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MTHFR C667T polymorphism association with male infertility risk in Pakistan

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Abstract: The methylenetetrahydrofolate reductase (MTHFR) gene is one of the main regulatory enzymes involved in folate metabolism, DNA synthesis and remethylation reactions. The objective of this study was to analyze the distribution of the MTHFR C677T variants using PCR-Restriction Fragment Length Polymorphism (RFLP) in a cross sectional study consisting of 160 infertile men including azoospermia, oligospermia, severe-oligospermia and normospermia infertile subjects compared to 90 ancestry-matched fertile normozoospermic controls. The genotype CT of C677T was highly significant frequency in controls and all infertility groups (28.75%; OR 1.983; 95% CI 1.117-3.522; P 0.012; chi-square 6.262) while TT homozygous variant is present statistically significant frequency in controls and azoospermic subjects and severe-oligozoospermic subjects with (P 0.065; chi-square 3.406 in azoosprimic) and (P 0.071; chi-square 3.267 in severe-oligospermic). The prevalence of C677T genotypes TT between azoospermic and severeoligozoospermic patients and controls was almost similar 6.67% and 7.4% respectively but high as compared to controls (1.11%). In conclusion, this analysis supports that the *MTHFR* C677T polymorphism acts as a genetic mutation risk factor and is capable of causing male infertility susceptibility in Pakistani population.

Keywords: MTHFR, RELP, polymerase chain reaction, male infertility.
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INTRODUCTION

Male infertility in humans is important cause of couple's inability to bear children in 20–50% of total cases[1]. In about 15% of male infertile cases, genetic abnormalities, including chromosomal aberrations and single gene mutations, could be present, which may affect spermatogenesis[2, 3]. Spermatogenesis is a complex process, and the mechanism is still unclear.

The folate cycle is essential for normal cell function and is involved in such processes as methionine production, and purine and pyrimidine synthesis. One crucial enzyme within the folate pathway is methylenetetrahydrofolate reductase (MTHFR). MTHFR irreversibly reduces 5, 10-methylenetetrahydrofolate (5,10-methylene-THF) to 5-methyltetrahydrofolate (5-methyl-THF), the primary methyl donor for remethylation of homocysteine to methionine. In turn, methionine provides the methyl group necessary for the formation of S-adenosylmethionine methylation. One of the these methylation reactions, that of cytosine residues within DNA, is essential for development; the majority of mice deficient in any one of the DNA methyltransferase (DNMT) enzymesDNMT1, DNMT3a, or DNMT3b die before or soon after birth[4].

A point mutation in its coding region (C677T) decreases the activity of the enzyme by about 30% in heterozygotes (CT) and 70% in homozygotes (TT)[8]. The frequency of T allele varies greatly among populations, ranging from 40–30% in Europe and Americas to 11–6% in Africans and Sri Lankans[5].

The most common phenotype of this mutation is the accumulation of homocysteine leading to homocysteinuria/homocysteinemia[6]. As DNA methylation is one of the most common repressor of tissue specific genes, inefficient methylation because of this mutation is likely to compromise gene regulation [7, 8]. Also, a low folate may cause inefficient DNA replication and DNA-repair. Therefore, it is not surprising that mutant MTHFR increases susceptibility to cardiovascular diseases, diabetes, neural tube defects (NTDs), cancers and other multifactorial disorders, either to the person concerned or the offspring, the latter being most evident in NTDs[9].

The phenotypic effect of the mutation is modulated by exogenous factors such as folate-supplementated food, providing a gene-environment interaction in phenotype development. This cross-sectional study carried out to determine the allele frequency of point mutation in the MTHFR gene, the C677T, examining the effect of this MTHFR mutation on normal sperm concentrations, and to assess association with male infertility in Pakistani population.

MATERIALS AND METHODS

Subjects

Using a cross-sectional strategy, the study population was selected from patients attending the different hospitals of Faisalabad, Peshawar, Islamabad, Jaranwala, Dera Ghazi Khan, Chiniot, Lahore and Karachi cities of Pakistan for couple

infertility and from volunteers participating in clinical studies. All were aged from 24 to 50 years and each subject gave informed consent before participating in this research. The subjects selected consisting of 90 men with normal spermatogenesis who had fathered at least one child by natural conception as control group and 160 men with infertility. The latter group included 43 men with azoospermia, 27 men with severe oligozoospermia, 35 men with oligozoospermia, and 55 men normozoospermia infertile men. Semen analysis was performed according to normal standard parameters using the World Health Organization (WHO) criteria. Normozoospermic infertile men had normal semen profile and infertility of unknown etiology.

Molecular analysis

Karyotyping

Cytogenetic study was carried out from each subject by sampling of peripheral venous blood tapping into a heparinized vacutainers. Chromosomal analysis was performed on phytohemagglutinin (PHA)-stimulated peripheral lymphocyte cultures using standard cytogenetic methods [10]. Thirty to 40 metaphases were analyzed per individual and the routine analysis was based on GTG-banded staining.

