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ORIGINAL ARTICLE

Epigenetic Modification in Methylene Tetrahydrofolate Reductase (MTHFR) Gene of Women with Pre-eclampsia

V. O. Osunkalu[1] · I. A. Taiwo[2] · C. C. Makwe[3] · A. A. Abiola[1] · R. A. Quao[4] · R. I. Anorlu[3]

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Abstract

Background Genetic and epigenetic factors play significant roles in the aetio-pathogenesis of pre-eclampsia (PE). The effects may vary across racial and geographical boundaries. The role of epigenetic modification in pre-eclampsia was studied among African populations in Lagos, Nigeria.

Aim and Objectives This study aimed to determine the pattern of Methylene tetrahydrofolate reductase gene (MTHFR) CpG island methylation in pre-eclampsia, and evaluate associated covariates.

Methodology This study was an observational, cross-sectional, study conducted at the Lagos University Teaching Hospital and the Lagos State Island Maternity Hospital. A total of 400 pregnant women consisting of 200 pregnant women diagnosed with pre-eclampsia (study group) and 200 pregnant normotensive and apparently healthy women (control group) were recruited for the study. Demographic and clinical histories were obtained through questionnaires. The DNA Methylation status of the CpG Island in promoter region of the MTHFR gene was assessed using bisulphite conversion and methylation specific PCR method. The biochemical parameters measured in the study were: red cell folate, vitamin B12, plasma homocysteine (Hcy) and methylene tetrahydrofolate reductase enzyme level.

Results Homozygous MTHFR CpG island hypomethylation pattern was significantly associated with pre-eclampsia ($\chi^2 = 22.96$; $p = 0.000$), Mean values of plasma homocysteine in PE women with homozygous hypomethylation (26.1 ± 9.1 umol/L) were significantly higher than (20.1 ± 4.2 umol/L) observed in PE subjects with homozygous hypermethylation ($p = 0.008$). Homozygous CpG island hypomethylated pattern of the MTHFR promoter region, was associated with the lowest median MTHFR enzyme level (72.8 ± 39.8 pmol/L) compared with heterozygous methylated pattern (91.3 ± 60.9 pmol/L; $p = 0.047$) and homozygous methylated pattern (82.3 ± 31.0 pmol/L; 0.047). Red cell folate and Vitamin B12 levels were not significantly associated with CpG island methylation status.

Conclusion Epigenetic modification plays significant role in the pathogenesis of pre-eclampsia.

Keywords Epigenetics · Methylation · Pre-eclampsia · Homocysteine · Methylene tetrahydrofolate reductase

Introduction

The International Society for the Study of Hypertension in Pregnancy (ISSHP) in 2014, defined and re-classified pre-eclampsia (PE). Pre-eclampsia as revised, is clinically characterized by new onset of hypertension occurring after 20 weeks of gestation, associated with either one, or a combination of proteinuria accompanied by maternal organ dysfunction including haematological and neurological complications such as eclampsia, and may include fetal growth restriction resulting from feto-placental dysfunction [1]. Pre-eclampsia undoubtedly remains a leading cause of morbidity and mortality for both pregnant women and neonates in

Osunkalu, Vincent Oluseye is an Associate Professor of Haematology and Blood Transfusion at the College of Medicine of the University of Lagos, Lagos, Nigeria. Idowu Taiwo, is an Associate Professor of Cell Biology and Genetics from the University of Lagos, Lagos, Nigeria. Makwe Christian is a Senior Lecturer in the Department of Obstetrics and Gynaecology at the University of Lagos, Lagos, Nigeria. Anorlu Rose is a Professor from the Department of Obstetrics and Gynaecology at the College of Medicine, University of Lagos, Lagos, Nigeria. Quao Rachel Abiana is a Graduate from the Physiology Department at the College of Medicine, University of Lagos, Lagos, Nigeria.

✉ V. O. Osunkalu
vosunkalu@unilag.edu.ng

Extended author information available on the last page of the article

sub-Saharan Africa and resource challenged countries over the years.

