HFE Gene Polymorphism and Iron Status in Preeclampsia

Lakshmiprabha S.[1], Suganthy K.[2], Shanmugapriya V.[3], Kalaiselvi K.[4]

[1]Associate Professor, Department of Biochemistry, Vinayaka Missions Medical College, Karaikal, Puducherry, (Affiliated to VMRF- DU, Salem). [2]Professor, Department of Biochemistry, Velammal Medical College, Madurai, Tamil Nadu. [3]Associate Professor, Department of Biochemistry, Vinayaka Missions Medical College, Karaikal, Puducherry, (Affiliated to VMRF- DU, Salem). [4]Professor and HOD, Department of Biochemistry, Vinayaka Missions Medical College, Karaikal, Puducherry, (Affiliated to VMRF- DU, Salem).

ABSTRACT

BACKGROUND

Over the past decade, with the development of the genome-wide association studies, a dramatic increase of identification of many biological candidate gene mutations and polymorphisms have been examined in association with preeclampsia in its molecular etiopathogenesis. Preeclampsia is one of the leading causes of morbidity and mortality in pregnant women, if not attended. This study attempts to determine the association if any between C282Y allele of HFE gene with preeclampsia, and evaluate the serum iron status in women with pre-eclampsia and third trimester healthy pregnant women, as this polymorphism is closely associated with haemochromatosis, a hereditary disorder in which serum iron is elevated, which is also seen in pre-eclampsia.

METHODS

This is a hospital-based case-control study. Study was performed on one hundred pregnant women of 28 to 34 weeks gestation. Fifty preeclamptic women and fifty healthy pregnant women were taken as cases and controls respectively. Whole blood was taken for the extraction of DNA, followed by PCR and gel electrophoresis for the HFE gene polymorphism study and complete blood count analysis. Serum was used for iron, Total Iron Binding Capacity (TIBC) and ferritin estimations. Statistical significance was determined by calculating odds ratio for HFE gene polymorphism study and students t-test for other parameters. p-value of less than 0.05 was considered statistically significant.

RESULTS

There is no significant association between preeclampsia and HFE gene and C282Y allele polymorphism. 92% of the cases and 98% of the controls do not show mutation in the C282Y allele. Odds ratio for wild type is 1.065 and that of heterozygote is 4.261. 95% confidence interval is large (0.021- 54.76), indicating a low level of precision in wild type. Z value for wild type is 0.031 and that of heterozygote is 1.275, p-value for both is not significant. Serum iron, ferritin and %age of transferrin saturation were significantly higher (p= 0.001) in preeclamptic women, in comparison with the control group. Unsaturated Iron Binding Capacity (UIBC) and TIBC were significantly lower (p=0.001) in preeclamptic group.

CONCLUSIONS

There are increased iron indices in preeclampsia when compared to the controls. Haemoglobin concentration and Haematocrit are raised in preeclampsia. There is no association of C282Y mutation of HFE gene with preeclampsia. Therefore, C282Y allele cannot be used as a molecular marker for preeclampsia.

KEYWORDS

Preeclampsia, C282Y Allele, HFE Gene, Iron, Ferritin, TIBC, UIBC

Corresponding Author:
Dr. Lakshmiprabha S,
Associate Professor,
Department of Biochemistry,
Vinayaka Missions Medical College,
Karaikal-609609, Puducherry,
(Affiliated to VMRF- Salem).
E-mail: agathiarlakshmi@gmail.com
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BACKGROUND

Preeclampsia is a multisystem disorder of pregnancy that contributes significantly to maternal and perinatal morbidity and mortality. The incidence of preeclampsia varies from 5 - 15%. The incidence of primigravidae is 16% and that of multigravidae is 7%.[1,2] In pregnancy, a decreased iron status is expected because of the increased requirement by the fetus. Contrary to this an increased iron status is observed in preeclampsia.[2,3] HFE protein which is encoded by HFE gene plays a very important role in the regulation of iron level in the body, at the level of intestinal absorption. A common mutation, C282Y allele, seen in HFE gene is one of the main causes of iron overload in hemochromatosis. Such a mutation which may not be manifested clinically under normal conditions may be well expressed under a stressful condition like pregnancy causing increased iron overload in preeclampsia. Therefore, C282Y allele, if present can serve as a very useful molecular marker for preeclampsia well before the onset of the disease, so that necessary preventive measures can be taken at an earlier stage of pregnancy.

