

## MTHFR A1298C POLYMORPHISM AND THE VASO-OCCLUSIVE CRISIS IN SICKLE CELL DISEASE

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### ABSTRACT

Vaso-occlusion is the most signs and symptoms of sickle-cell anemia (SCA). Elevated Homocysteine concentration contributes to form thrombosis, a frequent event in sickle cell anemia. Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme, which modifies homocysteine metabolism, and some polymorphisms of gene encoding this enzyme are accompanied with a decreased activity of the enzyme. The aim of the study was to study the association between the A1298C polymorphism and the incidence of Vaso-occlusive crisis. A case- control study was conducted over a period of one year from Jan- Dec 2014 inclusive, 50 patients were collected together with age, and sex matched healthy control 30 cases. Venous blood samples were collected from groups to estimate serum Homocysteine, folic acid and A1298C polymorphism identification through tetra primer ARMS PCR. Statistical analysis was done, using the student *t-test*, Pearson correlation analysis and  $\chi^2$  test. We found that, the homocysteine level was significantly upregulated in the patients group compared with control group with p value < 0.01. Moreover, a strong positive correlation between Homocysteine level and the frequency of Vaso-occlusive crisis was found ( $\chi^2= 4.836$  and p value 0.04). Association between vaso-occlusive events and polymorphism frequency showed a non-significant difference for the A1298C gene ( $\chi^2 = 1.720$ ; p = 0.4231). We conclude that Hyperhomocysteinaemia is positively correlated with the frequency of Vaso-occlusive crisis and the presence of A1298C MTHFR gene polymorphism is not a risk factor for vaso-occlusive crisis in the SCD patients.

**Keywords:** Vaso-occlusive crisis. Hyperhomocysteinaemia, MTHFRA1298C

### INTRODUCTION

Sickle cell anemia (SCA) is a disease disorder characterized by a single mutation of the six codon of  $\beta$ -globin chain ( $\beta 6\text{-GAG}\rightarrow\text{GTG}$ ), in which the glutamic acid was replaced by Valine. Despite this fact, the clinical course of individuals suffering from SCA is largely variable, the severity of the symptoms ranging from asymptomatic to a very severe course (Steinberg and Adewoye, 2006; Adams et al., 2003). The phenotypic variability of the SCA

may be elucidated by some genetic factors, those related to  $\beta$ -globin genes have been well-known (Adams et al., 2003). Many evidences suggest that, the SCA and other chronic hemolytic anemia are manifested by increased the coagulable state with promoted of thrombin and fibrin formation as well as platelet activation with an increased risk for thromboembolic complications (Ataga et al., 2003).

Homocysteine is a sulfur amino acid and involved in methionine metabolism as an

intermediate state. In many patients with inborn errors of homocysteine metabolism, liver or kidney disease, nutrient deficiencies, homocysteine levels may rise over normal levels and cause adverse health outcomes. It was reported that the elevated plasma tHcy is an indirect cause for cardiovascular-related as well as non-cardiovascular-related deaths (Vallset et al., 2001; Van der put et al., 1998).

MTHFR gene 1298A-C polymorphism resulted in a glu429-to-ala (E429A) substitution. This polymorphism destroys the MbolI recognition site and has an allele frequency of 0.33 (Van der put et al., 1998). Whereas the 677C-T polymorphism occurs within the predicted catalytic domain of the MTHFR enzyme, the 1298A-C transition is located in the supposed regulatory domain. The 1298A-C polymorphism resulted in lowering MTHFR activity, which was more proclaimed in the homozygous than heterozygous cases (Van der put et al., 1998).

So the aim of this study to investigate whether children with sickle cell disease have elevated concentration of serum Homocysteine with diminished levels of folate. Determine the correlation between Hyperhomocysteinaemia and Vaso-occlusive crisis. Further to assess an association between the A1298C polymorphism and the frequency of Vaso-occlusive crisis.

## PATIENTS AND METHODS

### PATIENTS

A case-control study was conducted over a period of one year from first of Jan. 2104 to the end of Dec. 2014, 50 cases of patients (sickle cell anemia and Sickle cell – Thalassemia) were collected from the Hematology center in Abo Elresh Hospital together with 30 healthy cases, age and sex matched, were taken from Menoufia Hospital. History of renal, hepatic or cardiac disease was considered as exclusion criteria. All the individuals were subjected to the following after signing an informed consent by one of the parents or the patient himself: full history taking laying stress on age, gender, residence, frequency of occurrence of Vaso-occlusive crisis, severity of pain and site, whether they took folic acid. Vaso-occlusive crisis severity was estimated according to the

pain scale (1-10) plus whether the patient uses hospital, emergency or random ambulatory care for pain relief in the previous day. Physical examination was performed. A 3cc of venous blood sample were collected from both groups and centrifuged, after centrifugation the serum were taken to the Medical Research Unit, in the Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat city, Menoufia, to estimate serum homocysteine and folic acid level.

