< **0.05**

MALB strip: urine microalbumin measured by strips. MALB: urine microalbumin measured by ELISA. Values are expressed as Mean ± SD.

Discussion:

It was reported by John et al. that poor glycemic control, and raised blood 4r52tqpressure as risk factors of microalbuminuria. A long and permanent derangement of carbohydrate metabolism, that is, in decompensation stage of diabetes mellitus and in the absence of adequate treatment, the level of HbA1c increases. At the same time, hemoglobin and other body proteins undergo an enzymatic glycosylation [15]. These can cause receptor dysfunction, thickening of membranes and metabolic disorders, which are typical for progression of diabetes mellitus [16]

Strong relationship was found between blood urea level and blood sugar level. To monitor the diabetic patients, estimation of blood urea level along with blood sugar level could be important. Shrestha et al. found the gender was not a determining factor for the diabetes. There was no relationship between gender and the blood sugar levels likewise significant relation between gender and urea level was also not observed [17]

There were no significant differences in cholesterol, Triglyceride and HDL while LDL showed significant difference. This result agreed with Iranparvar et al. who found serum cholesterol level, HDL and LDL-cholesterol were within the normal range in both groups with no significant differences between them, Because the patients were currently taking statins and did not find any relation between serum cholesterol level and microalbumin [18]

Microalbuminuria was described for more than three decades ago as a predictor of nephropathy and associated with higher cardiovascular risk [19].

However, it has also been considered as the first indication of renal injury in patients with diabetes. Thus screening for microalbuminuria is currently recommended for all patients with diabetes or kidney disease [20]

Measurement of albumin in urine has important role in secondary prevention, to decide treatment and monitor response to treatment. The measurement of albumin in urine is not standardized. There is a large variation for estimation of albumin in urine between different laboratories and between different methods. Furthermore, there is no consistency among laboratories regarding sample type, units of reporting, and reference intervals or cutoff values [21].

Leucocyte count can reflect the inflammatory condition of the whole system .This study was found whether leucocyte count is an indicator for the development of microvascular complication of type 2 diabetes like nephropathy which that confirmed with Moradi et.al [22]. Urinary ceruloplasmin excretion is higher in type 2 diabetic patients compared to controls that agreed with Narita et.al [23], it could be predicts for the development of microalbuminuria in normoalbumin patients [24]. Glycemic control [25] effected on increased urinary ceruloplasmin excretion in normoalbumin patients. And diurnal changes in the systolic blood pressure significantly correlate with urinary ceruloplasmin excretion [26].

In conclusion leucocyte count can be added to diabetes control program as an early predictor of diabetes microvascular complication, it is regulated as cost effective easily performed and available in every laboratory that can show inflammatory biomarker. Ceruloplasmin is a promising urinary marker for diabetic nephropathy patients, but further studies are needed.

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Original article

The prevalence of Hepatitis C Virus Infection in sample of Iraqi Patients With Non –Hodgkin's Lymphoma

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ABSTRACT

Background: Non –Hodgkin's Lymphoma (NHL) are monoclonal proliferation of lymphoid cells that may be of B-cell (70%) or T-cell (30%) origin and include many discrete entities with characteristic morphological ,immunophenotypical , genetical and clinical features . Hepatitis C Virus (HCV) is a hepatotropic and lymphotropic virus, several studies showed that HCV may chronically infect patients with Non–Hodgkin's Lymphoma with or without producing liver damage.

Objectives: To assess the prevalence of hepatitis C virus infection in Iraqi patients with non-Hodgkin's lymphoma and to compare with two control group healthy control and patients had general medical illnesses (unhealthy).

Material and method: A prospective case control study included 40 Iraqi patients with NHL. They were tested for the presence of anti-HCV using Enzyme Linked Immunosorbent Assay (ELISA). Positive results were subjected to confirmatory test using Recombinant Immunoblot Assay (RIBA).The diagnosis of NHL cases was confirmed by two consultants histopathologist and they were classified according to the Working formulation system (WF) .Two control groups was applied ; the first included 250 healthy individuals who were age and sex matched while the second group included 50 patients suffering from general medical diseases who were age and sex matched.

Result: Four out of 40 patients with Non–Hodgkin's Lymphoma (10%) were positive for anti-HCV, whereas only 1out of 250 healthy individuals (0.4%) were positive for HCV and no patient was positive in the unhealthy control group.

Conclusion : The current study revealed the high prevalence of HCV infection in Iraqi patients with Non-Hodgkin's Lymphoma compared to the healthy and unhealthy control groups which support the lymphogenetic role of chronic HCV infection in the pathogenesis of NHL.

Key word: NHL ,HCV , ELISA ,Immunoblot

Introduction:

Non-Hodgkin lymphomas (NHLs) are a heterogeneous group of malignancies arising from lymphoid tissue, with varied clinical and biological features [(1)]. According to the Iraqi Cancer registry for the years 2008 and 2012, NHL ranked the sixth most common cancer among the ten most common cancers in Iraq, with male predominance in all age groups. The majority of cases arise within the 6th and 7th decades.[(2)] Non-Hodgkin lymphoma (NHL) is more common in the developed countries with the highest incidence rate in USA, Australia and New Zealand and Europe and the lowest in Eastern and South Central Asia. [(3)]

However the ratios of mortality to incidence rate are higher in developing countries [(4)] .Moreover those countries display intermediate and high grade diffuse aggressive or peripheral T-cell NHL, extra nodal disease which is closely associated with Epstein-Barr virus (EBV) and Human T-cell Leukemia/ lymphoma virus type I (HTLV-1) infections. [(5)]

These findings indicate region specific differences in exposure to environmental factors especially infections and chemicals and coupled to a broad range of genetic polymorphism[(4)].

The etiology of many cases of NHL remain largely unknown, however many suggested and established risk factors have been applied including a positive family history of lymph proliferative malignancies , hereditary immune deficiency disorders , acquired immunosuppression such HIV and organ transplantation , some infectious agents such as EBV and H. pylori and some autoimmune disorders. [(3)] Thus more detailed studies and investigations should be performed to determine the exact mechanisms of the disease initiation and induction of molecular and genetic changes. [(6)]

Hepatitis C virus (HCV) is a positive, single-stranded RNA virus, member of the Flaviviridae family, during its replicative cycle it goes through a negative-stranded RNA, but not DNA, intermediate, so that integration of HCV nucleic acid sequences into the host genome seems unlikely. As such, it lacks a pivotal property of classical oncogenic retroviruses. The HCV genome produces a single polyprotein that is proteolytically processed by viral and cellular proteases to produce structural (nucleocapsid, E1, E2) and nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [(7)]

The laboratory tests used to diagnose hepatitis C virus (HCV) infection consist of serologic assays that detect human antibodies against HCV (anti-HCV) and molecular assays that detect HCV nucleic acid. [(8)]

There are three different types of assays that can detect antibodies to hepatitis C virus: enzyme immunoassay (EIA), chemiluminescent assay (CIA) and recombinant immunoblot assay (RIBA). The EIA test is now the dominant HCV screening test used in clinical practice and this assay detects antibodies against epitopes derived from the HCV core, nonstructural 3, nonstructural 4, and nonstructural 5 regions. The RIBA originally was developed as a high-specificity confirmatory test for patients with a positive EIA result, but the importance of the RIBA has diminished with the marked improvement in specificity of the 3rd generation EIA tests and with the more widespread use of molecular assays. The recombinant immunoblot assay (RIBA) identifies the specific antigens to which antibodies are reacting in the EIA, and the results are interpreted as positive (2 or more antigens) [8,9]

Over the past 2 decades considerable evidence has accumulated on the association between hepatitis C virus (HCV) and hepatitis B virus (HBV) and several hematologic malignancies, most notably B-cell non-Hodgkin lymphoma (NHL) [10]. The aim of this study was to assess the prevalence of hepatitis C virus infection in Iraqi patients with non-Hodgkin's lymphoma and to compare with two control group healthy control and patients had general medical illnesses (unhealthy)

Patients' material and methods :

This case control study was conducted on 40 adult patients (> 15 years) with diagnosis of NHL who were attending Imamein Kadhimein Medical City and Baghdad Teaching Hospital during the period from first February 2014 to October 2014 .Eight patients were newly diagnosed with NHL and the rest were seen during follow up visit.