The global prevalence of PE has been estimated to be between 5 and 14%. Prevalence of PE varies widely across different populations, with higher incidence reported in resource challenged countries [2] Countries like the United States of America, have reported prevalences between 2 and 6% among nulliparous women [2]. Prevalence figures of 5.4% and 1.8% have been documented in recent studies from Pakistan and Nepal [3, 4]. Pre-eclampsia has been reported to account for approximately 10–15% of cases of maternal morbidity and mortality globally [5]. Oladapo et al. [6] in a nationwide surveillance study in Nigeria, reported 23.4% of maternal deaths recorded as being attributed to pre-eclampsia [6] with varying degrees of adverse perinatal and neonatal outcomes [2, 3, 5].

Globally, efforts have been directed at identifying aetiological and predisposing factors for pre-eclampsia among pregnant women. However, the aetio-pathology of pre-eclampsia appears to be multifactorial. The interplay of genetic and environmental factors provides a wide range of opportunities for identifying candidate genes that may play significant roles in the occurrence or predisposition to pre-eclampsia in pregnant women [7]. While there are many published articles on the role of genetic and environmental factors in the predisposition to pre-eclampsia and other hypertensive disorders of pregnancy, the role of epigenetic modifications still requires further careful studies, especially as it relates to certain genes controlling key areas of the folate metabolic cycle, a micro-nutrient that has been evidently identified as key in the pathogenesis of PE.

The characteristic pattern of homocysteine elevation in women with pre-eclampsia has been well documented in the literature. However, the precise mechanism for elevated plasma homocysteine in pre-eclampsia is still largely controversial. While some researchers have documented significant association of hyperhomocysteinaemia with low plasma folate, polymorphisms involving genes regulating the activities of key enzymes in the folate metabolic pathways (such as methionine synthase gene, *CBS* and *DHF* genes together with methylene tetrahydrofolate reductase gene) in different study populations, some researchers have not been able to replicate similar findings. Reports from African populations however, are either not readily available or are biased due to low sample sizes.

The role of *MTHFR* gene polymorphism has been widely studied across different populations. As it plays a critical role in the modulation of physiological methylation processes involving lipids, proteins and nucleotides. Epigenetic modifications of CpG island in the regulatory region of *MTHFR* gene, could also affect gene expression even in the absence of classical genetic mutations. Such modification may subsequently influence the expression of MTHFR enzyme.

This study is aimed at describing DNA methylation pattern of CpG islands in the promoter region of the *MTHFR* gene in women with pre-eclampsia and determine other biochemical correlates.

Materials and Methods

Study Population, Inclusion and Exclusion Criteria

This is a hospital based study which included a total of 200 pregnant women with clinical and laboratory evidence of PE (B.P ≥ 140/90 mmHg + proteinuria) that were recruited into the study group according to the ISSHP classification of hypertensive disorders of pregnancy [1], and 200 apparently healthy normotensive pregnant females (B.P < 140/90 mmHg, negative proteinuria) as control group participants. However, only 196 women in the study group and 194 women in the control group had complete data.

Only pregnant women at gestation age above 20 weeks, and between 18 and 44 years of age were recruited. All women with chronic hypertension and other systemic disorders were excluded from the study including women with history of alcohol, tobacco and herbal concoction use.

Ethical approval for the study was obtained from the Lagos University Teaching Hospital Health Research and Ethical Committee, and the Lagos State Health Service Commission. Written informed consent was obtained from participants after due explanation of research objectives before recruitment.

Data and Sample Collection

Interviewer administered questionnaires were used to obtain demographic data, obstetric history, health and nutritional history. Blood pressure (BP) readings were taken twice in sitting position with a mercury sphygmomanometer 30 min apart, and mean blood pressure readings above 140/90 mmHg were considered as elevated BP. Urinalysis was done using the reagent strips for urinalysis by DFI Company Ltd, Republic of Korea. Subjects with detectable proteinuria (≥ 2 + on dipstick) with elevated BP ± complications were classified as PE according to ISSHP recommendation. Each recruited study group subject with pre-eclampsia was matched with a normotensive subject, for age, estimated gestational age and parity as control group participants. Venous blood (9 mL) was collected into two separate EDTA bottles (4.5 mL each) for analysis of biochemical and molecular studies. Plasma samples were separated from whole blood by centrifugation (3000 g for 10 min at 4 °C) and stored at − 80 °C until analyses. The plasma from the EDTA sample was used for the estimation of homocysteine based on Enzyme Immunoassay

