ASSOCIATION BETWEEN PLASMA HOMOCYSTEINE AND RIBOFLAVIN STATUS IN ACUTE LYMPHOBLASTIC LEUKEMIA IN CHILDREN

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ABSTRACT

Remethylation of homocysteine to methionine is dependent on an adequate supply of one or more of the B vitamins like folate, vitamin B₁₂ and vitamin B₆. Plasma total homocysteine (tHcy) is also influenced by genetic factors such as polymorphisms in the methylenetetrahydrofolate reductase (MTHFR) gene. MTHFR is a flavo enzyme and a key player in folate metabolism and changes in its activity could modify the susceptibility to Acute Lymphoblastic Leukemia (ALL). In this case – control study we have examined the effect of riboflavin status as measured by erythrocyte glutathione reductase activation coefficient (EGRAC) on homocysteine levels along with vitamin B₁₂ and folate in pediatric ALL. Folate and B₁₂ levels were significantly lower among cases as compared to controls while EGRAC and tHcy did not differ significantly among the groups. The multivariate regression analysis revealed that in the ALL group EGRAC significantly influences tHcy levels suggesting that riboflavin availability may be a predictor of tHcy levels in patients with ALL. This finding may have implications for tHcy lowering therapy.

KEY WORDS

Homocysteine, Riboflavin, MTHFR, Acute Lymphoblastic Leukemia.

INTRODUCTION

Homocysteine metabolism is rigorously controlled by two pathways, catabolism to cysteine and remethylation to methionine, which in turn is dependent on an adequate supply of one or more of the B vitamins like folate, vitamin B₁₂ and vitamin B₆. Concentration of total homocysteine (tHcy) is therefore inversely related to the status of these vitamins, in particular folate, the supplementation with which lowers tHcy approximately by 25% (1).

Concentration of tHcy is also influenced by genetic factors. Among them the methylenetetrahydrofolate reductase (MTHFR) gene involved in the metabolism of homocysteine

plays a key role. MTHFR converts 5, 10 methylenetetrahydrofolate to 5 methyltetrahydrofolate, the primary methyl donor in the transmethylation reaction in which homocysteine is converted to methionine. It can thus be perceived that defects in the MTHFR gene will result in elevated levels of plasma homocysteine. Two common polymorphisms in the MTHFR gene, 677 C→T and 1298A→C have been reported to decrease the activity of MTHFR, resulting in increased levels of tHcy (2). Folate and vitamin B₁₂ are critical nutritional factors, a deficiency of which results in hyperhomocysteinemia. There is a fourth B vitamin involved in the homocysteine metabolism albeit its role to date has received a little attention. In addition to folate, MTHFR requires riboflavin in the coenzyme form of flavin adenine dinucleotide (FAD).

In vitro studies have shown that the thermolabile MTHFR is approximately 10 times as likely as the wild type enzyme to dissociate from its FAD prosthetic group and thus become inactivated (3). What is unclear however is, whether riboflavin status affects the activity of the abnormal enzyme in vivo and consequently, tHcy concentrations.

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MTHFR is a key player in the folate metabolism and changes in its activity could modify the susceptibility to acute lymphoblastic leukemia (ALL). There are no published reports to date from India in which the effect of riboflavin status on homocysteine levels have been studied among pediatric ALL cases.

In this case – control study we have examined the effect of riboflavin status on tHcy concentration in children with ALL, the most common cancer among children.

MATERIALS AND METHODS

The study group included 86 patients aged ≤ 15 years with a confirmed diagnosis of ALL, prior to treatment and admitted to the Pediatric Oncology Department, Kidwai Memorial Institute of Oncology, Bangalore. 99 age and gender matched healthy children belonging to the same socioeconomic group were investigated as the control group. Subjects with altered renal function, hepatic dysfunction and hypothyroidism were excluded from the study.

The study protocol was approved by the Institutional Ethical Committee and informed consent was obtained from the parents /guardian of the subjects under study. Blood samples were collected from all subjects after an overnight fast into tubes with EDTA as an anticoagulant. The tubes were immediately placed on crushed ice, protected from light and centrifuged within 20 minutes at 2000xg for 10 minutes. Plasma samples were stored at -80°C until homocysteine estimation. 200 μL of EDTA blood was washed with cold saline and centrifuged (2000xg) thrice for 5 minutes. The washed cells were kept at -20°C till the assay of erythrocyte glutathione reductase activation coefficient (EGRAC).

Homocysteine was measured using a solid phase immunoassay, which measures total homocysteine in plasma or serum (Axis Shield, Norway). Protein bound homocysteine was reduced to free homocysteine and enzymatically converted to S-adenosyl- L- homocysteine (SAH) in a separate procedure prior to the immunoassay (4). A reference value of 5-15 $\mu\text{mol/L}$ was considered for comparison of test results. The samples were randomly checked by electrochemiluminescence method in an Elecsys 2020 analyzer and the values were comparable to that obtained by ELISA method.