The diagnosis of NHL was confirmed by two consultant's histopathologist and was classified according to the Working Formulation .Consent for the participation in the research was obtained from each patient. All patients were married with no history of alcohol abuse and were not receiving interferon therapy. Any patient who was diagnosed with HCV after the diagnosis of NHL and those who were HCV seropositive NHL patient with overt high risk for viral infection were excluded (ex: drug abuser , patient on dialysis , patients who had received blood product after diagnosis of lymphoma and patient with significant cirrhosis)

Two control groups were applied; the first included 250 age and sex matched healthy individuals while the second included 50 age and sex matched patients having general medical diseases such as diabetes mellitus, cardiac disease, dyslipidemia; this group was most matched with the patient group to exclude any possible risk of hospitalization or effect of treatment or the disease on the result of the

study. For both control groups the same exclusion criteria to that used in patients group was applied

Sample collection: From each patient and control, three milliliters of venous blood were aspirated and transferred to sterile plain tube. The sample was centrifuged at 1000 rpm for 5 minutes to separate the serum which was dispensed into tightly closed Eppendorf tubes and stored at -20 C° until the time of the assay.Anti HCV was tested for all NHL patients and control groups in 100 ul of serum using sandwich immunoassay ELISA technique manufactured by CUSABIO BIOTECH co. code reference 4250, LOT: S3C1/4 . The procedure was carried out for patients, control and standard samples in accordance with the manufacturer's instructions of the kit. The concentration of the anti-HCV in the sample was determine by comparing the O.D, of the sample to that on the standard curve by using spectrophotometer at a wave length of 450 ±10 nm . Patients with positive or intermediate results were tested by Recombinant Immunoblot Assay (RIBA) to confirm positive results .

The confirmatory Recombinant Immunoblot Assay (RIBA) test was done following the manual instruction of the Kit of RIBA 3.0 Strip Immunoblot Assay (Chiron RIBA HCV 3.0 SIA, Chiron Corp., Emeryville, CA, USA) 3.0 SIA #930600

Results

This study had revealed that the mean age of NHL patients was 58.2±9.13 years (mean±SD) with range of 35-81 years and it was more common in male than female as shown in table 1 and 2 .

The present study had revealed that 4 out of 40 patients with NHL (10%) were positive for anti-HCV using both ELISA and RIBA technique for diagnosis as shown in table 3. Regarding control groups ; the first healthy control group showed that one out of 250 healthy individuals (0.4%) was positive for Anti –HCV whereas no positive case was detected in the second unhealthy control group (0%). Table 3

By applying Chi square test , both control groups differed significantly from the patient group .Table 3 The distribution of NHL cases according to the Working formulation classification was shown in table 4 , which showed that the most frequent type was the intermediate subtype and the least subtype was the high grade. Regarding HCV positive NHL patients , three of them had intermediate grade and the other one had low grade lymphoma .P>0.05.

Two of the four HCV positive NHL patients gave history of previous blood transfusion and were diagnosed with HCV more than 10 years before the diagnosis of NHL. Whereas the other patient did not give any history suggesting the route of infection and was diagnosed with HCV six years before the diagnosis of NHL . Any patients who were diagnosed with HCV after the diagnosis of NHL were excluded from the study. This study revealed that HCV was detected in 5 out of 340 (1.47 %) Iraqi individuals from Baghdad.

Table 1: The age distribution of the three studied group

Groups	Mean age ±SD (Years)	P-Value
Patients group n =40	**58.2±9.13 (35-81 years)**	=
1st control group n =250	**57.9±8.5 (40-71 years)**	**>0.05***
2nd control group n = 50	**58.7±10.5 (40-68 years)**	**>0.05***

P-Value > 0.05 non- significant .

Table 2 : The gender distribution of the studied groups

Groups	Male		Female		P value
	no.	%	No.	%	
Patients	29	72.5	11	27.5	<0.05
Control 1st group	155	62	95	38	>0.05
Control 2nd group	32	64	18	36	>0.05

P-Value > 0.05 non- significant

Table 3- The incidence of HCV in the study groups

	Patients N=40		1st control N=250		2nd control N=50		P value
	no.	%	no.	%	no.	%	
HCV +	4	10	1	0.4	0	0	<0.0001
HCV_	36	90	249	99.6	50	100	<0.02

Table 4 : The distribution of the cases according to the WF classification of NHL

Grading	NHL n= 40	HCV +ve n= 4	%	p-value
Low	13	1	7.7	> 0.05
Intermediate	21	3	14.2	
High	6	-	-	
Total	40	4	10	

Discussion:

This study had revealed that NHL was more common in adult male than female which was consistent with Iraqi cancer registry and other Iraqi and non-Iraqi studies [2]

This study had demonstrated that the prevalence of HCV infection in sample of NHL Iraqi patients was significantly high reaching

10% as compared to normal healthy control population (0.4%) and unhealthy control patients (0%). A positive association between HCV and NHL was first described by Ferri et al [10] and Pozzato et al [11] , and was further confirmed in a large number of studies Mele et al [12] Duberg et al [13] and Anderson et al [14] as well as in Mezzaro et al study in 2005[9] ; those studies had found that the prevalence of hepatitis C virus infection in non-Hodgkin's lymphoma had ranged between 7.4 and 37.0%.

Since the pathogenic mechanism involved in hepatitis C virus-associated lymphomas remains considerably unknown, thus it is assume that the virus may exert its oncogenic potential via an indirect mechanism or utilizes other pathways directly and that several different pathogenic mechanisms may operate in the etio-pathogenetic role of hepatitis C virus in non-Hodgkin's lymphoma [9]

Zignego et al and Giordano et al studies had found that HCV-induced lymphomagenesis through three pathways ; (A) Chronic antigenic stimulation of a B cell through the interaction of B cell surface Igs with the cognate HCV antigen. (B) HCV-E2 protein will engage its high-affinity receptor CD81 which is expressed on B cells. (C) Direct infection of a B cell by HCV. [15,16] In the current study the NHL associated HCV cases were of the intermediate and low grade subtype ; this was similar to Mazzaro et al study who had state that lymphoproliferative disorders related to hepatitis C virus usually include the intermediate -grade lymphoma, and the more common indolent, low-grade lymphoma .[9]

The present study had tested HCV in 340 subject and it was positive in 5 of them (1.47 %) which can be considered as a limited number of community base study. HCV Infection occurs

throughout the world, however the incidence of HCV on a global scale is not well known, because acute infection is generally asymptomatic A review study was done by Hanafiah et al to estimate age specific sero-prevalence curves in 1990 and 2005, it found that globally the prevalence and number of people with anti-HCV has increased from 2.3% to 2.8% between 1990 and 2005. Central and East Asia and North Africa/Middle East are estimated to have high prevalence (>3.5%); South and Southeast Asia, sub-Saharan Africa, Andean, Central, and Southern Latin America, Caribbean, Oceania, Australasia, and Central, Eastern, and Western Europe have moderate prevalence (1.5%-3.5%); whereas Asia Pacific, Tropical Latin America, and North America have low prevalence (1.5%) [18] It is recommended that wide scale survey should be done to study the prevalence of HCV in Iraq .

Even though Hepatitis C virus (HCV) has been recognized as a potential cause of B-cell lymphoma but the management of them is still similar to that of conventional lymphoma ; thus recently trial therapy with antiviral therapy was applied for treatment of low grade hepatitis C viral –related lymphomas which had lead to regression of them.[17]

Conclusion:

References This study had show greater prevalence of HCV infection in NHL patients compared to that reported for the normal population .Moreover the intermediate and low grades are the most frequent subtypes

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Original article

C- Reactive protein and iron status in Iraqi patients with acute myeloid leukemia before and after treatment

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ABSTRACT

Background: Acute Myeloid Leukemia (AML) is a clonal hematopoietic disorder, leading to a premature arrest of the normal differentiation of stem cells. C - reactive protein (CRP) is a marker of inflammation. Serum level of C-reactive protein may be increase in patients with AML. This association between CRP levels and acute myeloid leukemia influenced by multiple factors. Acute myeloid leukemia commonly associated with iron overload. Many factors are participating to the hyperferritinemia associated with AML, inflammation chemotherapy, blood transfusion and ferritin hepatic clearance disorders

Objectives: To assess serum C-reactive protein (CRP) and iron status (serum Iron, Total iron binding capacity, serum ferritin levels) in patients with acute myeloid leukemia (AML) before and after chemotherapy

Materials and Methods: A prospective cohort study included 58 patients (30 male and 28 female) with acute myeloid leukemia with age range (15-65 years). Patients divided into two groups: Group (1) Patients with AML before starting chemotherapy. Group (2) the patients after 4 weeks of chemotherapy In addition to 43 healthy subjects (24 male and 19 female) were included. They were age and sex matched to patients group and considered as controls as (Group 3). This study conducted at the National Center of Hematology and Baghdad Teaching Hospital in the Medical City from February 2014 to June 2014. All patients were subjected to complete history and physical examination. Diagnosis as AML patients was established by complete blood count and blood film, bone morrow aspiration and biopsy. C-reactive proteins. iron, s.total iron binding capacity and s. ferritin were estimated for all .

