Study of Methylene Tetrahydrofolate Reductase enzyme Activity in Diffuse Large B-cell Non-Hodgkin's Lymphoma Patients

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ABSTRACT

Background: Diffuse large B-cell lymphoma is a heterogeneous disease with recognized variability in clinical outcome, genetic features, and cells of origin. It is the most common lymphoid malignancy in adults, comprising almost 40% of all lymphoid tumors.

Objective: To study of methylene tetrahydrofolate reductase in diffuse large B cell non-Hodgkin lymphoma patients.

Methods: This case control study was conducted during the period from April 2015 to December 2015 on 55 patients 19 patients before treatment and 36 patients after treatment. The patients were diagnosed as having diffuse large B cell non-Hodgkin lymphoma. Those patients were attending the Medical City Hospital in Baghdad and Immammain Kadhimain Medical City Hospital, in addition to another 30 apparently healthy individuals as control group. Both patient and control groups were examined and reviewed clinically and assessed by basic laboratory investigation. High pressure liquid chromatography technique was used for the estimation of methylene tetrahydrofolate reductase level (µg/dl) in diffuse large B cell non-Hodgkin lymphoma patients and controls.

Results: Methylene tetrahydrofolate reductase enzyme level showed significant difference between patients and control (P value ≤ 0.05). It was significantly lower in advanced compared to limited stage large B-cell non-Hodgkin's lymphoma; moreover methylene tetrahydrofolate reductase enzyme was significantly higher for both advanced and limited stage large B-cell non-Hodgkin's lymphoma patient after treatment compared to their level before treatment (P = 0.001) and (P value = 0.009), respectively.

Conclusion: Methylene tetrahydrofolate reductase enzyme level was inversely related to disease progression. Throughout course of treatment, the methylene tetrahydrofolate reductase activity was increased as the disease being under effect of chemotherapy. Therefore, folate supplementation may be useful to avoid metabolic disturbance and consequence of hyperhomocysteinemia and decrease of methionine.

Keywords: Diffuse large B-cell lymphoma, Methylene tetrahydrofolate reductase enzyme activity, B-cell lymphoma, Non-Hodgkin's lymphoma.

Iraqi Medical Journal Vol. 65, No. 1, January 2019; p.68-74.

Non-Hodgkin lymphomas are malignancies of the lymphoid tissue, which vary in histologic characteristics. B cell lymphomas making up majority of Non-Hodgkin lymphomas cases (about 85%). Diffuse large B-cell lymphoma and follicular lymphoma are the two major subtypes of B cell lymphomas[1].

They considered as the eighth most frequently diagnosed tumor type among men and the tenth among women worldwide[2].

Methylene tetrahydrofolate reductase is the rate-limiting enzyme in the methyl cycle, and it is encoded by the methylene tetrahydrofolate reductase gene. Methylene tetrahydrofolate reductase catalyzes the conversion of 5,10-methylene tetra-hydrofolate to 5-methyltetrahydrofolate, a co-substrate for homocysteine re-methylation to methionine, as well as susceptibility to malignant diseases like colon cancer, and acute leukemia had close association with methylene tetrahydrofolate reductase[3].

The active form of folate, may be appropriate to target for conditions affected

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by Methylene tetra hydro folate reductase polymorphisms[4]. Genetic instability, including chromosomal imbalance, is important in the pathogenesis of lymph proliferative disorders such as non-Hodgkin lymphoma (NHL). DNA synthesis and methylation, which are closely linked to folate metabolism and transport, may be affected by polymorphisms in genes involved in these pathways. Single nucleotide polymorphisms in (SNPs) is demonstrated in either (677 C>T and / or 1298 A>C) resulting in a 40% to 70% decrease in enzyme activity. They can cause increased availability of 5,10-MTHF for DNA synthesis along with a reduction in methionine availability for DNA methylation[5].

Due to the complexity of the folate metabolic pathway, several possible mechanisms exist by which variation in the genes involved may influence risk of non-Hodgkin's lymphoma subtypes. However, few studies have examined genetic variation in folate transport and metabolism and risk of NHL and the findings are inconsistent[6].