Riboflavin status was determined on the basis of EGRAC, a functional assay in which the activity of flavin dependent glutathione reductase (NADPH) (EC 1.6.4.2) is measured before and after in vitro reactivation with its prosthetic group

FAD (5). EGRAC was calculated as the ratio of FAD stimulated to unstimulated activity of erythrocyte glutathione reductase. The spectrophotometric assay for EGRAC was standardised and automated on the Hitachi 912 autoanalyser. An EGRAC of 1.0 was considered as adequate riboflavin, between 1.0 – 1.2 as normal, 1.2 -1.4 as deficient and above 1.4 as low.

Vitamin B₁₂ and folate were simultaneously analyzed using a solid phase no boil dual count radioassay kit (7). (Diagnostic Product Corporation- DPC, CA, USA). A reference value of 180- 900 pg/ml for vitamin B₁₂ and 3-17 ng/ml for folic acid were considered for comparison of results.

Biochemical levels were expressed as mean $\pm$ SD. Student's 't' test (two tailed, independent) has been used to find the significance of study parameters between controls and cases. Multivariate (ANOVA) analyses has been carried out keeping all study parameters as dependent set and the study parameters were compared between cases and controls. Multivariate regression analysis was carried out to find the association of study parameters with homocysteine levels in controls and cases.

RESULTS

The mean age in the control and case groups were 8.14 and 7.4 years respectively (range 2-15). There were 71 males and 28 females among controls and 59 males and 27 females among cases. Immunophenotyping was available for only 13 cases, most of which were pre B cell ALL. Morphologic classification of ALL was available for all cases. Based on morphology, cases were classified as ALL L1, ALL L2 and ALL L3. 16 cases were ALL L1, 69 cases were ALL L1/L2 and only one case was L3.

Folate and Vitamin B₁₂ were significantly lower among cases,

Table 1: Levels of EGRAC, Folate, Vitamin B₁₂ and Homocysteine in controls and cases

Study parameters	Controls	Cases	P Value
EGRAC	1.28 $\pm$ 0.15 (1.00-1.68)	1.26 $\pm$ 0.19 (1.00-1.68)	0.335
Folate (ng/ml)	9.25 $\pm$ 3.67 (3.85-20.0)	6.51 $\pm$ 3.36 (2.24-19.13)	<0.001*
Vitamin B ₁₂ (pg/ml)	400.69 $\pm$ 220.17 (128.60-1418.0)	340.77 $\pm$ 185.49 (38.85-900.90)	0.049*
Homocysteine ($\mu\text{mol/L}$)	12.58 $\pm$ 5.85 (2.0-28.30)	12.74 $\pm$ 7.13 (3.80-44.87)	0.874

Values are mean $\pm$ SD and range

whereas homocysteine and EGRAC levels were not significant among cases and controls (Table 1). Table 2 shows the homocysteine level in cases and controls in relation to the EGRAC levels. Results show that among cases homocysteine level significantly increases with increase in EGRAC or when there is a deficiency in the riboflavin status with a significant p value of 0.013.

Table 2: Homocysteine levels in controls and cases according to EGRAC

EGRAC	Homocysteine ($\mu\text{mol/L}$)	
	Controls	Cases
1.0-1.2	10.79 $\pm$ 5.44 (2.70-21.30)	10.62 $\pm$ 5.33 (3.80-28.70)
1.20-1.40	13.32 $\pm$ 5.66 (5.18-28.30)	13.78 $\pm$ 5.54 (4.48-27.80)
>1.40	13.78 $\pm$ 6.54 (2.00-23.00)	16.31 $\pm$ 10.80 (4.40-44.87)
Significance (P value)	0.104	0.013*

Table 3 depicts the correlation of homocysteine with EGRAC, folate and vitamin B₁₂. The 'r' value (Pearson correlation coefficient) indicates the correlation (positive or negative) between plasma homocysteine and other parameters studied. EGRAC is positively correlated with tHcy both among cases and controls suggesting that riboflavin deficiency increases tHcy levels. In the control group there was an inverse relationship between plasma Hcy and folate and Vitamin B₁₂ which was not seen among the cases.

Table 3: Correlation of Homocysteine with EGRAC, Folate and Vitamin B₁₂

Pair	Control		Case	
	r value	p value	r value	p value
EGRAC vs. Hcy	0.262	0.009*	0.396	<0.001**
Folate vs. Hcy	-0.335	0.001**	-0.061	0.586
B ₁₂ vs. Hcy	-0.280	0.005**	0.029	0.796

r = Pearson correlation coefficient

A multivariate regression analysis was used to find the influence of EGRAC, folate and vitamin B₁₂ on plasma homocysteine in controls and cases. The results are shown in Table 4. The influence of these parameters on tHcy levels was assessed by calculating the beta weight which works as a correlation coefficient for establishing the relationship between homocysteine and other study parameters. Apart from relationship beta weight gives the magnitude and direction of influence exerted by the study parameters on homocysteine

Table 4: Association of study parameters with Homocysteine levels in a multivariate analysis

Study parameter	Control		Case	
	Beta	P value	Beta	P value
EGRAC	0.192	0.048*	0.421	<0.001**
Folate	-0.312	0.002**	-0.060	0.564
Vitamin B ₁₂	-0.128	0.197	0.066	0.533

levels. In the control group tHcy levels were significantly influenced by folate followed by EGRAC whereas in the cases, only EGRAC significantly influenced tHcy levels.