Results: Serum CRP levels increased in AML patients before and after treatment , while there were a significant increase in mean serum ferritin levels observed in (Group 2) compared to newly diagnosis patients (Group 1)(P<0.002) and the levels were significantly higher in newly diagnosis group compared to healthy controls (P<0.015). Patients with (AML) during remission show significant decrease in iron levels compared to newly diagnosis group (P<0.0001), while levels in healthy controls recorded higher values than both (Group 2) and (Group 1) (P<0.0001). Serum total iron binding capacity (TIBC) levels showed a significant decrease in (Group 2) after treatment compared to (Group 1) before treatment(P<0.0001) but the levels were significantly higher in healthy controls compared to (Group 1) and (Group 2) (P<0.0001)

Conclusion: CRP does not predict response to chemotherapy while it may be of benefit in predicting infection or inflammation in patient with AML post chemotherapy. Regarding Iron status: s.ferritin increase significantly post chemotherapy while s.iron and TIBC decrease.

Keywords: CRP, iron status, AML

Introduction:

Acute Myeloid Leukemia (AML) is a clonal hematopoietic disorder arising from the acquisition of genetic and epigenetic alterations, leading to a premature arrest of the normal differentiation of stem cells and to the accumulation of immature neoplastic cells in the blood and bone marrow [(1).] Changes in white blood cells lead to impaired ability to fight infection and decrease the ability of the bone marrow to form red blood cells and platelets [(2)]. Rate of (AML) incidence raises in male than in female and with progressive of age [(3)]. The development associated with myelodysplastic syndromes (MDS), genetic disorders, acquired diseases, exposures to ionizing radiation and alkylating agents and exposure to anti-cancer chemotherapy [(4)]

Main induction therapy consists of cytarabine (Ara-C) and anthracycline based regimen. It has been found that the complete remission (CR) rate is approximately 60% to 80% in newly diagnosed younger adult patients with AML treated with 3+7" [(5)]. The remission induction therapy in leukemia produces normal bone marrow function, thereby complete remission is defined when the patients have full recovery of normal peripheral blood counts with recovery of normal bone marrow cellularity, and less than 5% blast cells are present in the bone

marrow" [6] Post remission therapy "consolidation therapy" is needed to damage remaining Leukemic cells and prevent relapse [7, 8]

C-reactive protein (CRP), a plasma protein of the pentraxin family and an acute phase reactant, which displays high sensitivity as a general inflammation marker [9]. It is produced and secreted mainly by liver in response to cytokines such as interleukin-6 [10], released from leukocyte within tumor microenvironment such as (location, age, gender). Blast cells growth could cause inflammatory response, thereby increasing CRP levels. Alternatively, chronic inflammation could lead to the development of cancer. CRP is a marker of inflammation, has a direct role in carcinogenesis [11].Serum level of C-reactive protein has a plasma half-life of 19 hours. [12] The association between CRP levels and acute myeloid leukemia risk influenced by multiple factors

During immune activation, ferritin is known as an acute phase reactant because of its intracellular iron storage abilities [13]. By hepatocytes, and also by other cell types, including macrophages and cancer cells it is produced and secreted [14]. Serum ferritin levels may be elevated in infection, systemic inflammation, and malignancies [15]. Evidences were suggested that, there was association between high body iron stores and the risk of developing cancer [16]. So, increased in serum ferritin might indicate the exists of malignant disease spatially in acute and chronic leukemia [17] Acute myeloid leukemia commonly associated with iron overload [18] Many factors are participating to the hyperferritinemia associated with AML , inflammation chemotherapy, blood transfusion and ferritin hepatic clearance disorders [19]. In other study on malignant cells predicted that malignant cells need a high requirement of iron due to the rapid division of the cells. Tumor cells were changed routes of the uptake of iron. These routes may be important in achieving raised iron levels under this condition [20].

Recent study predicted that iron is a risk factor for different types of cancers mainly due to its prooxidant activity. Non–protein-bound iron ("free" or catalytic iron) is a prooxidant, as its participation in the redox cycling which is associated with the generation of reactive oxygen species (ROS) such as the hydroxyl radicals. ROS are highly reactive molecules capable of oxidative damage to DNA [21]. Increased cellular iron may cause tumorigenesis. Neoplastic cells were higher iron requirements than normal cells; therefore decreasing iron level was developed as efficient strategies in chemotherapy and from malignant cells themselves [18]

Total iron binding capacity (TIBC) determines the maximum amount of iron that serum proteins can bind. TIBC assay measure the total number of transferrin binding sites per unit volume of plasma or serum. Normally, almost all the binding capacity is due to transferrin. One third of plasma TIBC is saturated with iron [22] Plasma TIBC rises in iron deficiency, but often tends to be low in patients with iron overload and in protein losing states such as infections, neoplasms, anemia of chronic disease and after trauma [23]. Elevated TIBC, were associated with increased risk for developing various types of cancer such as acute myeloid leukemia [21].

Materials and methods

The prospective cohort study conducted at the National Center of Hematology in Al-Mustansiriyah University and Baghdad Teaching Hospital in the Medical City from February 2014 to June 2014. This study was approved by scientific committee of Mustansiriyah Medical College. Questioner history and consent was obtained from all patients prior to study, fifty-eight (58) patients (30 male, and 28 patients female) aged between (15-65 years)

Inclusion criteria included patients with newly diagnosis of AML, age between (15-65 years), and no history of illness, while the exclusion criteria included patients of AML with subtype M3, age of patients was under 15 years and above 65 years, patients with relapse and refectory of AML and Frail patients not suitable for chemotherapy.

Fifteen patients (15) out of fifty-eight (58) were excluded from the study because preventing to take chemotherapy, went to another center outside Baghdad, loss of follow up or early death during period of study. After exclusion of fifteen patients, forty three patients (43) contained the study, (24 male and 19 female). Patients divided into two groups: Group (1): Patients with AML before starting chemotherapy. Group (2): Patients after 4 weeks of chemotherapy. Group (3): Forty-three (43) healthy subjects (24 male and 19 female) were included in the study mainly from medical staff and their families. They were age and sex matched to patients group and considered as controls.

All Patients were subjected to complete history and physical examination. The diagnosis was established by complete blood count and blood film, bone morrow aspirated and biopsy, liver function tests, and renal function tests. Other parameters were done in this study such as ferritin, CRP, S. iron, and total iron binding capacity.

Patient's treatment was done according to international protocol which is called (3+7)

when Daunorubicin was given in the first day to third day and Cytarabine (Ara-C) was given from the first day to seventh day then evaluation is done on twenty eighth day to evaluate response of patients. Five milliliters (5 ml) of venous blood were taken from each patients and controls. Blood samples were put in plain polyethylene tube and allowed to clot at room temperature for thirty minutes, then samples were centrifuged at (3000 rpm) for (10 min). The obtained serum were frozen at -20 C to be analyzed later, hemolyzed samples were discarded. Latex Agglutination Slide Test was used for the Qualitative and Semi-quantitative Determination of serum C - reactive protein (CRP) in Non-diluted (manufactured by Human-Germany). This measurement depends on the immunological reaction between C-reactive protein (CRP) of a patient specimen serum and the corresponding anti-human CRP antibodies bound to latex particles. In the test cell of the slide, the positive reaction is reflected by a visible agglutination of the latex fractions [24]

Estimation of serum ferritin levels was done by immunoenzymometric assay required essential reagents such as antibodies with affinity and specificity (enzyme and immobilization) using kit (manufactured by Monobind- UAS) [25]. Serum iron concentration was determined by iron Colorimetric test. Estimation of Serum Total Iron Binding Capacity (TIBC) was measured using kit manufactured by (Human-Germany) [27]. TIBC in serum is saturated with a further concentration of Fe+3 ions. Unbound iron (increase) is absorbed by aluminium oxide and precipitated

Statistical analysis: Data were analyzed using the available statistical package of SPSS-22 (Statistical Packages for Social Sciences- version 22), and presented in simple measures of frequency, percentage, mean, standard deviation, and range (minimum-maximum values)

The significance of difference of different means (quantitative data) were tested using Students-t-test for difference between two independent means or Paired-t-test for difference of paired observations (or two dependent means), or ANOVA test for difference among more than two independent means. The significance of difference of different percentages (qualitative data) was tested using Pearson Chi-square test with application of Yate's correction or Fisher Exact test whenever applicable. Pearson correlation was calculated for the correlation between two quantitative variables with its t-test for testing the significance of correlation.

