

Full Length Research Paper

Evaluation of methylenetetrahydrofolate reductase C677T gene polymorphism associated risk factor in the patients of recurrent pregnancy loss

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The etiology of human recurrent fetal loss is associated with methylenetetrahydrofolate reductase (MTHFR) C677T gene polymorphism. Several conflicting reports on MTHFR gene polymorphism increases the curiosity, with the aims to evaluate the prevalence of MTHFR gene in recurrent miscarriage cases using PCR-RFLP analysis. The present findings reveal that the highest (26.7%) incidence was observed in heterozygote (CT) cases when compared with controls (24.0%). The individual alleles (T) frequency (0.13%) was also calculated by Hardy Weinberg equilibrium showing lack of significant differences ($p < 0.05$). Biochemical analysis showed slight variation between homocysteine ($17.02 \pm 14.64 \mu\text{mol/l}$) and folates ($16.76 \pm 8.48 \text{ ng/ml}$). Cytogenetic study showed chromosomal association between D and G – groups, while karyotype of one case is mosaic and was also observed. However, the calculated O.R. (1.15) suggests that “risk factor” increased to confirm that genotypic variants of MTHFR C677T gene polymorphism are responsible for fetal viability.

Key words: Methylenetetrahydrofolate reductase, fetal loss, chromosome association.

INTRODUCTION

Pregnancy loss is the most complex disorder where one or the other parent acts as a carrier for genetic abnormalities (Clifford et al., 1994). Progress has been made to understand the pathogenesis of recurrent miscarriage at the molecular level. Variations in the chromosome number with and without abnormal structural abnormalities are the most acceptable etiology of miscarriage (Keefe et al., 1995; Tamarin 2002). Methylenetetrahydrofolate reductase (MTHFR) C677T gene polymorphism has been associated with fetal loss at early stages of pregnancy (Robertson et al., 2006).

Methylenetetrahydrofolate reductase (EC 1.5.1.20), a key enzyme, catalyzes the conversion of 5, 10-methylenetetrahydrofolate into 5-methyltetrahydrofolate. This is the predominating and circulating form of folate (5-MTHF) which participates in remethylation of homocysteine to methionine. Cystathionine β synthase

(C β S) is another key enzyme of folate metabolism which regulates methionine synthesis through condensation of homocysteine with serine to cysteine. Epidemiological studies reveal that high concentration of homocysteine act as a severe risk factor for recurrent fetal loss and development of neural tube defects (Ray and Laskin, 1999; Saxena et al., 2011). Due to several controversies in earlier reports on MTHFR gene polymorphism associated pregnancy loss; hence, curiosity has been generated several folds with the aim to evaluate the genotypic variants of MTHFR C677T gene polymorphism as a potential candidate gene in women having tendency of fetal loss.

MATERIALS AND METHODS

Blood samples (5 ml) were collected under sterile conditions, for both cytogenetic analysis as well as for molecular studies in prediagnosed women ($n=15$) with age range between 21 to 35 years with previous history of two or more miscarriage and their respective controls ($n=25$) included in the present study. These

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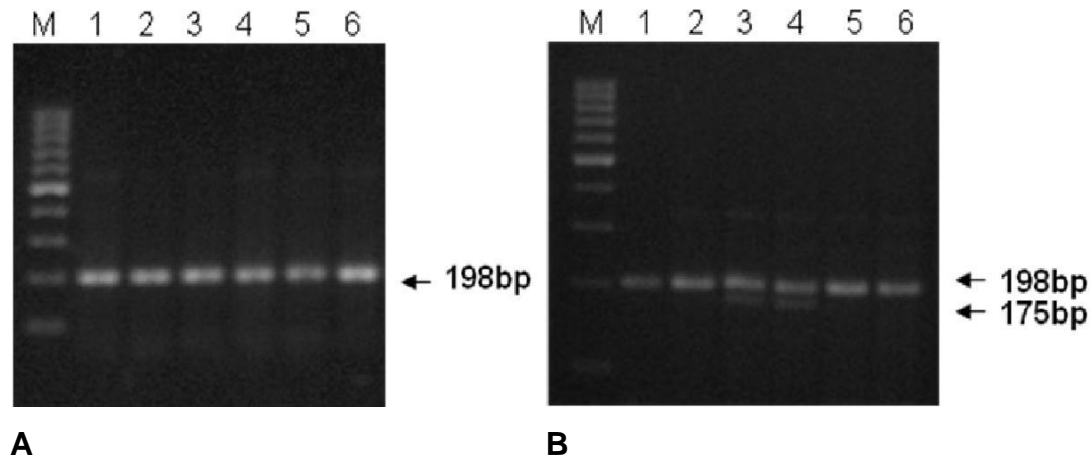


Figure 1. a) The PCR based amplified product of MTHFR C677T gene (198 bp) as shown in figure 1A. RFLP analysis after digestion with *Hinf-I* showing both the genotypes CC homozygous (lane 1, 2, 5 and 6) and CT heterozygous conditions (lane 3, 4) in Figure 1B.