### METHODS

Venous blood samples were drawn from each subject in three separate test tubes: one tube was used for biochemical testing. The others were collected on EDTA for complete blood picture, haemoglobin electrophoresis and DNA extraction.

**Homocysteine:** All blood samples and standard solutions of Homocysteine were measured chromatographically with C-18 column. Mobile phase solution linear gradient from 0.1M acetate buffer pH 4 including 2% methanol to 0.1M phosphate buffer including 6% methanol over 15min (0-100%) was used for chromatographic analysis, with flow rate 0.5ml/min and UV-VIS detection at wavelength 245 nm.

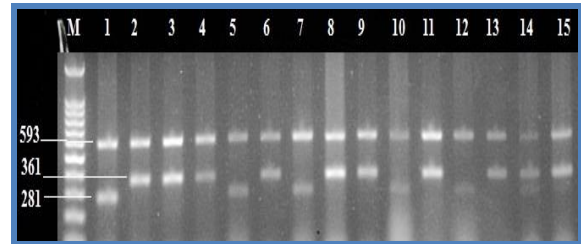
**Folic acid:** Folic acid was assayed using Mybiosource ELISA kits (San Deigo, california, U.S.A) The kit used a double-antibody enzyme-linked immunosorbent assay (ELISA) sandwich method to assay the level of Human Folic acid (FA) in samples.

**DNA Extraction:** Total genomic DNA was extracted from blood leucocytes pellets by salting out extraction method using a wizard genomic DNA extraction kit (Promega). Red blood cell was lysed by using red blood cell lysis buffer (20 mM tris-HCL pH 7.6) followed by centrifugation. Nuclei lysis was achieved by cell lysis solution containing (10 mM tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 0.1% (w/v) SDS) and proteinase K (20 mg/mL), and then centrifugation was done. Protein precipitation was completed by protein precipitation solution (60 mL of 5 M potassium acetate, 11.5 mL of glacial acetic acid, 28.5 mL of water). Finally isopropanol was used to precipitate the DNA and then washed by ethanol 70% and

rehydrated in Tris EDTA buffer (10 mM tris, 1 mM EDTA pH 8.0) and stored at -20 °C till assayed. DNA purity and concentration were estimated by the spectrophotometer at 260 and 280 nm absorbance.

**MTHR A1298C Genotyping:** The tetra-primer PCR procedure was used to genotype methylene tetrahydrofolate reductase (MTHFR) A1298C polymorphism. The method uses four primers to generate a larger fragment from DNA containing the SNP and the products representing each of the two allelic forms. Primers can be assigned to amplify fragments of differing sizes for each allele, and the bands can be easily identified by using agarose gel electrophoresis depending on size. The specificity of the reaction, could be increase by adding a mismatch at the 3' end of each of the two allele-specific primers. The primers were used as described in two studies by (Lajin et al., 2012 and Etlik et al., 2011). We utilized the 'BLAST' software at <http://www.ncbi.nlm.nih.gov/blast> to check the specificity of the primers. Gradient PCR was carried out using the Biometra T Professional thermal cycler (Biometra, Germany), to specify the proper annealing temperature for each primer set. The PCR reaction volume was of 20µL, containing 2X GoTaq® Green Master Mix (Promega, USA), genomic DNA (at a concentration of 50-100ng/µL), primers (primer sequences were as follows: The common forward primer is 5'/ GAAGAAGTTTGCATGCTTGTGGTTG 3', common reverse primer 5'/ CAGGCAAGTCACCTGGGAGAGA 3', for 1298-A 5'/ GCAAAGAACGAAGACTTCAAAGACACATT 3' and for 1298-C 5'/ GAGGAGCTGACCAAGTGATGC 3' and optimized concentrations of 0.5, 0.5, 1.0 and 0.1 µM respectively. The primer sets with the nearest optimal annealing temperatures were then incorporated into single mixes and gradient PCR was displayed, in try to optimize the procedures in multiplex reactions. PCR conditions were done as follow; an initial denaturation at 95°C for 5 min followed by 30 cycles of (denaturation at 96°C for 30 sec,

annealing at 54°C for 30 sec, extension at 72°C for 40 sec), and final extension at 72°C for 5 min. PCR products were separated by electrophoresis on a 2% agarose gel and the image was captured by gel documentation system under UV Transilluminator. The size of the amplicons obtained as follow; 593 and 281 bp bands for wild-type homozygote, 593, 361, 281, bands for heterozygote, and 593, 361 bands for mutant homozygote (**Figure 1**).