Results:

Regarding distribution of age and gender for AML patients and controls were shown in Table (1). Results of this study demonstrated that the screening test for C-reactive protein (CRP) levels in the most patients showed positive test while all controls showed negative test therefore the comparison is done between two groups of AML patients. CRP levels showed significant differences in two groups of AML : before starting treatment and after treatment in all titers of CRP in(mg/L) (≤6 x >6, ≤12 x >12, ≤24 x >24) with P-Value of 0.014, 0.045, 0.013 respectively as shown in Table(2)

Regarding serum ferritin levels changes in AML patients show a significant increase in patients during remission compared to newly diagnosis , values were statistically significant (849.1±777.6 ; 624.0±197.68 ng/ml , respectively [P<0.002] and the mean values were significantly higher in newly diagnosis patients compared to controls (624.0±197.68; 132.4±138.7 ng/ml ,respectively [P<0.015]) and significantly increase in the patients during remission compared to controls (849.1±777.6; 132.4±138.7 ng/ml , respectively [P<0.0001]) as shown in the Table (3).

In addition, results of iron study showed that the mean serum iron levels in acute myeloid leukemia patients during remission were significantly lower compared to newly diagnosis patients (12.57±1.98; 15.11±2.32 mmol/L), respectively [P<0.0001] .In the same time these mean iron values were significant lower in both newly diagnosis and during remission patients compared to controls (15.11±2.32;12.57±1.98; and 23.19±2.66 mmol/L, respectively [P<0.0001]) as shown in the Table (4) ,

Estimation of total iron binding capacity (TIBC) observed that there were a significant decrease in mean serum TIBC levels in acute myeloid leukemia patients during remission compared to newly diagnosis patients , values were statistically significant (37.63±7.63; 51.32±4.78 mmol/L, respectively [P<0.0001]) , and the mean significantly decrease in the newly diagnosis compared to controls (51.32±4.78; 58.24±5.27 mmol/L, respectively [P<0.0001]) and significantly decrease in the patients during remission compared to control (37.63±7.63;58.24±5.27mmol/L

Discussion:

the prevalence of age in AML group was(40-59) years and (≥ 65 years) (30.2%) were higher than other age groups. Our result results predicting that

AML is more common in elderly. So, AML is generally a disease of old age [28]. These findings consisted with previous report [29, 30]. The results of this study showed that there were a significant increase in serum C- reactive protein CRP) levels in patients with acute myeloid leukemia before treatment because of the response to tumor necrosis, local tissue damage, or associated inflammation. These results were agreed with other studies results [31,32], after treatment, infections in the immunocompromised host as a result of chemotherapy, were associated with elevation occurrence of neutropenic complication, which influences to response to chemotherapeutic, and cause morbidity and mortality. Additionally, malignant process itself causes increasing in CRP levels in spite of the presence of systemic bacterial infections [33]. These findings were in agreement with study done by Endo et al. [34]

Serum ferritin concentrations were estimated in this study for AML groups and it was found that there was an increase in values of serum ferritin in newly diagnosed patients and post chemotherapy when compared to healthy controls. These results were agreed with [35] results, who found that serum ferritin levels among patients newly diagnosed or on remission stage were significantly increased which may indicate that leukemia cell could affect iron metabolism leading to iron overload .Other study showed that the highest levels of ferritin were found in AML patients under chemotherapy course treatment [36].

There was a growing in evidences which predicted that iron overload is common in patients with hematologic malignancies [37], and the excessive iron body stores are known to interfere with natural body defenses and the increase in body stores of iron lead to increase growth rate of cancer cells [38]. Previous study suggested factors that may contribute to the increase in serum ferritin levels in acute myeloid leukemia including as followings:

1. All patients of acute myeloid leukemia are anemic and have an elevation in the amounts of iron storage which are presented by further serum ferritin levels. In large mass of leukemic cells elevated the production of ferritin, this leads to raise in serum ferritin levels.

2. Treatment by chemotherapy leads to damage most of the cells in the body, which lead to release of abnormal amounts of ferritin. There was no correlation between the elevation in the circulating of ferritin during chemotherapy with the amount of blood transfused or the degree of liver damage.

3. The elevation in ferritin levels is a marker of acute phase response, and this acute phase usually founds in the acute myeloid leukemia due to increase in the concentrations of ferritin in the body [39]

Regarding to serum iron levels investigation in this study, the results showed a significant decrease in serum iron levels in pre and post chemotherapy treatment comparing to healthy subjects. The evaluation of deregulations of iron metabolism is very important in serum iron studies, especially iron deficiency and iron excess. Physiological function of iron is importance to produce red blood cells and to use as antimicrobial defense [40] several previous studies also indicated that reducing in serum iron levels in AML patients may due to iron deficiency anemia and acute and chronic infections. These results are in agreement with previous results reported [41].

Other study agreed with these results was by sheikh et al [42], who observed that iron was thought to be a risk factor for cancer development in epidemiological studies in humans. The reducing in serum iron level leads to interfere with the vital functions and increased mortality risk. Before and after chemotherapy treatment, serum iron is effected by several factors including iron absorption from diets; infection, inflammation, and diurnal variation. Patients with AML have inflammation which caused reducing in the iron availability to cells [43]

Other related study showed that two biological effectors change the plasma iron concentration: infection and inflammation .Serum iron concentrations were affected by inflammatory factors that released from cells of immune system during the inflammatory process. Inflammation stimulate the movement of iron from the plasma pool into storage sites in macrophages, this explain the reduction in iron concentrations with the releasing of the inflammatory factors and lead to reduce in the hormone erythropoietin production, reduce response to erythropoietin, and interference with iron metabolism. Finally, anemia of inflammation caused reducing in serum iron levels [44]

In this study, the total iron binding capacity of acute myeloid leukemia patients before and during chemotherapy was lower than in the controls. These findings consisted with previous report which suggested that the production of iron binding proteins is became weak pre, during, and post chemotherapy and decrease the ability of the liver to absorb from the circulation non transferrin bound iron [45] Total iron-binding capacity (TIBC) presents the availability of iron-binding sites, which is influenced by factors: iron status, malnutrition, inflammation, chronic

infection, and cancer. Patients with hematologic disorder including AML cannot mobilized and utilize iron, which is stored in excess in reticuloendothelial system leading to decrease in serum (TIBC) [44]

Conclusion

CRP does not predict response to chemotherapy while it may be of benefit in predicting infection or inflammation in patient with AML post chemotherapy. Regarding Iron status: s.ferritin increase significantly post chemotherapy while s.iron and TIBC decrease

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Table (1): Age gender distribution of studied groups

Characterization		AML		Control	
		No.	%	No.	%
Age(years)	<20	7	16.3	8	18.6
	20-39	10	23.3	10	23.3
	40-59	13	30.2	12	27.9
	≥65	13	30.2	13	30.2
	Mean ±SD(Range)	43.0 ±18.6	(15-65)	43.0 ±18.6	(15-65)
Gender	Male	24	55.8	24	55.8
	Female	19	44.2	19	44.2
*Significant difference between proportions using Pearson Chi-square test at 0.05 level					

Table (2): Mean C - reactive protein Levels for Studied Groups.