Conde - Agadelo have grouped biomarkers of preeclampsia into 4 major categories.[4] A large scale study of genetic association with preeclampsia was studied in 190 genes, involving 775 Single nucleotide polymorphisms (SNPs) in around 350 preeclamptic women and their infants.[5] These studies show that preeclampsia is a genetic disorder involving multifactorial polygenetic inheritance. HFE Gene (hemochromatosis gene) is located at 6p21.3, approximately 4.6 mega bases, telomeric from HLA-A, and covers approximately 9,609 bp between 26,195,426 - 26,205,034bp within HLA class I region. Histone genes are present on either side of the HFE gene. The HFE genes' seven coding regions (exons) are scattered over about 10,000 base pairs of genomic DNA. After transcription, they are spliced to form an mRNA transcript about 2700 bp long. HFE protein is involved in the regulation of iron uptake in the diet.[6] It participates in the down regulation of intestinal iron absorption by binding to transferrin receptor (TR). HFE protein competes with transferrin-bound iron for the TR and thus reduces uptake of iron into cells. On the other hand, a lack of HFE protein increases the intestinal absorption of iron similar to iron deficiency.[7] Molecular analysis revealed that HFE variant allele (G) (rs1799945) was significantly associated with an adequate response to iron supplementation.[8] Mutation of HFE is associated with low hepcidin formation and hereditary iron overload.[9]

HFE Gene mutations**:** Many types of mutations have been seen in HFE gene.[6] The most common mutation is G to A at nucleotide 845 (G845A), which substitutes a tyrosine for a cysteine at aminoacid position 282 (C282Y). The allele frequency of C282Y in Caucasian population is 0.063.[6,10] C to G transversion at nucleotide position 187 (C187G) is seen in some individuals. This mutation is known as H63D. An aspartic acid is substituted for histidine and has an allele frequency of ~ 16% in European population.[6] A third type of mutation is replacement of serine to cysteine (S65C) is found in ~ 1.5% of European population. It is considered as a benign polymorphism**.** [10]

The C282Y polymorphism in this gene is associated with an increase in serum iron concentration. Rayman MP shows a correlation between increased iron status in mother and an unfavourable pregnancy outcome.[11] Therefore Ingrid P. Senden conducted a case control study which analysed the association of the C282Y allele and the development of pre-eclampsia.[12] The HFE C282Y mutation was detected by an automated method using minor-groove-binding DNA oligonucleotides (MGB probes).[13] The presence of C282Y allele was confirmed by conventional PCR with restriction fragment length polymorphism analysis.

Preeclampsia is one of the most common pregnancy related syndromes associated with major cause of fetal and maternal morbidity and mortality. In normal pregnancy also, many of the biomarkers proposed for preeclampsia are raised to some extent. If symptoms of abnormal placental endothelial dysfunction could be diagnosed well before the manifestation of the clinical disease, it would be useful for therapeutic strategies. So, this study is done in search of molecular biomarker in diagnosing preeclampsia.

Objectives

1. To study the association of C282Y mutation in HFE gene with preeclampsia and compare it with healthy third trimester pregnant women.
2. To analyse and compare the level of serum Iron, TIBC, UIBC, ferritin, and percentage transferrin saturation in pre-eclampsia with normal healthy pregnant women.