DISCUSSION

Elevated plasma tHcy and deficiency of vitamins related to its metabolism such as vitamin B₁₂ and folate have been associated with various disorders including cancer. Concentration of tHcy is also influenced by age, sex, lifestyle and genetic factors such as inborn errors and polymorphism in enzymes involved in Hcy metabolism (7). The most prominent polymorphism is the MTHFR polymorphism.

The flavo enzyme MTHFR catalyses the conversion of 5, 10 methylenetetrahydrofolate to 5- methyl tetrahydrofolate, which serves as a methyl group donor in the conversion of homocysteine to methionine. In rats, experimental riboflavin deficiency is associated with low MTHFR activity and reduced levels of 5-methyltetrahydrofolate. In humans, reduced enzyme activity caused by the commonly occurring 677 C→T substitution of the MTHFR gene is associated with elevated plasma tHcy. The mutant enzyme has lower affinity for its flavin cofactor than the wild type enzyme, and recent studies show that plasma homocysteine is inversely related to riboflavin in subjects with the T-allele. This indicates that the metabolic effect of the 677 C→T polymorphism is related to riboflavin status, which may have implications for further studies on the relation between this polymorphism and various clinical and biochemical endpoints (8).

An inverse relationship between plasma concentrations of riboflavin and tHcy was reported by Hustad et al in a study of 423 Norwegian blood donors (9). Plasma tHcy was 1.4 $\mu\text{mol/L}$ higher in the lowest compared to the highest riboflavin quartile in a multiple regression model adjusted for folate and other determinants of tHcy. The riboflavin- total homocysteine relationship was not significantly modified by levels of serum folate. Jacques et al (10) studied 450 subjects from the Framingham offspring cohort and found an inverse association between plasma concentration of riboflavin and

plasma tHcy in subjects with the TT genotype and plasma folate below the median. There was no relationship between concentrations of flavin cofactors and tHcy.

In a recent study by McNulty et al (1) of 286 healthy individuals from Northern Ireland EGRAC was used to determine riboflavin status. The authors demonstrated a significantly inverse association between riboflavin status and plasma concentration of tHcy which was modified by the MTHFR genotype. However, when riboflavin status was higher, tHcy was independent of genotype. The genotype-tHcy relationship was also dependent on folate, and was only observed in subjects with concentrations of erythrocyte folate below the median.

The relationship between plasma tHcy and riboflavin status has also been investigated in end-stage renal disease. (11). The authors found a positive association between EGRAC and tHcy, which is consistent with high tHcy when riboflavin status is low. Riboflavin was significantly related to plasma tHcy in multivariate models, which included folate and other B-vitamins. They did not study the riboflavin-homocysteine relationship in relation to the MTHFR 677C→T polymorphism. Several studies in different populations have reported that the MTHFR 677 TT genotype is associated with high levels Hcy (>20 µmol/L). However, in the Indian population this polymorphism is present at a much lower frequency (2). Furthermore, although Hcy levels in plasma were found to be higher in subjects with TT genotype of MTHFR C677T polymorphism in the Indian population, this was not statistically significant. Thus, it can be perceived that the impact of this genotype will be low in the Indian population. (2)

In this case-control study we have evaluated the influence of riboflavin status on plasma tHcy levels along with vitamin B₁₂ and folate. Folate and B₁₂ levels were significantly lower among cases as compared to controls while EGRAC and tHcy did not differ significantly among the groups. The multivariate regression analysis to evaluate the influence of EGRAC, folate and vitamin B₁₂ on tHcy levels revealed that while EGRAC levels were significantly influenced by folate among the controls, in the ALL group EGRAC significantly influenced tHcy levels.

A potential limitation to our study is that the MTHFR genotype was not analysed. However, the reported numbers of MTHFR 677 TT genotype among the Indian population is very low and may not impact on the results. Although much of the interest in the MTHFR 677C→T polymorphism stems from its association with moderate hyperhomocysteinemia it is still not

clear whether tHcy is a risk factor for several diseases or if it is mainly a surrogate marker. Inconsistent reports on the MTHFR 677C→T polymorphism and disease risk could be explained through effect modification by nutritional factors. The association between tHcy and riboflavin has been documented only in a few studies.

This case control study is a pilot study with a small sample size. The objective of this study was to correlate the Hcy levels with riboflavin status, as there are no published reports from India on the relationship between Hcy and riboflavin status. Our results indicate that riboflavin availability may be a predictor of tHcy plasma levels in patients with ALL and may have implications for tHcy lowering therapy.

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