		AML Before treatment		AML After treatment	
		No.	%	No.	%
CRP (mg/L)	0	8	18.6	3	7.0
	6	6	14.0	1	2.3
	12	-	-	2	4.7
	24	7	16.3	4	9.3
	48	22	51.2	33	76.7
P value comparing (CRP=<6 x >6)	0.014*				
P value comparing (CRP=<12 x >12)	0.045*				
P value comparing (CRP=<24 x >24)	0.013*				
*Significant difference between proportions using Pearson Chi-square test at 0.05 level					

Table (3): Mean Serum Ferritin Levels for Studied Groups

Serum Ferritin (ng/ml)	AML Before treatment	AML After treatment	Controls
Number	43	43	43
Mean+SD	624.0±197.68	849.1±777.6	132.4±138.7
Standard Error of Mean	30.146	118.588	21.157
Range	300-1200	410-5704	40.9-982
P value compared to Control	0.015*	0.0001*	-
P value compared to AML After	0.002*	-	-

*Significant difference between two independent means using Student-t-test at 0.05 level

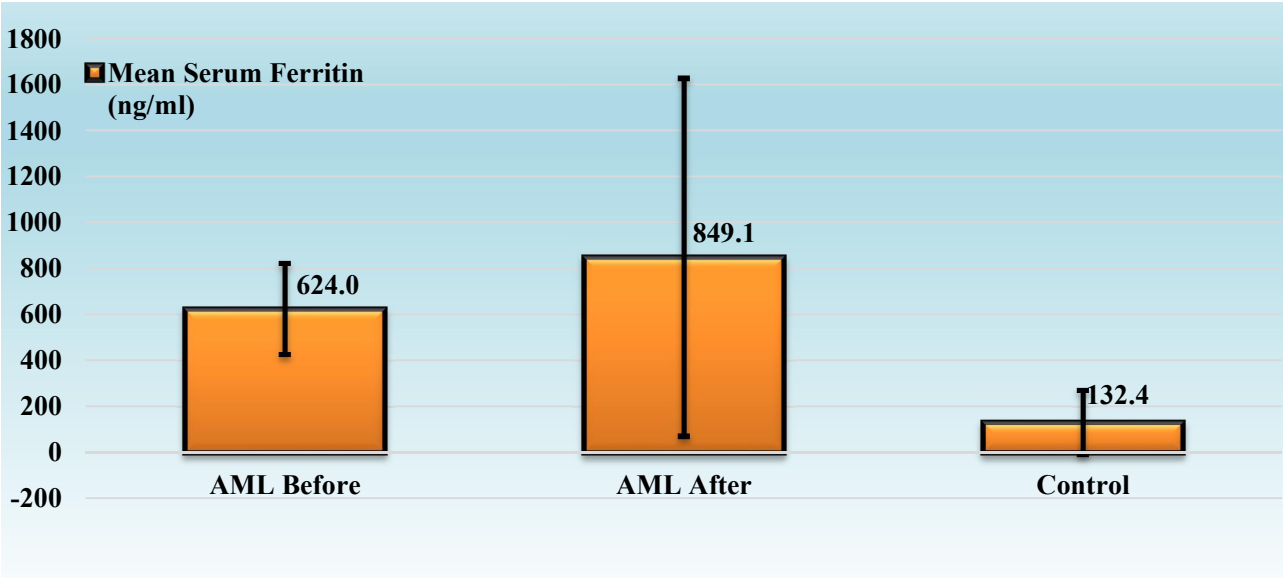

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Table (4): Mean Serum Iron Levels for Studied Groups:

Serum Iron (mmol/L)	AML Before	AML After	Controls
Number	43	43	43
Mean±SD	15.11±2.32	12.57±1.98	23.19±2.66
Standard Error of Mean	0.354	0.301	0.406
Range	10.60-20.10	8.81-16.30	19.02-28.00
P value compared to Control	0.0001*	0.0001*	-
P value compared to AML After	0.0001*	-	-
*Significant difference between two independent means using Student-t-test at 0.05 level			

Table (5): Mean Serum TIBC Levels for Studied Groups.

TIBC (mmol/L)	AML Before	AML After	Controls
Number	43	43	43
Mean±SD	51.32±4.78	37.63±7.63	58.24±5.27
Standard Error of Mean	0.729	1.163	0.804
Range	42.00-69.00	15.00-60.20	49.00-69.00
P value compared to Control	0.0001*	0.0001*	-
P value compared to AML After	0.0001*	-	-
*Significant difference between two independent means using Student-t-test at 0.05 level			

Original article

Assessment of serum ferritin levels in thalassemia and non-thalassemia patients presented with anemia

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ABSTRACT

Background: Thalassemia is an autosomal genetic disease leading to anemia and remains one of the major health problems in Southeast Asia and other parts of the world. Almost 100,000 patients with major thalassemia need regular transfusion. Human hemoglobin (Hb) is the molecule that carries and transports oxygen all through the body. Ferritin is the principal iron storage protein, found in the liver, spleen, bone marrow, and to a small extent in the blood.

Objectives: The aim of this study was to assess serum ferritin levels in B-thalassemia patients, and to compare it with non-thalassemic anemia and healthy control.

Materials and methods: A prospective cross sectional study conducted at Thalassemia Center in Ibn Al- Baladi Hospital for Children and Women during the period from1st February to 30th May 2014 during their attendance to out-patient clinic. A total .number of 101 patients complaining of anemia (51 patients with thalassemia, 50 with non-thalassemia) in addition to 50 healthy subjects considered as control. All patients were tested for Serum ferritin levels and all results were obtained through automated quantitative test for use Vidas machine

Results : The mean serum ferritin levels in cases of thalassemia was 9542 $\pm$ 782 ng/ml while serum ferritin levels in control sample was 138 $\pm$323 ng/ml in male and 28$\pm$ 108 ng/ml in female . in patients with non-thalassemia anemia, the levels of serum ferritin was 1$\pm$80 ng/ml. Age of all patients in this study ranged from 3day-9year .the age of thalassemia patients ranged from 1-6 year and p-value was(0.23) while the age of patients with non-thalassemia anemia was 1-5 year and p-value was (0.11).

Conclusion: This study confirm that serum ferritin is high in patients wih thalassemia than non thalassemia.

Keywords: thalassemia, serum ferritin**.**

Introduction

Thalassemia is an autosomal genetic disease leading to anemia and remains one of the major health problems in Southeast Asia and other parts of the world where malaria is or has been endemic [(1)].

The thalassemia are a group of inherited hematologic disorders caused by defects in the synthesis of one or more of the hemoglobin chains [(2)].

Beta-thalassemia major is a common inherited hematological disorder in Asia. Almost 100,000

patients with major thalassemia need regular transfusion [3]. Iron deficiency is the most prevalent nutritional deficiency and the most common cause of anemia in the United States [4] Iron deficiency anemia is characterized by a defect in hemoglobin synthesis, resulting in red blood cells that are abnormally small (microcytic) and contain a decreased amount of hemoglobin (hypochromic) [5] ,The capacity of the blood to deliver oxygen to body cells and tissues is thus reduced. Anemia is seen in many chronic diseases such as heart failure, rheumatoid arthritis, cancer and chronic renal disease [6,7]

Human hemoglobin (Hb) is the molecule that carries and transports oxygen all through the body. It is made up of two parts: heme and globin. Heme is a porphyrin containing iron. Globin is made up of four polypeptide chains of two types two alpha and two non-alpha chains (beta â, gamma ã and delta ó). Adult and fetal hemoglobins have á-chains combined with â-chains. In normal adults, Hb A is the main type of hemoglobin (96.98%) while HbA2 and Hb F are only present in 2.3% and less than1%, respectively [8,9].

Cardiac disorders related to ventricular failure are the most frequent causes of death in this syndrome [10,11] . According to literature, heart failure from iron overload causes 71% of deaths in thalassemia major patients [12] Ferritin is the main iron-storage protein in the body. Its synthesis is regulated by quantities of iron by means of the interaction of cytoplasmic proteins bound to the messenger ribonucleic acid (mRNA), currently identified as iron regulatory proteins with specific structures of the mRNA, called iron-responsive elements [13]. Ferritin is the principal iron storage protein, found in the liver, spleen, bone marrow, and to a small extent in the blood (serum ferritin - SF) [14] the standard method of evaluating the total amount of body iron is measurement of the SF concentration in the blood [15,16] ,However the correlation between SF and body iron is not sufficiently precise to be of high prognostic value, especially when associated with inflammation or tissue damage. Moreover, alterations in the relationship between blood serum ferritin concentration and body iron content by chelation and vitamin C treatment are complex. For example, the relationship between serum ferritin and body iron appears to be singular for different hematologic conditions[3] .A variety of tests are currently used to assess iron overload, including serum ferritin level, computed tomography(CT), magnetic resonance imaging (MRI), and liver iron content (from biopsy) [17]. Among these procedures, serum ferritin level is a commonly used measurement as it is minimally invasive, inexpensive, and widely available and can be performed frequently allowing regular monitoring,and the values correlate with total body iron store [15,18]. Serum ferritin levels consistently >1000 μg/L are indicative of iron overload [15,19,20].

Materials and methods

A prospective cross sectional study conducted at Thalassemia Center in Ibn Al- Baladi Hospital for Children and Women during the period from1st February to 30th May 2014 during their attendance to out-patient clinic. A total .number of 101 patients complaining of anemia (51 patients with thalassemia, 50 with non-thalassemia) in addition to 50 healthy subjects considered as control. All patients were tested for Serum ferritin levels through Blood Samples collection by EDTA blood samples tube from thalassemia, non-thalassemia patients and healthy controls. The age of the patients ranged between 3days – 9 years.