METHODS

All pregnant women of age ranging between 18-35 years and having gestational age between 28 to 34 weeks constitute the study population. 50 pre-eclampsia cases and 50 normal healthy third trimester pregnant women were taken as controls. Institutional ethical committee approval was obtained. Informed consent was taken from the study subjects.

Exclusion Criteria

- Hypertension without proteinuria in third trimester pregnant women.
- Essential hypertension before 20th week of pregnancy.
- Hypertension that has not normalized by 12-week post-partum, in the previous pregnancy.
- Subjects having past history of diabetes mellitus, renal diseases and liver diseases are excluded.

A brief clinical history of the subject was taken and systematic examination including weight, height and pulse rate was done. Blood pressure was recorded on two occasions, 6 hours apart. The information obtained was recorded in a structured protocol (a proforma was prepared for the same).

Collection of Blood Sample

8 ml of venous blood sample was collected after overnight fasting and 24 hours urine sample was collected and analysed. All the estimations were done by standard methods as given below.

1. Serum iron and TIBC - Ferrozine colorimetric endpoint method using semi auto analyser (Photometer 5010 V 5+)**.**[15,16]
2. Serum ferritin - Enzyme immuno assay (ELISA).[17,18]
3. Complete blood count: Automated haematology analyser (Celenium Junior).
4. Urine - 24 hr urinary protein (pyrogallol red colorimetric endpoint method) and creatinine (modified Jaffe's method) were quantitatively estimated. Routine urine analysis was done.[19]
5. Calculated parameters- UBIC in µg/dl = (TIBC- Iron) in µg/dl, % Transferrin saturation = (serum iron / TIBC) x 100%.

HFE Gene Polymorphism Study (SNP Identification) by PCR.[13,20]

DNA purification kit (PureFast® genomic DNA purification from human blood kit), HELINI 2X PCR master mix, agarose gel electrophoresis consumables Agarose, 50X TAE buffer, 6X gel loading buffer and ethidium bromide and primers purchased from HELINI biomolecular, Chennai, India. Genomic DNA was extracted from 3 ml of whole human blood. The purified DNA was stored at -20°C. Quality and quantity of extracted DNA is checked by loading in 1% agarose gel. 1 µl of extracted DNA was used for PCR amplification. Genomic DNA was amplified using 25µl of master mix containing 10X Taq buffer, 2mM $MgCl_2$, 0.4mM dNTPS mix, 2 units of proof reading Taq DNA polymerase, 10 pmol/ µl C282Y of each forward and backward specific primer. The contents were mixed gently and spun down briefly and placed into PCR machine. After the DNA was denatured for 3 minutes at 94℃, the reaction mixture was subjected to 30 cycles of denaturation for one minute at 94°C, 1 minute of annealing at 60℃ and 1 minute of extension at 72°C. Final extension was carried over at 72°C for 5 minutes. The PCR amplification products were purified using HELINI PCR clean up kit and digested with Rsa1 (HELINI biomolecules) restriction enzyme for C282Y mutation. After digestion with restriction enzyme, products were separated in 2% Agarose gel, stained with ethidium bromide. Gel was viewed in UV transilluminator and the bands patterns were observed.

Figure 1

Figure 1. Extracted DNA was tested on 1% Agarose gel electrophoresis with QuickRef 250bp DNA Ladder (Lane 2). Lane 1: Purified DNA Lane 3: Lambda DNA / Hind III digest.

