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ROLE OF CELL FREE FETAL DNA IN MATERNAL BLOOD - A PROSPECTIVE ROLE OF CBS AND MTHFR GENE AS ANTENATAL GENETIC MARKER IN PRE-ECLAMPSIA PATIENTS

GARIMA GUPTA, JYOTI GUPTA, SULEKHA PANDEY, PANDEY L.K., SAXENA A.K.*
Human Cytogenetic and Molecular Genetic laboratory, Center of Experimental Medicine and Surgery, Department of Obstetrics and Gynecology, Institute of Medical sciences, Banaras Hindu University, Varanasi-221005, India
*Corresponding Author: Email- draksaxena1@rediffmail.com

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Abstract- The pathophysiology of cell - free fetal DNA in maternal blood of pre eclampsia patients remain inexplicable because of two main reasons- first presence of very minute quantity of cell free fetal DNA in plasma required sophisticated equipments & techniques for its identification and second is the variation in gestational age of the patients. Because of lacking molecular biology and associated "risk factors" involvement of CβS and MTHFR gene mutation is remain unidentified in such patients. Hence, the curiosity has been developing to understand the role of these genes in such cases to explore etiology of increasing risk in patients. In the present study, n=151 case were selected and 80% cases showed Sry positive (Y-chromosome specific sequence) used as marker for confirmation of cell free fetal DNA in maternal blood. Genetic study reveals, >20% case showing mutation of CβS gene and MTHFR C677T gene polymorphism showing lack of transition between C/T alleles. Statistical analysis showing significant difference due to hence, these findings could be useful to assess as prenatal diagnosis marker in genetics lead to altered single gene folic acid metabolism (homocysteine) and may increased as an independent "risk factor" in pre eclampsia patients.
Key words- Cell free fetal DNA, Cystathionine β Synthase, Methylene Tetrahydrofolate reductase and Pre eclampsia.
Short Title- Cell free fetal DNA in maternal blood as genetic marker in pre-eclampsia patients

Introduction

Globally, a hypertensive disorders such as pre-eclampsia of pregnancy responsible for 50,000 maternal and 9, 00,000 prenatal deaths annually [1]. In human pregnancy, it seems to be a unique disease and require extensive investigations whose underlying etiology at molecular level remains obscure. Approximately 3% of pregnant women in the western world suffer from pre-eclampsia condition, a potentially life-threatening multi-system disorder [2]. Pre-eclampsia & eclampsia remains one of the leading causes of prenatal mortality and morbidity selected as model for such clinical study. Clinical feature includes hypertension, loss of protein in the urine, headache and visual disturbances. The burden of pre-eclampsia on reproductive health care resources and substantial progress in the prevention and treatment will require advance knowledge to our understanding of the pathophysiology of the disease at the molecular level. Because pre-eclampsia resolves postpartum, premature delivery of the baby may be essential to safeguard the mother's life. The relationship between the entry of fetal nucleated cells into maternal circulation and cell- free fetal DNA remains to be elucidated. Several studies have been shown that both intact fetal cells and cell-free fetal DNA cross the placenta circulate in blood stream of mother [3]. Placental membrane is separating the fetal and maternal circulations during pregnancy. Numerous evidence has accumulated that placenta forms an impermeable barrier between mother and child. The bidirectional cellular trafficking of genomic materials between the fetus and the mother during pregnancy becomes an essential prenatal genetic marker for clinical research as non-invasive assessment methods [4,5].The tremendous knowledge of human genetics has been accumulated dramatically for the last two decades on pre eclampsia has been accumulated regarding analysis of circulating fetal DNA in maternal blood plasma or serum as a metabolic markers was not realized until 1996, when DNA with tumor-specific characteristics was demonstrated in cancer patients[6].

Methylenetetrahydrofolate reductase (MTHFR), an important regulatory enzyme of folate metabolism associated plasma homocysteine concentration increased risk factor for atherosclerosis and other clinical lesions. MTHFR regulate folic acid metabolism and to maintain the genomic instability during DNA methylation [7]. Epidemiological studies of hyperhomocysteinemia have been reported as a risk factor during pregnancy complication in pre eclampsia patients [8-11]. Environmental factors including intake of folate as dietary supplements are essentials molecules for growing fetus and polymorphic variation in MTHFR alleles may

increase "risk factor" for severe disorders such as neonatal congenital malformations of neural tube defects, trisomy-21, Alzheimer disease, cardiovascular disorders and cancer [12, 13]. However, apart from its biological complications and risk associated gene mutation or polymorphic variation of allele's frequency may raise the possibility of circulating DNA in maternal blood to prove non - invasive tool as a genetic marker for prenatal diagnosis.