The beta-thalassemia trait identified through clinical evaluation and laboratory tests, Hemoglobin electrophoresis

(Hb electrophoresis) is used as a screening test to identify variant and abnormal hemoglobin, including hemoglobin A_1 (HbA_1), hemoglobin A_2 (HbA_2), hemoglobin F (HbF; fetal hemoglobin), hemoglobin C (HbC), and hemoglobin S (HbS). analyzed using HPLC (high-performance liquid chromatography), (VARIANT β-thalassemia Short Program, Bio-Rad Laboratories) .

Serum ferritin levels were obtained through automated quantitative test by using vidas machine or using ELFA technique (enzyme linked fluorescent assay),using vidas biomerieux france. The limits for normal ferritin levels 138-321 in man and 27.15-102 ng/ml in woman.

Results

A total of 151 cases, 51 cases of B- thalassemia, 50 cases non-thalassemia and 50 healthy control sample major were enrolled in this study

In B-Thalassemia group there were 31(69.78%) males and 20 (39.22%) females with a male to female ratio of 1.5: 1. The age of patients B-thalassemia at the time of diagnosis ranged from 10 months to 6 years, Age of male in this study was from10 month to 6 years where's female age was from1 years to 4 years , Table 1 describe age of all patients.

The mean serum ferritin levels in cases of B-thalassemia were 9542 ±782 ng/ml. Only one patients(1.96%) had serum ferritin levels of less than 1000 ng/ml. Sixteen patients (31.37%) had serum ferritin levels between 1000 – 2000 ng/ml, while 23 patients (54.90%) had values levels between 2000-4000ng/ul ,five patients (9.80%) had serum ferritin levels between 4000-8000 ng/ml and one patients (1.96%)more than 8000 ng/ml (Table 2). Serum Ferritin Levels in control group (50 sample), 25(50%) was male 25(50%) female , mean Serum Ferritin Levels in male was 138 ± 323 and 28 ±108ng/ml in female table3.

Table1: Age and gender Distribution in Beta Thalassemia group and healthy controls group .

age	Thalassaemia pateints (n=51)	Male:female	Healthy (n,50)	Male:female	p-value
<1year	1(1.96%)	(1,0)	6(12%)	(4,2)	
1-2year	8(15.69%)	(5,3)	10(20%)	(6,4)	
2-3year	16(31.37%)	(11,5)	14(28%)	(8,6)	
3-4year	14(27.45%)	(6,8)	11(22%)	(3,8)	
4-5year	11(21.57%)	(7,4)	5(10%)	(2,3)	
>5year	1(1.96%)	(1,0)	4(8%)	(2,2)	
					0.23695

Table 2: Serum Ferritin levels in Beta Thalassemia group

Levels	Patients no.	Percent	Male	female
<1000ng /ml	1	1.96%	1	0
1000-2000ng /ml	16	31.37%	11	5
2000-4000ng/ml	28	54.90%	16	12
4000-8000ng/ml	5	9.80%	2	3
>8000ng/ml	1	1.96%	1	0

Table 3: serum ferritin levels in control sample (healthy).

Age	Number of control		percent	Serum ferritin level
5m-5years	Male	25	50%	324+138
5m-5.5year	female	25	50%	108+28

In patient with non-thalassemia group there were 26(52%) male and 24(48%) female ,the age of patient ranged from 3month-6years for female and 9days - 9 years for male table 4. The serum ferritin levels in this group was as follows : one patient (2%) had value < 1ng/ml ,sixteen patients (32%) had serum ferritin level between 1-10 ng/ml ,14 patient (28%) 10-20ng/ml table 5 .

Table 4: Age Distribution of the Patients with non-thalassemic anemia and healthy controls.

age	Non-Thalassaemia pateints (n=50)	(male,female)	Healthy Controls(n,50)	(male,female)	p-value
<1year	5(10%)	(3,2)	6(12%)	(4,2)	
1-2year	13(13%)	(7,6)	10(20%)	(6,4)	
2-3year	9(18%)	(4,5)	14(28%)	(8,6)	
3-4year	8(16%)	(4,4)	11(22%)	(3,8)	
4-5year	10(20%)	(5,5)	5(10%)	(2,3)	
>5year	5(10%)	(3,2)	4(8%)	(2,2)	
					0.112275

Table 5:serum ferritin levels in patients with non-thalassemic anemia

Levels	Number of patients	percentage	number of male	numbre of female
<1ng/ml	1	2%		1
1-10ng/ml	16	32%	8	8
10-20ng/ml	14	28%	5	9
20-40ng/ml	10	20%	7	3
40-80ng/ml	4	8%	2	2
<80ng/ml	5	10%	4	1

Discussion

Ferritin is the main source of stored iron whereas hemosiderin is described as degraded form of ferritin [21] appearing as blue intracellular granules that are large enough to be viewed by a light microscopy [22]. Iron was taken from plasma to cytosol of young erythroid cells in the bone marrow for heme synthesis via transferrin-transferrin receptor pathway [23] Certain previous studies have shown that patients with beta thalassemia have levels of serum ferritin far more than the patients presented with anemia due non-thalassemic causes as shown in (Table 6).

Table 6: studies of serum ferritin in thalassemia

Nadeem *et al*(24)	2004	3390 ng/ml
Zahra *et al* (25)	2007	3200 ng /ml
Ivan et al(26)	2008	4930 ng/ml
Sonali *et al*(27)	2012	2543ng/ml
Present study	2014	8000ng/ml

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Treatment of low serum ferritin in females with alopecia by oral iron.

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ABSTRACT

Background: Hair loss affects over 25% of women in developed countries. Three hair disorders, androgenetic alopecia, telogen effluvium and alopecia areata which account for most cases of nonscarring alopecia in women. Iron deficiency has been reported in the majority of women presenting with diffuse hair loss. Iron has important function in oxidation-reduction reactions, collagen synthesis, and as a co-factor for enzymes.

Objectives: To assess the percentage of the low serum ferritin hair loss females and their response to oral iron replacement therapy.

Materials and methods: a prospective cohort study conducted in at Marjan teaching hospital from August 2013 to January 2015. It included 72 female complained from diffuse hair loss were enrolled in this study. Full history was taken from all patients and physical examinations were done both general examination and local examination of the scalp including pull test. Patients were sent to complete blood examinations, serum ferritin. Patients divided in to three groups according to their response to treatment. Before the treatment the severity of hair loss was assess by VAS.

Results: Fifty two patients met criteria of inclusion. Their serum ferritin levels range between (1.4-14.4) with mean 6.16±3.30 their hemoglobin levels were (8.3-13.4) with mean ±SD 11.98±1.22. Group **I** patients included 27 patients (51.9%), their severity of hair loss before treatment was assess by VAS which was 9.18±0.84, after two week of treatment the score become 7.33±1.03, P value is < 0.0001; confidence interval 95% = (1.477 to 2.277). in group **II** patients which included 12 patients (23%), their severity of hair loss before treatment was assess by VAS which was 9.33±0.49, after two week of treatment the score become 9.08±0.66, P value is 0.081 considered not significant; confidence interval 95% = (-0.037 to 0.537). The other thirteen patients (25%) represented the third group. All the patients in this group didn't show any response to treatment

Conclusion: Measurement of serum ferritin level should be done to all patients with chronic telogen effluvium before starting other anti-hair loss modalities. Iron replacement is a safe drug with a few side effects and indicated to all females with low serum ferritin level.

Keywords: ferritin, female, hair loss

Introduction

Hair loss affects over 25% of women in developed countries [1]. Three hair disorders, androgenetic alopecia, telogen effluvium and alopecia areata account for most cases of nonscarring alopecia in women ([1, 2]. Diffuse hair shedding is distressing. In many cases, the patient notes an increase in hair on the pillow, or when brushing, or in the shower drain. [3] It is usually recognized more readily by women than men. [4] Few dermatologic complaints carry as much anxiety and emotional distress as hair loss. Equally, evaluation and management of hair loss are challenging. [5]

Kligman's hypothesis was that whatever the cause of the hair loss, the follicle tends to behave in a similar way, the premature termination of anagen. The follicle is precipitated into catagen and transforms into a resting stage that mimics telogen. [6] Acute telogen effluvium presents as a diffuse, non-patterned hair loss from the scalp that occurs around 3 months after a triggering event, and is usually self-limiting within 6 months. A host of different triggers have been implicated and identify the cause, e.g., post-febrile, postpartum, accidental trauma or surgical operations with large blood loss, a crash diet, or severe emotional distress are among the most common causes [7]. While chronic telogen effluvium is diffuse shedding of telogen hairs that persists more than 6 months either represents a primary disorder and is then a diagnosis by exclusion[8], It can also be secondary to prolonged, sequential, or repeated triggers, such as a nutritional deficiency or underlying systemic disorder, and shedding can be less pronounced than in acute telogen effluvium.[9] Apart from iron deficiency as a cause of chronic diffuse hair loss, all others are less common, although the literature concerning iron deficiency remains controversial. Iron deficiency has been reported in the majority of women presenting with diffuse hair loss [10, 11].