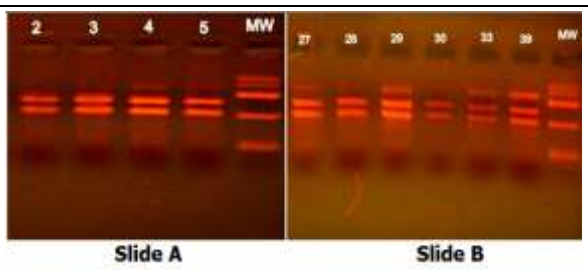

Figure 2

Figure 2. PCR product amplified with the primers C282Y forward and C282Y reverse and digested with RSA, ethidium bromide stained in 2% Polyacrylamide gel electrophoresis. MW:HELINI Quick Ref 250bp DNA. Slide A & B represents DNA fragments. Controls Lanes: 2,3,4,5 Preeclamptic cases Lanes: 27, 28, 29, 30, 33, 39. Lane 29, 33 and 39 shows the presence of positive control mixture of undigested 350 bp amplicon of C282Y allele. In the absence of C282Y mutation (wild type), Rsa I cleaves the 388 bp PCR products into fragments of 248 and 142 bp. While the presence of C282Y mutation (homozygous mutant) results in three fragments, 248 bp, 140 bp, 116 bp and 29 bp (not visible).

Statistical Analysis

The data obtained was entered systematically in MS Excel sheet. Statistical analysis was done using standard software. Average, standard deviation and Student's t-test were done and P - value was calculated to verify the significance of the data. P value less than 0.05 is considered as significant. Statistical significance was determined by calculation of odds ratio for gene polymorphism study.

RESULTS

A broad knowledge of the mechanisms involving preeclampsia at molecular level has opened up various areas of investigations in recent years towards identifying molecular biomarkers. This study constituted 50 cases and 50 controls. The average age of preeclampsia women is 25.56 ± 5.66 years and that of normal pregnant women is 24.72 ± 3.9 years. As per the definition of preeclampsia, PE has ≥ 140 mm Hg of systolic pressure and ≥ 90 mmHg of diastolic pressure**.** 84% (n=42) of PE have systolic BP of 140 – 160 mmHg and 16% (n=8) have ≥160 mmHg. The mean systolic blood pressure of PE of 140 - 160 mmHg range is 143 ± 4.41 mmHg and that of >160 mmHg is 163 ± 7.07. The mean systolic blood pressure of control women (n=50) had BP of 100 - 140 mm Hg with mean of 111.4 ± 5.3 mmHg. The p-value is statistically very significant in both mild PE (140 - 160 mmHg) (p=0.038) as well severe PE (>160 mmHg) (p=0.004) when compared to the control. All the preeclamptic women (n=50) have diastolic pressure >90 mmHg and all the controls (n=50) between 60-90 mmHg. The mean diastolic pressure of PE is 95.2 ± 1.2 mmHg and that of control is 73.6 ± 7.49 mmHg and is statistically extremely significant (p=0.0001).

Biochemical Parameters Reference Range (units)	Cases (Mean ± SD)	Controls (Mean ± SD)	t	p
Iron 35 -140 μg/dl	89.7 ± 30.62	58.71 ± 22.28	2.957	0.0039*
TIBC 250 – 400 μg/dl	385.6 ± 47.34	453 ± 37.6	1.343	0.1824
Ferritin 15 – 240 ng/ml	115.5 ± 57.62	30.66 ± 7.2	6.459	0.0001*
UIBC 160 -360 μg/dl	295.9 ± 60.32	394 ± 35.0	4.26	0.001*
Transferrin Saturation 12-45%	23.7 ± 8.83	12.9 ± 4.61	3.099	0.0025*
Hb 12-15.5 g%	10.6 ± 0.84	9.08 ± 1.00	1.221	0.221
HCT 37-48%	38.24 ± 3.46	32.10 ± 4.04	3.527	0.0006*

Table 1. Comparison of Serum Iron Indices in Pre-Eclamptics and Controls

(* p -value <0.05 considered to be statistically significant)

Geno Type	Cases		Controls		Total	Odds Ratio (95% CI)	Z	p
	No. (n=50)	%	No. (n=50)	%				
Wt/Wt	46	92	49	98	95	1.065 (0.021-54.76)	0.031	0.975 (NS)
Wt/C282Y	4	8	1	2	5	4.261 (0.459-39.55)	1.275	0.202 (NS)