This study aimed to study of methylene tetrahydrofolate reductase activity in diffuse large B cell non-Hodgkin lymphoma patients with correlation with clinical parameters and response to treatment.

Methods

In a case control study, fifty-five patients with diffuse large lymphoma B-cell. Nineteen of them were before treatment, while the rest 36 assessed after complete chemotherapy treatment were enrolled. They were diagnosed and followed by consultant hematologist from hematology unit of both Baghdad teaching Hospital in Medical City Complex and Emmammain Kadhimain Medical City Hospital during the period of from (April 2015 to December 2015). They aged between (14-80) years and they were interviewed and assessed for the baselines clinical characteristics (age, gender, presenting compliant, past medical history as well as the method of histological diagnosis and radiological staging of the disease). All patients were classified according to their stages as: Limited stage disease (stage I A, II A) and Advanced stage disease (I B, II B, III A, III B, IV A and IV B)[7].

All patients treated with R-CHOP chemotherapy every 21 days, for eight cycles over 3-4 months duration[8,9].

Those patients were subdivided into two further groups, which are

- Group 1 (before treatment group): including all patients at time of diagnosis before receiving any treatment.
- Group 2 (after treatment group): including those patients who received 6-8 cycles of chemotherapy (for advanced stage disease).

In addition to Group 3 who considered as an age and sex, matched (control group) included 30 apparently healthy volunteer individuals.

All groups were assessed for the same laboratory parameters.

Inclusion criteria including cases of definitive diagnosis of diffuse large B-cell non-Hodgkin's lymphoma confirmed by histopathology of LN biopsy or BM biopsy with typical immunophenotyping markers by either immunohistochemistry (IHC) or flow cytometry.

Exclusion criteria included all other types of non-Hodgkin lymphoma like (T cell type NHL, Follicular type NHL, Marginal zone NHL, Mantle cell lymphoma, Chronic lymphocytic leukemia or small lymphocytic lymphoma) or relapsed diffuse large B-cell lymphoma cases that received multiple prior courses of treatment.

All patients were assessed according to international prognostic score IPI, which include five adverse prognostic risk factors:
1. Age >60 years.
2. Ann Arbor stage III/IV.
3. >1 extra nodal site.
4. Serum lactate dehydrogenase (LDH) level >normal.
5. Eastern Cooperative Oncology Group (ECOG) performance status ≥2.

The total score will be classify the patient prognosis into 4 groups that help in predicting their survival as: Low risk =0-1 (five-year overall survival of 73%), Low-Intermediate =2, High- intermediate= 3 and High= 4-5 (five-year overall survival of only 26%)[9].

Venous blood sample aspirated as 5 ml in plain tube from each patient and control that left to stand for 5 minutes after which centrifugation at (3000 rpm for 10 minutes) was done to get the serum. The serum complete underwent de-proteinization directly at the same time draw the blood to determine the level of Methylene tetra hydro folate reductase enzyme activity, then preserved at (-20 C°).

Measurement of MTHFR enzyme activity: The deproteinization of serum sample was achieved directly by adding 50µl of 15% 5-sulphososalsic to 400µl of deprotienzation frozen serum, then mixed and centrifuge at 3000 rpm for 10 min. The supernatant was taken and diluted three folds with ethanol and filtrated using minipore filter paper[10].

Preparation of calibration standard curves: A stock solution of 100 µg/ml of standard methyltetrahydrofolate and 5-methyltetrahydrofolate were prepared by dissolving 0.0001g of methyltetra-hydrofolate in 10 ml of N-hexane and diluted to 100 ml with N-hexane. The same procedure for 5, methyltetrahydrofolate was followed in the preparation of their stock solutions. Other standard solutions were prepared by subsequent dilution of the stock solutions and draw the calibration curve of these standards, (Figure 1). The solvent used to prepare these solutions before injection into HPLC.