The total iron content of an adult man is 4–5 g [12]. The major role of iron in mammals is to carry oxygen as part of the heme protein that, in turn, is part of hemoglobin. Oxygen is also bound by a heme protein in muscle, myoglobin [13].

Also it has important function in oxidation-reduction reactions, collagen synthesis, and as a co-factor for enzymes (such as succinic dehydrogenase, monoamine oxidase, and glycerophosphate oxidase [14], also including in the cytochrome system in mitochondria. Without iron, cells lose their capacity for electron transport and energy metabolism. [13]

Iron deficiency is a major risk factor for disability and disease worldwide, affecting about two billion people. General symptoms include fatigue, palpitations on exertion, sore tongue with atrophic filiform papillae, angular cheilitis, dysphagia and

koilonychias. Generalized itch may occur and hair loss with or without morphological changes of the hair shaft may be seen [12]. Free iron is toxic to cells, and the body has established an elaborate set of protective mechanisms to bind iron in various tissue compartments. Within cells, iron is stored complexed to protein as ferritin or hemosiderin. [13] The Aim of study was to assess the percentage of the low serum ferritin hair loss females and their response to oral iron replacement therapy.

Materials and Methods

A prospective cohort study included 72 female complained from diffuse hair loss were enrolled in this study. The study conducted in Marjan teaching hospital in Hilla / Babylon from Their age ranged 15-45 years. All patients were complaining from diffuse hair loss for more than 6 months. Full history was taken from all patients and physical examinations were done both general examination and local examination of the scalp including pull test. Telogen shed may be estimated by the pull test: grasping 40 hairs firmly between thumb and forefinger, followed by a slow pull that causes minimal discomfort to the patient. A count of more than 4–6 club hairs is abnormal, but the result is influenced by recent shampooing (a count of 2–3 hairs being abnormal in a freshly shampooed scalp), combing. Patient were send to complete blood examinations, serum ferritin, patients with identifiable causes of hair loss were excluded from the study e,g thyroid disease was measured by thyroid function test, or hyperandrogenism was measured by serum testosterone, deydroepiandrosterone levels. Twenty patients (27.7%) were excluded as their serum ferritin level was within the normal range (40-134) with a mean ±SD of 83.75±28.19 and their hemoglobin levels within normal range (12-15.5) with mean ±SD 13.36±0.98.Our patients divided in to three groups according to their response to treatment. Group **I** good response included those patients with more and or equal to 50% reduction in their hair loss. Group **II** mild response included those patients with less than 50% reduction in their hair loss. Group **III** none response included those patients without response.

Before the treatment the severity of hair loss was assess by VAS. The scale consisted of a 10 cm horizontal line marked from 0 (denoting absent of hair loss) to 10 (denoting worst symptoms). Serum ferritin was measured before treatment, after one month and thereafter three months (after finish course of treatment). Patients were assigned to receive 4 weeks of ferrous sulfate tab 200 mg three times daily for one month for those patients whose their serum ferritin less than 15 ng/mL, their after 200mg once daily for two months. A reduction in scores of ≥ 50% was considered as the desired improvement in symptoms during treatment.

Results

Fifty two patients were their serum ferritin levels range between (1.4-14.4) with mean 6.16±3.30 their hemoglobin levels were (8.3-13.4) with mean ±SD 11.98±1.22. Twenty one (40.3%) patients were their hemoglobin levels within normal range (12-14.9).

Group **I** patients include 27 patients (51.9%), their severity of hair loss before treatment was assess by VAS which was 9.18±0.84, after two week of treatment the score become 7.33±1.03, P value is < 0.0001; confidence interval 95% = (1.477 to 2.277). This score continued to decrease reaching to 4.63±1.36 by the end of first month; P value is < 0.0001 confidence interval 95% = (3.922 to 5.189). At the end of 2nd month the score become 2.92±1.03; P value is < 0.0001; confidence interval 95% = (5.693 to 6.825). The score continued to decrease reaching to 2.11 ±1.08 in the end of 3rd month; P value is < 0.0001; confidence interval 95% = (6.526 to 7.622), considered extremely significant as showed in table (1), figure (1).

While in group **II** patients include 12 patients (23%), their severity of hair loss before treatment was assess by VAS which was 9.33±0.49, after two week of treatment the score become 9.08±0.66, P value is 0.081 considered not significant; confidence interval 95% = (-0.037 to 0.537). This score continued to decrease reaching to 8.5±0.0.52 by the end of first month; P value is <0.0001; confidence interval 95% = (0.586 to 1.08). At the end of 2nd month the score become 8.33±0.49; confidence interval 95% = (0.729 to 1.27). The score continued to decrease reaching to 8.16 ±0.38 at the end of 3rd month. P value is < 0.0001; confidence interval 95% = (0.919 to 1.414). As showed in table (2), figure (2)

The other thirteen patients (25%) represented the third group. All the patients in this group didn't show any response to treatment, and their severity of hair loss was 9.23±0.72 and remains constant during all period of treatment. No side effect was reported except in seven patients (13.4%) develop mild gastric upset which controlled by taken the drug with food.

Discussion

Hair loss has been associated with iron deficiency. [15, 16] However, the role of iron deficiency in hair loss continues to be a controversial topic. [17, 18]

In our study twenty patients (27.7%) were excluded as their serum ferritin level was within the normal range (30-134) with a mean ±SD of 83.75±28.19 and their hemoglobin levels within normal range (12-15.5) with mean ±SD 13.36±0.98.

Other fifty two patients were their serum ferritin levels range between (1.4-14.4) with mean 6.16±3.30 their hemoglobin levels were (8.3-13.4) with mean ±SD 11.98±1.22. Twenty one (40%) patients were their hemoglobin levels within normal range (12-14.9). So we should keep in our mind that one of the important investigations to assess anemia is serum ferritin and not depend on the hemoglobin level only. Iron deficiency anemia is the condition in which there is anemia and clear evidence of iron deficiency. However, iron deficiency can be divided into three stages. [13] The first stage is *negative iron balance*, in which the demands for (or losses of) iron exceed the body's ability to absorb iron from the diet. This stage can result from a number of physiologic mechanisms including blood loss, pregnancy, rapid growth spurts in the adolescent, or inadequate dietary iron intake. Under these circumstances the iron deficit must be made up by mobilization of iron from reticuloendothelial storage sites. During this period measurements of iron stores such as the serum ferritin level or the appearance of stainable iron on bone marrow aspirations will decrease. As long as iron stores are present and can be mobilized, the serum iron, total iron-binding capacity (TIBC), levels remain within normal limits. As long as the serum iron remains within the normal range, hemoglobin synthesis is unaffected despite the dwindling iron stores. The 2nd stage when iron stores become depleted, the serum iron begins to fall, TIBC increases, transferrin saturation falls to 15 to 20%, so hemoglobin synthesis becomes impaired. This is a period of *iron deficient erythropoiesis*. Careful evaluation of the peripheral blood smear reveals the first appearance of microcytic cells later gradually hemoglobin and hematocrit begin to fall, reflecting *iron deficiency anemia*. [13] So from the above the normal hemoglobin and hematocrit not exclude iron deficiency. In our study there are twenty one (40%) patients with normal range hemoglobin levels (12-14.9) in spite of low serum ferritin.

The serum or plasma ferritin concentration is an excellent indicator of iron stores in otherwise healthy adults and has replaced assessment of bone marrow iron stores as the gold standard for the diagnosis of iron deficiency in most patients .[12,19-22]

The ferritin concentration ranges from 40 to 200 ng/mL (mcg/L) in normal subjects, and is markedly elevated in states of iron overload, due to stimulation of hepatic ferritin synthesis and release by iron. [23]
There is no clinical situation other than iron deficiency in which extremely low values of serum ferritin are seen.[20,22] By definition, marrow iron stores are absent when the serum ferritin level is <15 mcg /L.[13]

In our study the 1st patients include 27 patients (51.9%), their severity of hair loss before treatment was assess by VAS which was 9.18±0.84, after one month of treatment the score become 4.63±1.36, P value is < 0.0001 confidence interval 95% = (3.922 to 5.189). At the end of 2nd month the score become 2.92±1.03; P value is < 0.0001; confidence interval 95% = (5.693 to 6.825). The score continued to decrease reaching to 2.11 ±1.08 in the end of 3rd month; P value is < 0.0001; confidence interval 95% = (6.526 to 7.622), considered extremely significant. while the 2nd group patients include 12 patients (23%), their severity of hair loss before treatment was assess by VAS which was 9.33±0.49, after one month of treatment the score become 8.5±0.52 by the end of first month; P value is <0.0001; confidence interval 95% = (0.586 to 1.08). The score continued to decrease reaching to 8.16 ±0.38 at the end of 3rd month. P value is < 0.0001; confidence interval 95% = (0.919 to 1.414).