Wt - Wild type, CI - Confidence Interval, NS - Not significant

Table 2

DISCUSSION

The improvements in research in obstetrics and paediatrics have led to a decrease in morbidity and mortality due to preeclampsia, but the possibility to predict the preeclampsia is still difficult, easier methods to diagnose women at risk still remains a clinical and epidemiological challenge All pregnant women between 28 to 34 weeks of gestation and age ranging between 18-35 (25.56 ± 5.66) years constitute the study population. The mean systolic blood pressure of preeclamptic women is 151.87 ± 7.01 mmHg and that of control is 112.00 ± 5.50 mmHg and is statistically very significant (p = 0.0074). The mean diastolic blood pressure of preeclamptic women is 106.17 ± 7.27 mmHg and that of control is 75.00 ± 7.30 mmHg. The pathophysiology of preeclampsia mainly involves reduced placental perfusion and ischemia, which results in the release of pressor agents, which in turn generate dysfunction of vascular endothelium. Oxygen reactive species and iron free radicals released also bring about damage to the endothelium. There is decreased formation of vasodilators. These alterations lead to hypertension and multiorgan dysfunction.[21]

Haemoglobin concentration is lower in both PE and control. The mean value in PE is 10.6 ± 0.84 g% and that in control is 9.08 ± 1.00 g%. No significant difference in the concentration of haemoglobin in PE and in the control group. Haematocrit value (HCT) observed to be higher in preeclampsia than that of the controls. The mean HCT in PE is 38.24 ± 3.46% and in controls 32.10 ± 4.04% (p = 0.0006). The mean serum iron level in PE is 89.7±30.62 μg/dl and that of control was 58.7±22.28 μg/dl. Serum iron was significantly elevated in PE when compared to the control (p = 0.003). In normal pregnant women, there is a decrease in serum iron and ferritin, during the third trimester of pregnancy, as their body stores are used up by the fetus. In preeclampsia, elevated serum iron, % transferrin saturation, Hb, haematocrit and ferritin is observed. There is a decrease in TIBC and UIBC**.** [1] Thus serum iron status is very much increased in PE, when compared to controls. Giving iron supplementation to such patients can do more harm than benefit Therefore, iron level of pregnant women should be assessed before prescribing iron supplements. [1]

Serum ferritin observed around 35 times elevated in PE when compared to the control. (p=0.0001). Increased concentration of ferritin during third trimester may be part of an acute phase response, increased risk of poor pregnancy outcome**.**[22] Serum TIBC and UIBC is lower in PE when compared to the controls. The mean UIBC in PE was 295.9±60.32 μg/dl and that of control is 394 ± 35.0 μg/dl (p value = 0.001). The results allude to the possible contribution of released iron free radicals from ischemic placenta in preeclampsia to its aetiology.[23,24]

Percentage transferrin saturation is higher in preeclampsia when compared to the normal pregnant women. The mean% transferrin saturation in PE is 23.7 ± 8.83% and that of control was 12.9 ± 4.61% statistically significant (p = 0.0025). So, there is an increased iron status in preeclampsia, shown by, increased serum iron, ferritin and percentage transferrin saturation. There is decreased total iron binding capacity and unsaturated iron binding capacity. According to a model for the Iron regulatory function of HFE explained an increase in transferrin saturation triggers the release of HFE from TfR1 and stabilizes TfR2.[25,26] In that way TfR1 becomes accessible for the binding and endocytosis of holo-transferrin, resulting in cellular iron uptake. At the same time, HFE associates with stabilized TfR2 and possibly other proteins, such as hemojuvelin and bone morphogenetic proteins (BMPs) and their receptor (BMPR), to form a putative iron signalling complex that induces hepcidin transcription via Smad proteins. Thus, an increase in the iron content of hepatocyte is indirectly translated into a systemic regulatory response via hepcidin. Iron dependent degradation of TfR, mRNA by iron regulatory proteins, would terminate this process in a feedback loop. According to this model, HFE serves to sense alterations in transferrin saturation. Mutation of HFE is associated with low hepcidin formation and hereditary iron overload.[27] Under low serum iron conditions, hepatocyte HFE is predominantly bound to TfR1.