Table 1. Statistical evaluation of genotype frequency in cases and their controls in the patients of recurrent pregnancy loss.

Genotypes	Percentage frequency		Cases/Controls		
	Cases (n=15)	Controls (n=25)	O.R	C.I at 95%	P value
CC	73.5	76.0	0.86	0.21-3.52	1.00
CT	26.7	24.0	1.15	0.28-4.75	1.00
C	0.86	0.88	1.04	0.37-3.43	0.93
T	0.13	0.12	1.13	0.63-2.02	0.79

patients were referred to the Cytogenetic and Molecular Genetics laboratory, Centre of Experimental Medicine and Surgery from O.P.D of S.S Hospital, Department of Obstetrics and Gynecology, Institute of Medical Sciences, BHU, and Varanasi, India. This study was duly approved by the ethical committee of the Institute and written informed consent was obtained prior to participation of either relatives or attendants before collection of blood samples.

Specific forward and reverse primers for C677T (F-5'TGAAGGAGAAGGTGTCTGCGGGA3') and (R-5'AGGACGGTGCGGTGAGAGTG3') were selected for PCR reaction (Van der Put and Blom, 2000). The final reaction volume of 25 μ l containing 100 ng of genomic DNA, 25 mM of $MgCl_2$, 3 U of Taq DNA polymerase, 10 mM of Tris-HCl (pH 8.4), 50 mM of

KCl, 2.5 mM of dNTPs, 0.2 μ M of each primer. Amplification was carried out in a thermo-cycler (Bio Red, USA) consisted of a 5 min denaturing step at 95°C, followed by 35 cycles of 1 min at 95°C (denaturing), 1 min of annealing at 61°C and 1 min at 72°C (extension), followed by a final extension of 7 min at 72°C. For polymorphism analysis of C677T allele of MTHFR gene, the amplified product of PCR (15 μ l) was digested using (10 U) restriction enzyme *Hinf-I* (Van der Put and Blom, 2000). The digested products were separated on 3% agarose gel stained with Et.Br and visualized on Gel Doc system (SR Biosystem) for further analysis of genotypes (Figure 1A and B).

For cytogenetic study short- term lymphocytes cultures were set up and lymphocytes were grown in RPMI-1640 having 5% FBS with or without supplement of folic acid (1 mg/ml), L-glutamine (0.5 mg/ml), phytohemagglutinin-M and antibiotics at 37°C. Colchicine (0.01 mg/ml) was used as mitotic (inhibitor) arresting agent added to culture 30 min before harvesting of the culture. Cells were fixed

in methanol and acetic acid mixture (3:1). Air dried slides were used for GTG banding using trypsin (0.001%) and visualized by 5% Giemsa stain with standard protocol of Yunis et al., (1978).

RESULTS AND DISCUSSION

There are a number of retrospective and case-control studies demonstrating a higher frequency of MTHFR mutations in patients with recurrent pregnancy loss (Lissak et al., 1999; Zetterberg et al., 2002; Behjati et al., 2006). The highest frequency of CT genotype was observed, that is, 26.7% in cases with respect to controls (24.0%) in heterozygous condition, while in homozygous condition that is CC genotype (wild type), the frequency was quit higher as documented in Table 1. The etiology of recurrent fetal loss is highly complex because of the interaction between gene and environment. The individual allele frequency was also evaluated using Hardy Weinberg Equilibrium between homozygous and heterozygous genotypes showing lack of a significant difference. Although, TT genotype (rare type) was not observed in the present case control study due to a small sample size. The allele frequencies of C677T are quite variable in Canadian population (Frosst et al., 1995). Similarly, our study also reveals higher frequency of

Table 2. Miscarriages patients showing details of genetic variation during different gestation periods.