Chromatographic analysis: All prepared samples and standard solutions have been chromatographically analyzed by using C-18 column and gradient mobile phase N-hexane: orthophosphoric acid 0.3M (70:30). The flow rate of 1mL/min and UV-visible detector at 290nm. The retention time of the product 5-methyltetrahydrofolate was 7.2 min. The large peak at 2 min is the 5, 10-methylenetetrahydrofolate, (Figure 2). Then measured activity of Methylene tetra hydro folate reductase; by divided differences of two concentrations on deferent of retention time of each beaks.

Kinetic of MTHFR: Kinetic measured by using Michaelis-Menten kinetics and Eadie-Hofstee plots Dependence on Methylene tetra hydro folate reductase activity and the concentrations of 5,10-methylenetetra-hydrofolate, (Figure 3).

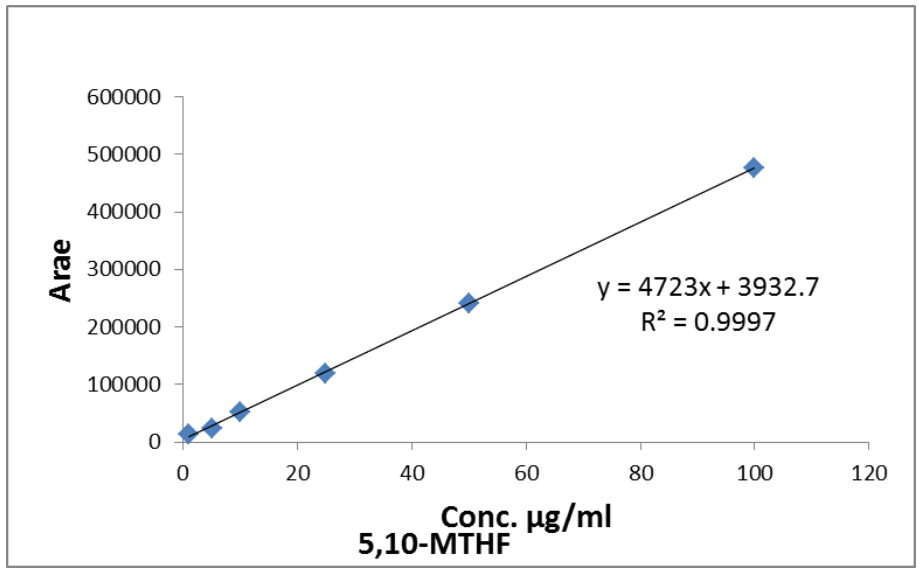

Figure 1: Calibration curves of 5,10-MTHF.