There are only few studies involving HFE gene mutation in hemochromatosis during pregnancy and anaemia.[28,29] In 2004, Ingrid Seden had done a case control study on preeclampsia and C282Y mutation in HFE gene.[12] There are not many published studies, to show the association of HFE gene polymorphism in preeclampsia. In the present study in preeclampsia, even though preeclampsia women were associated with increased iron indices, 92% (n=46) of the cases and 98% (n=49) of the controls do not show significant mutation in the C282Y allele gene. The observation that 8% (n=4) and 2% (n=1) of the cases and controls respectively have mutation was not sufficient to come to any significant conclusion. Odds ratio for wild type was 1.0645 and that of heterozygote is 4.261. The 95% confidence interval is large (0.021-54.76), indicating a low level of precision in wild type, as well as in PE (0.459-39.55). Z value for wild type is 0.031 and that of heterozygote is 1.275, p-value for both is not significant. Sood and Jain was the first report of a female patient of hemochromatosis associated with porphyrins in urine.[30] Thakur et al., studied the prevalence of C282Y mutation in Chronic Liver Diseases

(CLD) patients and healthy subjects in a tertiary care referral center in India found that almost 10% of non-alcoholic CLD patients have iron overload, but this is independent of C282Y mutation of HFE gene.[31]

Rekha Athiyarath et al, had studied, association of genetic variants of eleven genes including HFE, TFR2 and others with response to iron supplementation in pregnancy.[29] The two most common mutations in the HFE gene are C282Y and H63D mutations studied in various studies. In another observational prospective study by Virgenie Scotet suggested pregnancy to be one potential factor responsible for later manifestation of hemochromatosis in women. Due to difference in expression of genotype in men, C282Y homozygous women are diagnosed at a later stage. This protective nature for late genetic expression in women may be attributed to iron loss during menstrual cycle and pregnancy. Phlebotomy, the treatment usually performed in hemochromatosis, occurs naturally during menstrual cycles in women. Also increased iron requirement by growing fetus during pregnancy reduces the complications of hemochromatosis in women.[28] Alisa Sokoloff reported that clinical diagnosis of hemochromatosis, is suspected in females when transferrin saturation is greater than 45% in females and >50% in males.[32] As Iron overload is possible with this mutations in C282Y gene, but genetically and clinically not expresses may remain asymptomatic to multi-organ comprise in those women with pre-eclampsia. So C282Y mutation which may not be manifested clinically under normal conditions may be well expressed under a stressful condition like pregnancy causing increased iron overload in preeclampsia. HFE genetic testing is not a usual modality for clinical diagnosis of hemochromatosis or in pregnancy, still we had considered it for a search of biomarker for preeclamptic women. This study shows no significant difference in C282Y allele distribution between preeclampsia and controls of normal healthy pregnant women.

Limitations

Due to limitations of time and cost, the study was done in a small sample. Still the fact that 4% of our cases and 2% of our controls do have C282Y allele is an important observation in preeclampsia. Preeclamptic women are diagnosed only after they have developed the full-blown manifestations of the condition. So, if a large sample is studied, a significant finding may be obtained, and it would be very helpful in the research of future therapeutic strategies.

CONCLUSIONS

There is no association of C282Y mutation of HFE gene with preeclampsia. Haemoglobin concentration was less than normal reference range in both the study groups. But haemoglobin concentration and haematocrit were raised in preeclampsia compared to the controls. Although mean value of iron indices was within normal reference range there is an increased iron status supported by increased serum iron, ferritin, percentage transferrin saturation and there is significantly decreased total iron binding capacity and unsaturated iron binding capacity in preeclampsia.

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