**Figure (1):** Agarose gel electrophoretograms showing MTHFR A1298C SNP genotyping by duplex tetra-primer ARMS PCR. The outer primers amplified a large fragment of the target gene (593 bp), irrespective of MTHFR genotype, which served as an internal control. The inner primers combined with the respective opposite outer primer to generate smaller allele-specific amplicons. **M**, Sizer™-100 DNA Marker (iNTRON Biotechnology, Korea). Samples no 14 and 15 showed two amplicons which denoted heterozygous state while samples no 1,5,7,10 and 12 showed one amplicon (281 bp) which denoted homozygous state, samples no 2,3,4,6,8,9,11 and 13 showed one amplicon (361 bp) which denoted mutant state.

#### Statistical analysis

Data were statistical analyzed using SPSS Win statistical package version 18. Numerical data were calculated as mean, range and standard deviation. Qualitative and categorical data were analyzed as frequency and percentage. Pearson correlation test was used between serum Homocysteine and Folic acid level, as well as between Homocysteine level and the frequency of Vaso-occlusive crises. *r*. value range from -1.0 to 1.0 referees and reflects the extent of a linear relationship between two data sets. *P*-value was calculated, a value < 0.05 indicates statistical significance.

#### RESULTS AND DISCUSSION

The clinical parameters of study group are summarized in **table (1)**. A non-significant

difference is noted regarding age, sex, SBP, and DBP. Homocysteine level was higher in the patients group compared with control group with mean and standard deviation of (44.68 ± 9.096) while in the control group standard deviation of (18.81 ± 3.76). In addition, folic acid level was lower in the patients group compared with control group with mean and standard deviation (12.02 ± 2.76) while in the control group (14.68 ± 2.99) ng/ml. Pearson correlation shows a negative significant correlation between

Homocysteine level and folic acid level (r. value =- 0.1, p. value 0.04) as it is shown in **(Figure 2)**.

The correlation between the SCD genotypes and the incidence of vaso-occlusive event was significant ( $\chi^2 = 4.836$ ;  $p = 0.04$ ), with the majority of the cases occurring in patients with the Hb SS genotype, as shown in **table (2)**.

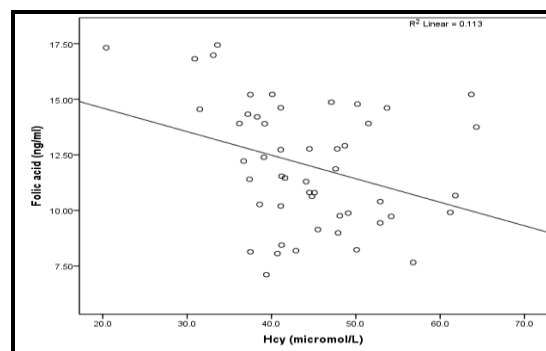
Association between A1298C polymorphism and biochemical parameters of study group are summarized in **table (3)**.

Variables	SCD (N=50) Mean ± SD	Controls (N=30) Mean ± SD	p value
Gender	Male: 33 (66%) Female: 17 (34%)	Male: 17 (60%) Female: 13 (40%)	0.797
Age (years)	6.20± 2.55	6.03 ± 2.64	0.781
Weight (kg)	19.28 ± 8.01	21.73 ± 7.41	0.169
Height (centimeters)	103.90 ± 15.98	111.67 ± 16.99	0.079
Heart rate/minute	88.42 ± 13.92	90.10 ± 11.26	0.577
Systolic BP	101.74 ± 9.50	104.83 ± 8.95	0.154
Diastolic BP	66.90 ± 6.98	66.17 ± 5.97	0.633
Haemoglobin (g/L)	7.97 ± 1.66	10.947 ± 1.1383	< 0.01
MCV	82.08 ± 11.04	75.28 ± 4.74	0.004
WBCs (thousands /cm)	11.528 ± 5.18	8.64 ± 3.43	0.009
Platelets (thousands /cm)	307.24 ± 191.35	295.63 ± 76.37	0.752
HbA (%)	13.89 ± 13.33	95.79 ± 1.366	< 0.01
HbA2 (%)	3.47 ± 1.59	3.473 ± 0.80	0.992
Homocysteine(μmol/L)	44.68 ± 9.096	18.81 ± 3.76	< 0.01
Folic acid (ng/ml)	12.02 ± 2.76	14.68 ± 2.99	0.002

**Table (1):** The clinical parameters of study groups.

Where a non-significant difference is noted regarding all parameters including homocysteine and folic acid level.