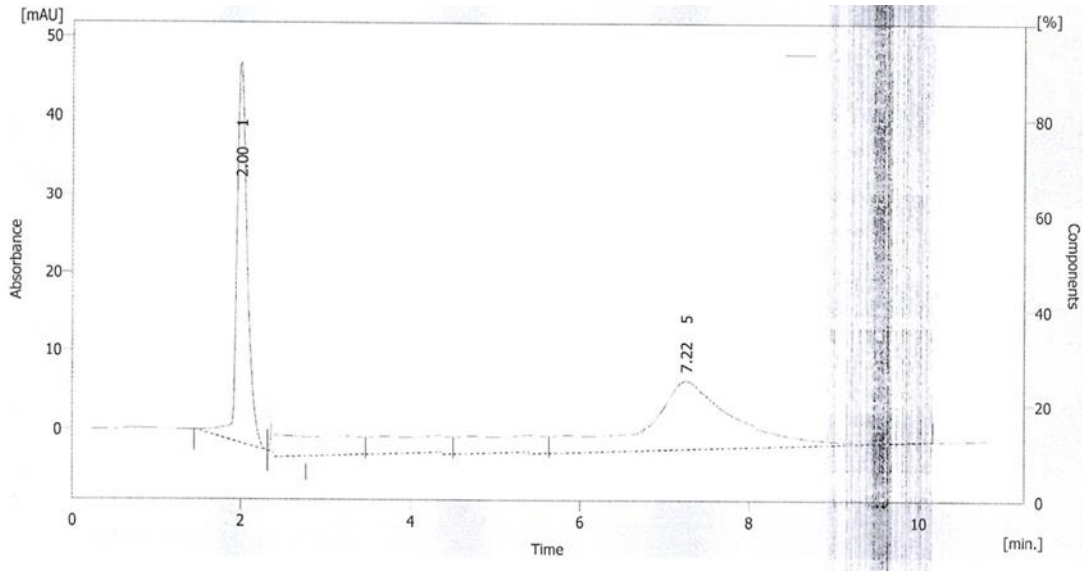

Figure 2: Chromatogram of 5, 10-MTHF and 5-MTHF using C-18 column and gradient mobile phase N-hexane: orthophosphoric acid 0.3M (70:30). The flow rate of 1mL/min and UV-Visible detector at 290nm.

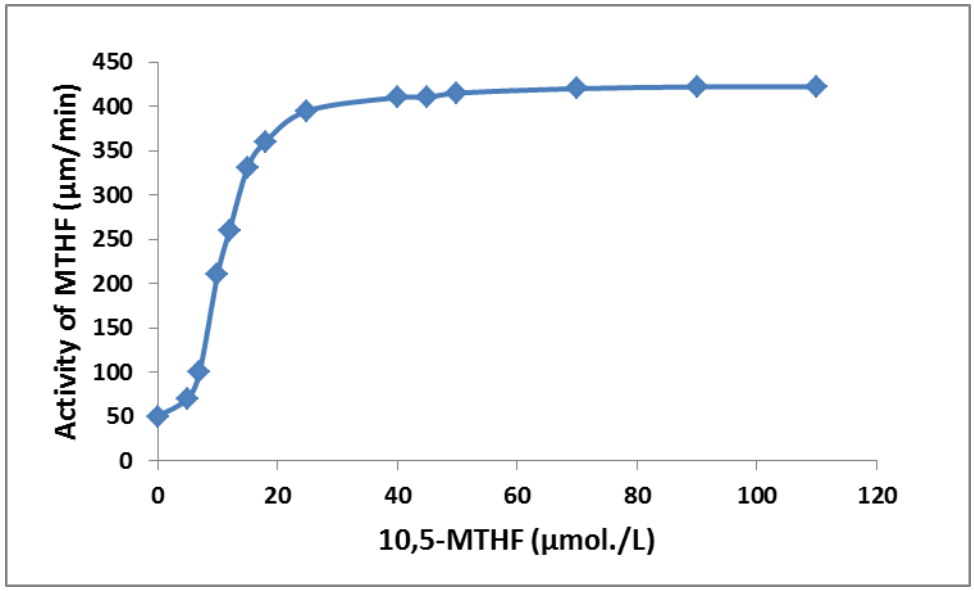

Figure 3: the relation between MTHFR activity and 5,10 MTHFR.

Results

Sample description: Fifty-five patients enrolled in this study, with age from 14 to 80 years (mean 51.6 ±18.6 year), in addition to thirty (age and gender matched) control healthy volunteer, (Table 1).

Clinical characteristic of patient group: Eighty-seven percent (87.27%) of non-Hodgkin's lymphoma were in advanced stage (I B, II B, III B, IVB, III A, I VA). Who have nodal involvement in 44 of them (80%), (Table 2). Bulky NHL described in four patients (7%) only. They were mostly in

intermediate IPI consisting of thirty-four patients (61.83 %), (Table 2).

Methylene tetra hydro folate reductase enzyme activity in diffuse large B-cell non-Hodgkin's lymphoma: When comparison Methylene tetra hydro folate reductase enzyme activity (µg/dl) between patients and controls group. There was statistical significant difference (P value =0.05) as the methylene tetra hydro folate reductase enzyme activity was significantly lower in patients than in healthy controls, (Table 3).

The enzyme activity (µg/dl) in relation to disease stage between limited stage and advanced stage showed a highly significant difference (p value = 0.001), while when comparison between before treatment and after treatment in limited stage showed significant difference (p value = 0.009), as well as when comparison between before treatment and after treatment in advanced stage showed significant difference (p value = 0.001), (Table 4).