technique using the AxisR- homocysteine EIA kit (LOT No: 802896074, Axis-Shield Diagnostics Ltd, Scotland, United Kingdom). Final reading was done by ELX 800TM absorbance microtiter reader at 450 nm (Biotek Ltd, UK serial No: 205808). Detection range for the plasma homocysteine was 2–50 µmol/L. Vitamin B12 and Red cell folate were determined using electrochemiluminescence binding assay method on Cobase E 411 immunoassay auto-analyzer (Roche, Switzerland). The MTHFR level was determined using commercial enzyme-linked immunosorbent assay (ELISA) kits by MyBiosource, Inc. San Diego (USA) with a detection range of 46.88–3000 pg/ml.

DNA Extraction

Extraction of DNA from whole blood was done by spin column method using Jena Bioscience Blood-Animal-Plant DNA preparation kit (Thuringia, Germany). The concentration, purity and yield of the DNA samples were checked using Unico 2100 Spectrophotometer (Unico, USA) at 260 nm and 280 nm, respectively. Eluted DNA samples were stored at − 20 °C till they were used (Table 1).

Epigenetic Study

The processes of bisulphite deamination, sulphonation and hydrolytic deamination of the CpG island within the promoter sequence of the MTHFR gene were done using methylation specific PCR method as described by Ge et al. [8].

Data Analysis

Statistical analyses were performed using SPSS 25.0 software (Armonk NY: IBM Corp. USA) Kolmogorov–Smirnov test was used to assess normality of data distribution for continuous variables, and Box plots were used for identification of outliers. Continuous variables were presented as mean ± standard deviation (SD) for normally distributed data. Categorical variables were expressed as percentages, and Chi square test was used to compare proportions for categorical variables. Analysis of variance was used to compare mean values across the three categories of methylation profiles for normally distributed data followed by Sidak post hoc test, while Kruskal–Wallis statistics was used to compare nonparametric data, using Mann–Whitney-*U* as post hoc. The interactions and association of covariates with the risk of CpG island hypomethylation were determined using a multivariate logistic regression model.

Results

Mean plasma homocysteine level differed significantly across the different CpG island methylation patterns as shown in Table 2 ($F = 5.011$; $p = 0.008$). Study group participants with homozygous unmethylated loci, had higher mean plasma homocysteine level (26.1 ± 9.1 µmol/L) compared to study group participants with homozygous methylated loci (20.1 ± 4.2 µmol/L). Similarly, median MTHFR

Table 1 Primers and sequences used for the study

Primers	Sequences
MTHFR-: Methylated forward Primer	ATTGAGATTAGGAGTGGTTGTAGAC
MTHFR-: Methylated reverse Primer	CTAAAAAAAACGAACCTACAAAACGA
MTHFR-: Unmethylated forward Primer	TGAGATTAGGAGTGGTTGTAGATGA
MTHFR-: Unmethylated reverse Primer	TAAAAAAACAAACCTACAAAACAAA

Table 2 Mean values of biochemical variables stratified by CpG Island methylation pattern in women with Pre-eclampsia

	Red cell folate Mean ± SD(ng/mL)	Hcy level Mean (umol/L) ± SD	Vit B12 Median ± IQR(pg/mL)	MTHFR level Median ± IQR(pg/mL)
U	U 373.9 ± 64.9 CI:357.5–390.4	26.1 ± 9.1 CI:17.7–22.4	445.4 ± 89.6	72.8 ± 39.8
M	377.9 ± 72.7 CI:347.2–408.7	20.1 ± 4.2 CI:20.0–32.0	497.9 ± 66.2	82.3 ± 31.0
Ht	397.1 ± 80.7 CI:382.1–412.0	20.81 ± 8.7 CI:19.2–22.4	458.6 ± 109.9	91.3 ± 60.9
F	2.12	5.011	–	–
P	0.123	0.008*	0.090**	0.047**
Post hoc		*U* > *M*; *M* < Ht		*U* < *M*&Ht

Key: Homozygous Unmethylated loci (U); Homozygous methylated loci (M); Heterozygous loci (Ht); Interquartile range (IQR). *P* value for ANOVA (*); significant *p* value for Kruskal–Wallis statistics (**)

enzyme levels were lowest among study group participants with homozygous unmethylated promoter CpG islands (72.8 ± 39.8 pg/mL) compared with heterozygous methylated pattern (91.3 ± 60.9 ± IQR: 79.9–102.6; $p = 0.047$) and homozygous methylated promoter CpG island pattern (82.3 ± 31.0 ± IQR: 59.1–85.5). Conversely, no significant difference was observed in the distribution of mean red cell folate and median vitamin B12 across the different methylation patterns ($p = 0.123$ and 0.09, respectively).