The other thirteen patients (25%) represented the third group. All the patients in this group didn't show any response to treatment, and their severities of hair loss were 9.23±0.72 and remain constant during all period of treatment.
Hair follicle matrix cells are one of the most rapidly proliferating cells in the body. Ferritin levels are increased in non-dividing cells, such as stem cells and terminally differentiated cells, whereas rapidly proliferating cells appear to have lower levels of ferritin and higher levels of free iron [24-28]. This balance of ferritin and iron is at least partially controlled by the transcription factor c-myc [28]. C-myc is one the proto-oncogenes associated with apoptosis, change immediately prior to or coincident with the onset of catagen. [29]
The apoptosis-inhibitory proto-oncogene bcl-2 is expressed in cycling follicular epithelium during anagen, disappears during catagen and is absent in telogen.[30]
Over expression of c-myc in the cutaneous epithelium results in loss of follicular differentiation and a decrease in stem cells [31], but whether this phenotype is related to abnormal iron metabolism remains to be determined.

Iron has an important function as co-factor for enzymes (such as succinic dehydrogenase, monoamine oxidase, glycerophosphate oxidase [14] and ribonucleotide reductase, the rate-limiting enzyme for DNA synthesis which has an important function in the hair growth stem cell.

The depletion of iron could prevent proper functioning of this enzyme resulting in inhibition of proliferation [32]. Inhibition of other iron-dependent enzymes, such as stearoyl-CoA desaturase activity in mammals would be expected to affect a variety of key physiological variables, including cellular differentiation, insulin sensitivity, and metabolic rate. [33]

In spite of our patients were complained from chronic telogen effluvium, certain study demonstrate iron supplementation has been recommended as an enabler of response to other treatments in patients androgenic alopecia with low serum ferritin. [34]

In this study more than 50% of the patients with chronic telogen effluvium showed dramatic improvement in severity of their hair loss after three months of therapy with iron, while only 23% of the patients showed mild improvement.

Conclusion

Measurement of serum ferritin level (as a marker of iron state) should be done to all patients with chronic telogen effluvium before starting other anti-hair loss modalities.Iron replacement is a safe drug with a few side effects and indicated to all females with low serum ferritin level.

Table (1) showed the response to treatment in group I patients

	Time	No.	Mean of hair loss severity	SD	P	95% Confidence Interval
Pair I	Before	27	9.25	0.81	P<0.001	1.150-2.554
	2nd week	27	7.40	1.07		
Pair 2	2nd week	27	7.40	1.07	P<0.001	2.039-3.443
	One month	27	4.66	1.38		
Pair 3	One month	27	4.66	1.38	P<0.001	1.150-2.554
	2nd month	27	2.81	0.96		
Pair 4	2nd month	27	2.81	0.96	P<0.05	0.0385-1.443
	3rd month	27	2.07	1.07		
Pair 5	Before	27	9.25	0.81	P<0.001	6.483-7.887
	3rd month	27	2.07	1.07		

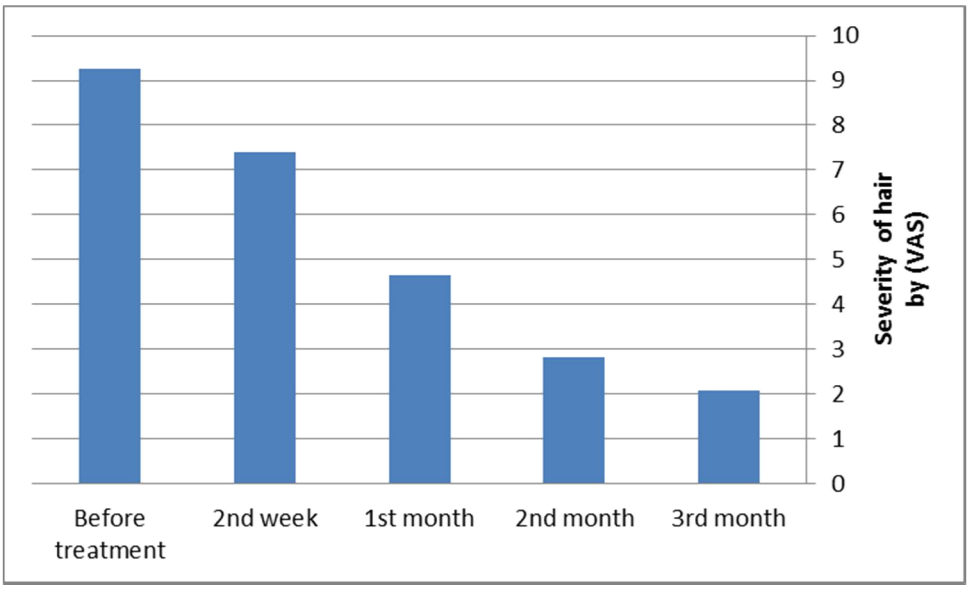

Figure (1) showed the response to treatment in group I patients

Table (2) showed the response to treatment in group II patients

	Time	No.	Mean of hair loss severity	SD	P	95% Confidence Interval
Pair I	Before	27	9.25	0.45	P<0.05	0.0367-0.7966
	2nd week	27	8.83	0.57		
Pair 2	2nd week	27	8.83	0.57	P<0.01	0.1201-0.8799
	One month	27	8.33	0.49		
Pair 3	One month	27	8.33	0.49	P>0.05	-0.2966-0.4632
	2nd month	27	8.25	0.45		
Pair 4	2nd month	27	8.25	0.45	P>0.05	-0.1299-0.6299
	3rd month	27	8	0.42		
Pair 5	Before	27	9.25	0.45	P<0.001	0.8701-1.630
	3rd month	27	8	0.42		

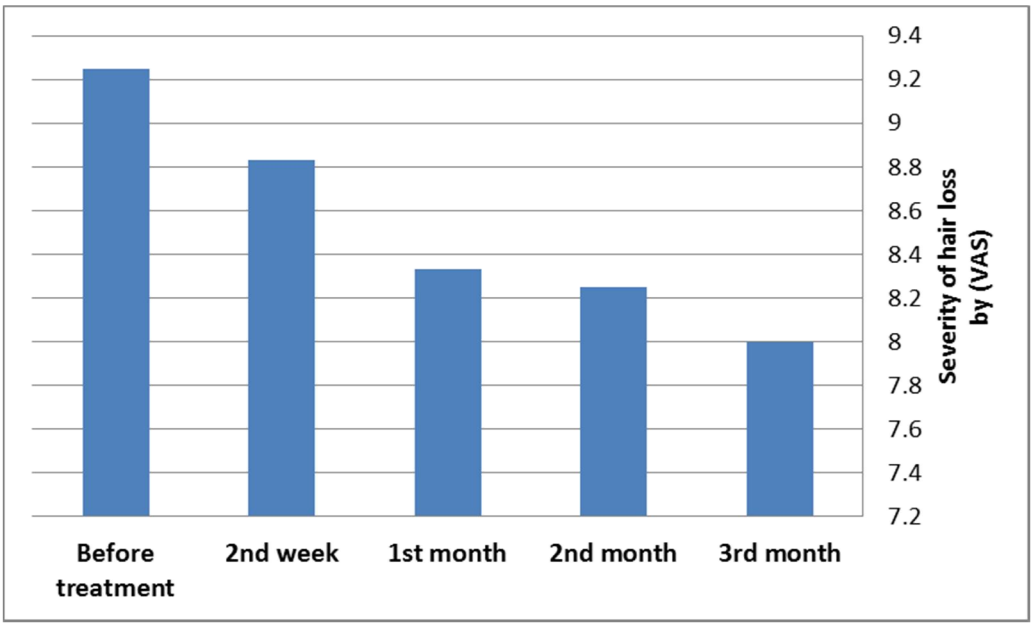

Figure (2) showed the response to treatment in group II patients

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