Table 1: Demographic parameters for patients and control groups.

Parameter		**Patients (n=55)**	**Control (n=30)**	**P-value**
Age (y)	Mean ±SD	51.64±18.62	51.37±16.81	0.946
	(Range)	(14-85)	(16-80)	
Gender	Male no. (%)	30 (54.55)	20 (66.67)	0.358
	Female no. (%)	25 (45.45)	10 (33.33)	

Table 2: Clinical characteristic of patients group.

Parameter	**Patients n=55**	**%**
Stages	Limited (I A, II A) no.	7 (12.73)
	Advanced (I B, II B, III B, IVB, III A, IVA) no.	48 (87.27)
Extra nodal involvement	No. (%)	11(20)
Bulky diseases	No. (%)	4 (7.27)
IPI score	low = 0-1	17 (30.90)
	Low-intermediate= 2	23 (41.83)
	Intermediate-high = 3	11 (20)
	High = 4-5	4 (7.27)
Hypertension	No. (%)	17 (30.91)
Diabetes mellitus	No. (%)	10 (18.18)

Table 3: Comparison of MTHFR activity (µg/dl) between patients and controls by unpaired T test.

Parameters	**Patients (No. 55) Mean ±SD(Range)**	**Control (No.30) Mean ±SD(Range)**	**P-value**
MTHFR (µg/dl)	3.28 ± 0.92 (1.75-4.87)	3.57±0.44 (2.65-4.38)	0.05

Table 4: Comparison of MTHFR (µg/dl) among different diseases stages and treatment effect by ANOVA.

Parameter	**Limited stage**		**Advanced stage**		**P-value**
	Before Rx (No.3) Mean ±SD (Range)	After Rx (No.4) Mean ±SD (Range)	Before Rx (No.16) Mean ±SD (Range)	After Rx (No.32) Mean ±SD (Range)	
MTHFR(µg/dl)	2.4±0.52 (2.06-3.0)	3.54±0.17 (3.29-3.66)	2.09±0.36 (1.75-3.29)	3.93±0.38 (3.11-4.87)	0.001
	0.009		0.001		

Discussion

Majority of patients with non-Hodgkin's lymphoma were in advanced stage (I B, II B, III B, IVB, III A, IVA) which is consistent with late presentation and this can be explained that delayed presentation due to nonspecific systemic symptom manifestation.

Activity of methylene tetra hydro folate reductase in diffuse large B-cell Non-Hodgkin's lymphoma patients was lower in comparison with normal activity in control which is clearly demonstrate the effect of folate metabolism secondary to methylene tetra hydro folate reductase enzyme activity in non-Hodgkin's lymphoma pathogenesis that can be due to different gene polymorphisms as demonstrated by[5,6] in both Caucasian[11,12] and Japanese people[13]. it is also possible to consider enzyme activity changes in relation to disease extension and the different metabolic activity between tumor cell compared with normal cells which may recommenced again to the normal activity by increasing the activity after treatment when the tumor cell get regression and the normal cell regain normal activity with normal metabolism for float substrate and DNA synthesis to keep normal body hemostasis as there is statistical significant difference (P value =0.009) between the Methylene tetra hydro folate reductase mean activity level in limited stage before treatment and the mean activity level of limited stage after treatment, respectively. As well as similar statistical significant difference in advanced stage disease before treatment and after treatment courses (P value =0.001).

This increased enzyme activity after treatment courses may suggest a resuming activity of normal cell Methylene tetra hydro folate reductase enzyme similar to normal cell metabolism and therefore this may recommend the need for the use folic acid supplement after end of chemotherapy courses to maintain the normal cell supplementation for DNA synthesis and avoid block in folate metabolism with subsequent hyperhomocysteinemia and its clinical consequences.

In conclusion, level of methylene tetra hydro folate reductase enzyme activity is inversely related with disease progression disease while it get gradually increasing with treatment response resulting with possible folate depletion if it isn't supplied by diet or as pharmacological agent.

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IMJ 2019;65(1):68-74.