Materials and Methods

In the present study we selected n=151 case attending antenatal clinic, Department of Obstetrics & Gynecology, S.S Hospital, I.M.S., B.H.U.,Varanasi in age group of 20-30 yrs with live intrauterine pregnancy and clinically diagnosed pre-eclampsia & eclampsia (according to Williams Obstetrics 22nd edition) with their respective controls (n=50), Maternal plasma was collected from pregnant patients for cell free fetal DNA. Maternal blood (10 ml) was collected into EDTA sterile vials and centrifuged within 4 hrs at 1500 rpm for 15min. Isolated plasma was transferred into another vial of polypropylene and immediately stored at –80°C till further study. The study was dually approved by ethical committee of the Institute of Medical Sciences and samples were collected after written consent either from the patients or their attendant.

Analyses of Cell free fetal DNA (cffDNA) from maternal blood

The identification and characterization of cell free DNA from maternal blood is a very difficult task. The genomic DNA was extracted from maternal blood (plasma) using a standard protocol Miller et al. 1994 of chloroform - phenol method [14]. Starting volume and experimental conditions fluctuate during standardization of our protocol was as follows - initially 400 µl of plasma sample were collected from maternal blood (10 ml) samples and out of which 0.5µg-1µg (20 µl) DNA was finally extracted for genomic study, Sry gene (a single copy Y- chromosome specific sequence) primers (F=5'-GGTAAGTGGCCTAGCTGGTG-3' and R=5'-CACAGAGAGAAATACCCGAA-3') was used as a specific marker to determined the fetal DNA in maternal blood. For CβS gene forward=5'-GTTGTTAACGGCGGTATTGC-3' and reveres 5'-GTTGTCTGCTCCGTCTGGTT-3' primers were selected. For MTHFR C677T allele analysis selected primers are F=5'-TGA AGG AGA AGG TGT CTG CGG GA-3' and R=5'-TGA GAG TGG GGT GCA GGG AGC TT-3'. We have developed PCR specific strategies using forward and reverse primers in total volume of 25 µl contain 50-100 ng of plasma DNA , 20 pmole of each primer, 200µM of each dNTPs mix with Taq buffer (10mM Tris HCl pH 8.3, 50mM KCl), 3.0mM $MgCl_2$ and 3 unit of Taq polymerase (New England Biolab). PCR conditions were 4 min at 94ºC for initial denaturation, 58 ºC/1min for annealing followed by 35 cycles and 72 ºC/7min for final extension. RFLP analysis was carried out for the polymorphism analysis of MTHFR C677T allele. PCR product (5 µl) were digested at 37ºC for 3hr. in reaction volume of 25 µl containing 1U of *Hinf-I* restriction enzyme (New England, Biolabs) and NEB buffer (2.5 µl). The digested product of RFLP was separated on 3% agarose gel stained with Et.Br and visualized on Gel Doc system (SR Biosystem).

Statistical Analysis was carried out between cases and controls using chi square fisher exact probability test to find out the level of significance differences ($p<0.05$).

Results

We have selected (n=151) pre-eclampsia cases for isolation of cell free fetal DNA and 62.75 % cases were evaluate successfully for the identification and characterization of cell free fetal DNA using Sry specific primer of Y- chromosome sequences corresponding to male DNA serve as internal control . Those patient's (35.29%) having lack of clinical history of pre-eclampsia or eclampsia symptom were excluded from the present study. Figure 1A & B shows 80% cases were Sry positive while 20 % were Sry negative confirming females serve as negative controls. In the present study, we have also evaluate the mutation of 171 bp DNA fragment of CβS gene as one of the important candidate gene for pre-eclampsia with variability in expression in different cases of pre eclampsia, up regulation (increased intensity of band) as observed in lane -2, while down regulation i.e. decreased intensity of band was observed in lane 3-6 as shown in fig-2.The most interesting finding were 3.6 % patients showing complete disappearance of 171bp amplified product of CβS gene as shown in lane-1(arrow head). These findings were repeated thrice to confirm the disappearances of 171bp DNA fragment of CβS gene. Simultaneously, the polymorphic variation of MTHFR C677T gene was also evaluated to identify the genotype frequency in pre eclampsia cases. The highest frequency of CT allele was 37.5% in pre eclampsia cases and 24.0% frequency was observed in controls as shown in fig-3. The individual frequency of T allele (0.187%) was calculated using Hardy Weinberg Equilibrium (HWE). Besides this we have also noticed first time that complete disappearance of 198 bp fragment of MTHFR gene in one case (0.18 %) confirming the role of folate metabolism associated risk factor in pre eclampsia patients. Statistical analysis were carried out between cases and controls using fisher exact probability test shows significant ($p<0.05$) differences.