Case	Age	Fetal Loss	Normal (Child)	MTHFR C677T Genotype	Karyotype Status
1	22	2 in first trimester	1	Homozygous (CC)	46,XX (Normal)
2	28	3 in first trimester	0	Heterozygote (CT)	46,XX (Normal)
3	35	2 in first trimester	0	Homozygous (CC)	46,XX (Normal)
4	38	1 in first trimester	1	Homozygous (CC)	46,XX (Normal)
		1 in second trimester			
5	25	3 in first trimester	0	Heterozygote (CT)	46, XX+ D/G
6	34	3 in first trimester	0	Heterozygote (CT)	46, XX+ D/G
7	24	3 in first trimester	0	Homozygous (CC)	46, XX+ D/G & G/G.
8	25	3 in first trimester	0	Homozygous (CC)	46, XX+ D/G
		1 in second trimester			
9	24	2 in third trimester	0	Homozygous (CC)	46,XX (Normal)
		1 in 2nd trimester			
10	30	3 in first trimester	1	Homozygous (CC)	45,X0/46,XX(Mosaic)
		1 in 3rd trimester			
11	23	2 in first trimester	0	Homozygous (CC)	46,XX (Normal)
		1 in 2nd trimester			
12	25	1 in second trimester	0	Homozygous (CC)	46,XX (Normal)
		1 in first trimester			
13	25	3 in first trimester	1	Homozygous (CC)	46,XX (Normal)
14	23	2 in THIRD TRIMESTER	0	Homozygous (CC)	46,XX (Normal)
		1 in 2nd trimester			
15	28	3 in first trimester	1	Heterozygote (CT)	46,XX (Normal)

C677T allele in heterozygote when comparing to controls, although such variation in frequency is probably either due to a heterogeneous group of population which belong to different ethnic background or unknown environmental factor. Simultaneously, factors affect homocysteine concentration either as dietary supplement or gene polymorphism involved in folate and vitamin B₁₂ dependent metabolism. The contribution of MTHFR C677T gene polymorphism is to reduce fetal viability and may be difficult to determine as the majority of pregnancies unexpectedly terminate before the 6th week of gestation.

However, it is difficult to interpret our data when compared with other studies because of variability in the timing, karyotypic variation and age of gestational fetal loss (less than 12 weeks to less than 24 weeks) as also documented in Table 2. The biochemical analysis of homocysteine and folic acid were also observed in the present study and reveals a higher value that is 17.02 ± 14.64 $\mu\text{mol/l}$ of plasma homocysteine (tHcy) as compared to folates (16.76 ± 8.48 ng/ml) recorded in the

same cases of recurrent fetal loss. The increase of tHcy and decrease of folate is also associated with the development of risk factors for congenital neural tube defects (Botto and Yang, 2000). The most common genetic factor is numerical karyotypic variation that is monosomy/polyploidy or loss of one paternal sex chromosome during early cleavage (Chandley, 1981).

However, the present study also shows similar findings (numerical karyotypic variation) with high frequency of association perhaps due to changes in physiological conditions of cell-division leading to the breakage and reunion with involvement of the repetitive DNA sequences and Robertsonian translocation during gametogenic impairments (Therman et al., 1989) which might have been responsible for increased risk for recurrent fetal loss. The cytogenetic study shows numerical variations (6.7%) in total cases of fetal loss, while the other important feature was the chromosomal association between D and G group chromosomes, making triradial and tetrad structures with 46, XX karyotypes, while one case shows mosaic with karyotypes

45, XO/46, XX as documented in Table 1. These studies when combined with case reports confirmed the significant involvement of a variable frequency of MTHFR alleles in different population but still lacking in Indian population. However, it is evident that large numbers of cases are required to fully elucidate the mechanism of MTHFR gene polymorphism in contributing to decreased fetal viability especially during period of high folate requirement. About half the general population carries at least one mutated allele frequency in homozygous mutated genotype (677TT) ranges vary from 1 to 20% (Botto, 2000). The statistical analysis shows lack of significant difference after using chi square test ($p=1.00$) probably due to small sample size or an unknown factor. The odd ratio was 1.15 at 95% confidence interval (C.I. 0.28 to 4.75) also calculated to observe the "risk factor" for heterozygous condition. Interestingly, one patient shows karyotypic variation (mosaicism) along with MTHFR C677T gene polymorphism clearly evident of gene and gene interaction, which might have been responsible for increasing high risk in early pregnancy loss.

In conclusion, the present study demonstrates the interaction of MTHFR C677T gene polymorphism involving an "independent risk factor" during early embryogenesis. From our small study, it is very well evident that such genetic associations are responsible to reduce the fetal viability and pregnancy complications. However, these finding are promising but small, needs further evaluation to determine allele frequency by collecting large samples size to confirm the exact role of such candidate gene in cases of recurrent fetal loss.

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