In Table 3, a total of 88 homozygous unmethylated *MTHFR* gene loci (*U*) were identified in the study and the control groups, respectively. Of these, 61 (69.3%) *U* loci were observed in the study group and only 27 (30.7%) were observed in the control group ($\chi^2 = 22.96$; $p = 0.000$; Cramer's $V = 0.243$, $p = 0.000$). Conversely, of the 75 homozygous methylated (*M*) loci characterized in this study, 51 (68%) occurred among participants in the control group, and 24(32%) among study group participants, ($\chi^2 = 22.96$, $p = 0.000$; Cramer's $V = 0.243$, $p = 0.000$).

In a multivariate logistic regression (Table 4), occurrence of homozygous CpG island hypomethylation methylation was significantly associated with risk of hyperhomocysteinaemia (OR = 1.003, 95%CI: 1.001–1.013; $p = 0.047$). Similarly, low MTHFR enzyme level was significantly ($p = 0.019$) associated with increased odds for CpG island hypomethylation in women with pre-eclampsia. Conversely, the occurrence of CpG homozygous hypomethylation was not significantly associated with variations in the red cell folate levels (OR = 1.002, 95%CI: 0.988–1.005; $p = 0.345$) and was also not significantly associated with changes in vitamin B12 level (OR = 1.001, 95%CI: 0.999–1.004; $p = 0.320$).

Table 4 Regression analysis predicting association of some covariates with occurrence of CpG Island homozygous hypomethylation in women with pre-eclampsia

Variables	β	OR (CI)	p
Red cell folate	0.002	1.002 (0.988–1.005)	0.345
Vitamin B12 level	0.001	1.001 (0.999–1.004)	0.320
Plasma Homocysteine	−0.014	1.003 (1.001–1.013)	0.047
MTHFR enzyme activity	0.004	1.009 (1.002–1.017)	0.019*

(significant association)

Discussion

Whereas, many researchers have presented conflicting reports on the role of folate in the aetio-pathogenesis of pre-eclampsia [7, 9], majority of these reports were mainly from Caucasian populations. Few attempts have been made to report the role of folate cycle gene defects among African populations where there have been reports of folate deficient diets [10]. To the best of our knowledge, the role of epigenetic modification of folate cycle genes has not been documented for African populations.

The extent of epigenetic modification (methylation) within the CpG island of the regulatory regions of genic sequences, ultimately results in significant modulation in the level of gene expression, especially as it relates to MTHFR enzyme level in this study. Significant hypomethylation was found to be closely associated with occurrence of PE in this study. It was observed from the study, that unmethylated CpG alleles (*U*) were associated with significant increase in the risk for pre-eclampsia within the study population. DNA methylation is an epigenetic modification critical to normal genome regulation and development, and this process involves the vitamin folate, as a key source of the one carbon group used to methylate genome DNA. Published epigenetic studies of DNA sequences within the *MTHFR* gene regulatory region using peripheral blood, are extremely rare. One of the few documented studies in a Chinese population using the methylation specific PCR method on 117 pre-eclamptic women [8], reported marginally higher frequencies of homozygous methylation pattern among pre-eclamptic women compared to normotensive controls. A further critical evaluation of this report, indicated that the proportion of pre-eclamptic women with unmethyated CpG alleles in that study was as high as 62.6%. However, many other published studies on *MTHFR* gene methylation have used placental tissues, maternal omental arteries and umbilical cord blood in place of maternal venous blood. These other studies have also reported significant hypomethylation of the CpG islands as observed in this study [11, 12].