Association between vaso-occlusive events and polymorphism frequency (table 3) showed a non-significant difference for the A1298C gene ( $\chi^2 = 1.720$ ;  $p = 0.4231$ ).



**Figure (2):** homocysteine & folic acid correlations

	Positive Crisis	Negative Crisis	Total
<b>SS</b>	12	6	18
<b>S/B</b>	11	21	32
<b>Total</b>	23	27	50
<i>Chi-squared: 4.836 DF: 1</i>			
<i>Significance level: 0. 040</i>			

**Table (2):**  $\chi^2$  test between vaso-occlusive crisis and sickle genotype.

	AA (no=17)	AC (no=25)	CC (no=8)	p value
<b>Biochemical parameters (Anova Test)</b>				
<i>Hcy</i> ( $\mu\text{mol/L}$ )	43.3 $\pm$ 6.1	45.1 $\pm$ 9.0	43.6 $\pm$ 12.8	0. 828
<i>Folate</i> (ng/ml)	12.2 $\pm$ 3.0	11.7 $\pm$ 2.6	12.2 $\pm$ 2.8	0. 800
<b>Vaso-occlusive Crisis (Chi Square Test) Total</b>				
<i>Negative</i>	7	15	5	27 (54.0%)
<i>Positive</i>	10	10	3	23 (46.0%)
<i>Total</i>	17 (34.0%)	25 (50.0%)	8 (16.0%)	50
<i>Chi-squared: 1.72 DF: 2 Significance level: 0.4231</i>				

**Table (3):** Association between A1298C polymorphism and biochemical parameters.  $\chi^2$  test between vaso-occlusive crisis and A1298C polymorphism.

In the present study, patients with homozygous sickle cell have a higher frequency of vaso-occlusive crises than patients with sickle cell- $\beta$ +thalassemia genotype. This observation was consistent with the results of other previous studies (Platt et al., 1994; Nagel et al., 1985).

In this study, we found that the mean values of homocysteine in AA genotype, AC genotype and CC genotype were not statistically significant. This result comes in accordance with (Hanson et al., 2001; Friedman et al., 1999 and Friso et al., 2003) who observed that there was no significant difference in mean homocysteine concentrations in individuals who were either heterozygous carriers or homozygous for the 1298C allele compared with homozygous individuals for the 1298A allele. This means that the A1298C mutation may have no significant influence on plasma homocysteine levels and can be explained by the fact that the A1298C polymorphism is located within the C-terminal regulatory domain of the MTHFR gene, while the C677T mutation is located within the catalytic domain of the gene.

Moreover in this study, MTHFR A1298C allele was not associated with vaso-occlusive complications in patients with SCD. This can be explained by the absence of correlation between A1298C polymorphism and homocysteine level among sickle cell disease patients. There are a few studies were done on the A1298C MTHFR polymorphism in children with strokes, but their results are contradicted (Rook et al., 2005; Biswas et al., 2009; Akar et al., 2001; Sirachainan et al., 2008; Komitopoulou et al., 2006; Morita et al., 2009).

Two studies reported a positive association between A1298C polymorphism and vascular strokes. The first study was performed by (Rook et al., 2005) on the group of 21 young individuals after stroke, ethnically heterogeneous (people from 4 different ethnic populations). Such group tends to be extremely small and insufficient for an analysis of genetic polymorphisms. Additionally, the authors had no control group; so they compared the patient's results with known population controls. The second finding with positive result was made by (Biswas et al., 2009) on an Asian-Indian population. The incidence of the C allele in the control individual was exceptionally low (.08), which could be the cause of the observed significant difference between studied patients and controls in the Biswas et al.<sup>(14)</sup> findings. The problem of each of these studies is a relatively small individual size of the study groups.

On the other hand, many studies (Akar et al., 2001; Sirachainan et al., 2008; Komitopoulou et al., 2006; Morita et al., 2009) reported that, there was no association between the A1298C polymorphism and pediatric stroke.

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