Discussion

The anatomy of cffDNA in reproductive medicine is quite relevant. The presence of fetal DNA in maternal plasma was first discovered in 1997 through the detection of Y-chromosome specific sequences in the plasma of women who were carrying male fetuses [15]. Maternally inherited fetal DNA and native maternal DNA is being used as a diagnostic obstruction. A comparable equivalent to Y-specific DNA that could serve as a facile internal control to verify presence of fetal DNA in male while lacking consider as negative control (female fetus) as present study significantly (80 %) documented Sry

positive as shown fig-1A. The presence of fetal DNA in maternal plasma was through the detection of Y-chromosome specific sequences (primers) in the plasma of women who were carrying male fetuses [15]. The numbers of copies of Sry (a single-copy of Y chromosome-specific sequence) and the ratio of fetal to maternal DNA was increased (775–970 fold) in the plasma than amount of DNA derived from intact fetal cells. Several reports have confirmed that gestational age correlates positively with amount of fetal DNA in plasma and concentrations to be low in the first trimester rising in the second and third trimester [16].

Inability of PCR to distinguish readily between maternally inherited fetal DNA and native maternal DNA is clearly a diagnostic obstacle. Identification of circulating cell free fetal DNA can lead to selection or creation of more effective methods of enrichment during DNA isolation from plasma for improved clinical application. Therefore, understanding the molecular structure and dynamics of fetal DNA in plasma of normal individuals will be necessary to achieve further advancement in the field of reproductive medicine. Fetal cells appear early in the maternal circulation during the first trimester and continue to be present throughout gestation till birth [3]

The origin of the cffDNA has remained uncertain and evidence suggest that the placenta is also the most liable source. Three sources of circulating DNA can be plausibly hypothesized- (i) dying cells (necrotic or apoptotic); (ii) active secretion of DNA; (iii) terminal differentiation. Present study dealt with the variation in CBS gene expression might be due to different gestational age of the pre eclampsia cases correlates positively with amount of fetal DNA exist in plasma [17]. In recent studies, represent that 3-6 % of total DNA in maternal plasma release during 2^{nd} -3^{rd} trimester. However, the isolation of cell free fetal DNA from the maternal blood circulation is stills a difficult task which requires extensive exercise / labor [3]**.**

Another gizmo has been added to further confirmation of cffDNA to evaluate the polymorphism of MTHFR alleles in pre eclampsia patients because, a common C to T transition at nucleotide 677 (C677T) associated as a risk factor for various clinical lesions including pre-eclampsia [18]. Interestingly, the transition between 677C -> T of MTHFR gene C677T polymorphisms was unable to observed in the present study due to either small sample size or unknown biological factors such variation of gestational age/or ethnicity. Embryonic development of homozygous pregnant female lacking the enzyme appears to be normal but there is an increased mortality during postnatal development in third trimester. Cystathionine ß-synthase gene mutation not only induces complications during pregnancy but extend their effects to increase “risk factor” for congenital neural tube defects [19]. However, the present study confirm the disappearance of 171bp fragment of CBS gene in two patients might have responsible for increasing as an independent “risk factor” in pre eclampsia patients. Epidemiological studies have shown that consumption of vegetables / fruit as dietary supplements (rich in folate) might have reduced risk of pre- eclampsia due to protective effect [20]. However, the high degree of genetic heterogeneity is quite striking in the present study, each serve as complexity of mutation has been present in only one or two family of pre eclampsia. These findings in conjunction with observed clinical variability suggested genetic heterogeneity in such cases.

Earlier, it was observed that variation the frequency of alleles of MTHFR gene between homozygous (TT), mutant subjects had significantly higher concentration of plasma total homocysteine, whereas in CC alleles and in heterozygous (CT) subjects were indistinguishable [19]. Such diversity in the frequency of alleles between homozygous (CC) / heterozygous (CT) of MTHFR may be either due to ethnic variation in populations or unknown biological reason/ factors in neural tube defects, early pregnancy loss and venous thrombosis [21] .