Table 3 Association between MTHFR gene methylation status and pre-eclampsia

Subject Category	*MTHFR* gene (CpG) methylation status			χ^2 (p)
	Homozygous Unmethylated (*U*)	Homozygous methylated (*M*)	Heterozygous methylated(Ht)	
Study group ($n = 196$)	61 (69.3)	24 (32.0)	111 (48.9)	22.96 (0.000)
Control group ($n = 194$)	27 (30.7)	51 (68.0)	116 (51.1)	
Total	88	75	227	

Cramer's $V = 0.243$; $p = 0.000$

The methylation process involving the principal methylation product, S-Adenosyl methionine (SAM) is under strict physiological control where excess S-Adenosyl methionine exerting negative feedback on MTHFR enzyme level. It may be implied from available evidence in the literature, that *MTHFR* polymorphisms which result in decreased MTHFR enzyme activity, could precipitate a global hypomethylation state due to reduced generation of physiological methyl donors. The resulting hypomethylation in the *MTHFR* genome could inadvertently trigger an attempt at a compensatory, but inadequate increase in MTHFR expression by the epigenetic modifications of the promoter CpG islands. It has been documented that the genic promoter methylation pattern appears to be affected by race and geographical origin [8], such that the degree and pattern of hypomethylation in pre-eclampsia, may better be studied and interpreted within the context of a given population. Therefore, evaluation of *MTHFR* gene methylation profile may act as useful marker in early detection of pregnant women at risk of pre-eclampsia in a given population.

Previous studies have been able to establish an association between rising plasma homocysteine and reduction in MTHFR enzyme activity [13]. In this study, an association was established between CpG islands hypomethylation and lower levels of MTHFR enzyme. Consequentially, lower MTHFR enzyme levels could be responsible for the rising plasma homocysteine levels [14], and therefore, explains the observed association between hypomethylation and plasma homocysteine in this study.

Though many researchers have attributed global hypomethylation to deficiencies of folate, vitamin B12 or other related co-factor deficiencies [15], low serum folate or vitamin B12 levels were not associated with the hypomethylation in pre-eclamptic subjects from this study, and therefore, may not have contributed significantly to the hyperhomocysteinaemia associated with pre-eclampsia in African populations. A limitation to this study however, was the inability to obtain preconception levels of folate and vitamin B12 for the participants, prior to ante-natal care folate supplementation. Subtle preconception micronutrient lack has been implicated in many obstetric disorders [16].

Conclusion

MTHFR CpG island methylation appears to be a significant epigenetic event associated with pre-eclampsia, and may occur independent of nutritional status of pregnant women.

Author Contributions Osunkalu Vincent: Conceptualization, data analysis and manuscript writing. Idowu Taiwo: Conceptualization, Genetic analysis and study design, literature review. Makwe Christain: Article review, literature review, project supervision. Abiola Ann Ogbenna: Article review, literature review, project supervision Quao Rachel: statistical analysis and editing. Anorlu Rose: Article review, literature review, project supervision.

Compliance with Ethical Standards

Conflict of interest All authors declare no conflict of interest whatsoever.

Human and Animal Rights Research involving Human participants only. The study has been performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helenski and its amendments or comparable ethical standards.

Informed Consent Study was approved by the Human Research and Ethical Committee of the Lagos University Teaching Hospital, Nigeria. Written and oral informed consent were obtained from all participants before recruitment into the study.

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About the Author

Osunkalu, Vincent Oluseye is an Associate professor of Haematology and Blood transfusion at the College of Medicine of the University of Lagos, Nigeria. He has special interest in folate metabolism, a subject that has formed the basis of his research effort spanning over 13 years. He is credited with 43 publications in peer-reviewed journals. In addition to his basic medical degree (MBBS) and postgraduate specialization in Haematology (FMCPath), He holds Master degree in both public health and genetics and he is currently writing up his Ph.D. dissertation on 'genetic and epigenetic modifications of MTHFR gene in the pathogenesis and prediction of pre-eclampsia'.

Affiliations

V. O. Osunkalu[1] · I. A. Taiwo[2] · C. C. Makwe[3] · A. A. Abiola[1] · R. A. Quao[4] · R. I. Anorlu[3]

1 Department of Haematology and Blood Transfusion, College of Medicine, University of Lagos, Lagos, Nigeria

2 Department of Obstetrics and Gynaecology, College of Medicine, University of Lagos, Lagos, Nigeria

3 Department of Cell Biology and Genetics, University of Lagos, Lagos, Nigeria

4 Department of Community Health and Primary Care, Lagos, Nigeria