Inability of PCR to distinguish readily between maternally inherited fetal DNA and native maternal DNA is clearly a diagnostic impediment. However, studies of epigenetic changes resulting in distinguishable patterns of DNA methylation between fetal and maternal DNA provide promise in the development of unique gene or global fetal specific sequence detection assays [22]. Although, our observations could be useful to assess future studies on genetic determinants of folate and homocysteine levels with an independent “risk factor” in patients like preeclampsia. Thus, an important goal in this study should be to identify the predominant form of cell free fetal DNA in maternal plasma among normal pregnancies and its correlation to the other gene mutation as non- invasive tool for genetic marker in prenatal diagnosis .

Conclusion

We conclude from the present study that **-** (1) Cell free fetal DNA from maternal blood should be used as “genetic marker” for programmed cell - death, (2) Single gene mutation (CβS and MTHFR genes) may also help to assess as an independent “risk factor” for several disorders such as pre-eclampsia & eclampsia condition, and (3) cffDNA may act as non- invasive device for antenatal diagnosis.

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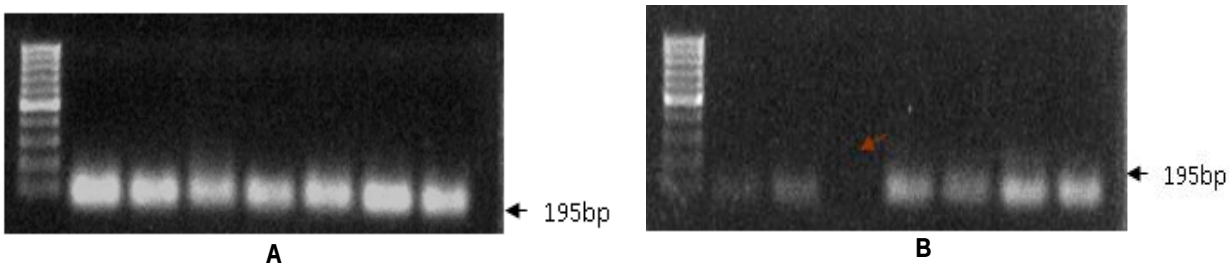

Fig.1- Identification of cell frees fetal DNA in maternal plasma using Sry marker. Lane (M) ladder 100 bp, lane 1 to 7 showing all patients are SRY positive (Fig. A) while lane-1& 3 showing absence of Sry gene act as negative control while lane- 2, 4,5,6 & 7are Sry positive (fig.B).

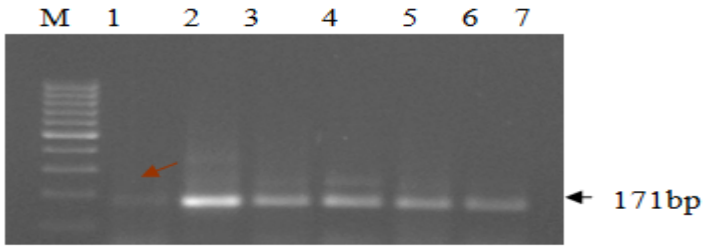

Fig.2- Representative photograph of CβS gene in pre-eclamsia patients. M - 100bp ladder, Lane-1 showing mutation of CβS gene (indicated by arrow), Lane-2-4 cases showing differential expression of CβS gene, Lane-5 & 6 act as controls.

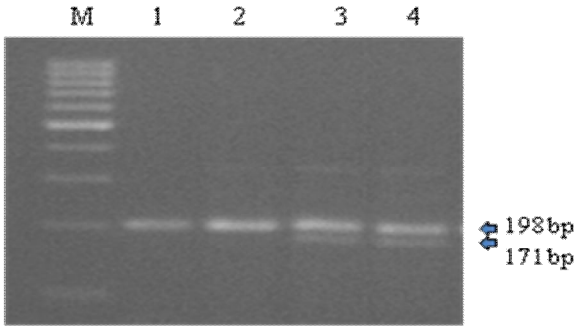

Fig.3- MTHFR-C677T gene analysis in pre-eclampsia patients. M-ladder 100bp, lane 1& 2 showing CC genotype. Lane 3 & 4 CT genotype.