Genomic DNA extraction

Each subject was submitted to blood sampling through peripheral blood collecting into EDTA vacutainers separately, shake thoroughly and were stored at -20°C until further analysis. Genomic DNA was extracted from peripheral blood by the standard phenol-chloroform technique described by Sambrook et al. [11] with little modification and optimizations. In an sterile eppendorf tube, 700µl of human blood was taken and 700µl of solution A (0.32M sucrose, 10mM Tris (pH 7.5), 5mM $MgCl_2$, and Triton X-100 1% (v/v) in distilled water was added to it. It was mixed by inverting at room temperature for 10 minutes and centrifuged at 13000rpm for 10 minute. The supernatant was discarded and nucleated cellular pellet was re-suspended in 400µl of solution B [10mM Tris (pH 7.5), 400mM NaCl and 2mM EDTA (pH 8.0)], 12µl of 20% SDS and 5µl of proteinase K and incubated at 37°C overnight in water bath. Then 500µl of a fresh mixture of solution C (buffer saturated phenol) and solution D [Chloroform (24 volumes): Isoamyl alcohol (1 volume)] were added, mixed thoroughly and centrifuged at 13000rpm for 10 minutes. Aqueous phase (upper layer) was transferred in a new tube and precipitated the DNA by adding 55µl of 3M sodium acetate (pH 6.0) and equal volume (500µl) of iso-propanolin chilled form and inverted the tubes several times to precipitate the DNA. DNA pellet washed by 200µl of 70% ethanol was added, centrifuged at 13000rpm for 7 minutes and the ethanol was discarded. The DNA pellet was dried for half an hour at room temperature 37°C for 30 minutes. The precipitated DNA was dissolved in autoclaved distilled water. DNA quantitative analyzed with spectrophotometer and stored in aliquots at 4°C.

Genotyping of SNP A1298G in the MTHFR gene: Polymerase chain reaction

The primers 5'-CTTTGGGGAGCTGAAGGACTACTAC-3' (forward) and 5'-CACTTTGTGACCATTCCGGTTTG-3' (reverse) were used to amplify the 163bp fragment around the polymorphic site studied. Polymerase chain reaction (PCR) was carried out in reaction mixture contacting approximately 70ng of genomic DNA, 1X *Taq* buffer with KCl (Fermentas), 2.5mM $MgCl_2$, 0.2mM deoxynucleotide triphosphates (dNTPs), 1U of *Pfu* DNA polymerase (Fermentas # EP 0501). A negative control reaction containing everything except DNA i.e., 5 µl distilled water was used in place of DNA and included in every PCR experiment to rule out PCR contamination. Amplification was carried out in a Bio-Rad thermocycler with following thermal profile-initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 51°C for 1sec, extension at 72°C for 1 sec followed by final extension at 72°C for 10 minutes. Amplified PCR products along with 50bp DNA ladder (Fermentas # SM0371) were separated on a 3.5% agarose gel by electrophoresis in TAE buffer at 80 volts for 60 minutes and required sized PCR products were analyzed under UV light in Gel documentation system (GDS) (Syngene, USA).

RFLP for A1298C MTHFR

The PCR product was digested with *Mbo*II (Fermentas, catalogue #ED1214). The restriction sequence of *Mbo*II (*Mbo*IIR gene from *Moraxella bovis*) is given as follows.

5´….GAAGA $(N)_8$▼…3´

3´….CTTCT $(N)_7$ ▲…5´

A total of 15µl reaction mixture was prepared as follows: 8 µl of water, 1 µl of 10x digestion buffer Tango, 1 µl of restriction enzyme, and 5 µl of PCR product. Digestion was carried out by incubation at 37°C overnight.

The products of the restriction digest were run on 8% native poly acrylamide gel electrophoresis (PAGE) along with ultra-low DNA ladder (Fermentas # SM1223) at 80 volts for 2-3 hours to identify the base pair change. Normal healthy individuals homozygous (AA) generated five fragments 56, 31, 30, 28, and 18bp. The mutated homozygous (CC) created four fragments 84, 31, 30 and 18 bp; whereas in case of heterozygous (AC) 3 fragments of 84, 56,

and 30bp, were observed after ethidium bromide staining.

Statistical analysis

The allelic frequencies were calculated by direct counting the alleles. The observed genotype frequencies for fertile and infertile men were compared with each other using Hardy-Weinberg Equation (observed allele frequencies) using the Chi squared test. Comparisons of genotype, allele, combination genotype and haplotypes frequencies between control and infertile men were done by Chi square test, all p-values were based upon two-tailed tests and Odds ratios (ORs), 95% confidence intervals (CIs) were carried out, P values <0.05 were taken as statistically significant. This analysis was done with SPSS 16 statistical software (SPSS Inc., Chicago, Illinois, USA).

RESULTS

Frequency and distribution of the MTHFR SNP C677T among fertile and infertile men

In order to determine a successive effect of ***MTHFR*** variants on the spermatogenesis phenotype, a bi-allelic distribution analysis of the entire coding sequence of two SNPs in ***MTHFR*** gene, SNP 677 and 1298 has been performed in 250 subjects enrolled in this cross-sectional study.

Genotyping of SNP C677T in the MTHFR gene

PCR amplification was carried out with specific primer pair and amplification profile to amplify 265 bp fragments in all the subjects enrolled in this study (Figure 1).The mutation C677T leads to a Ala222Val change (A222V) of the MTHFR protein and C677T transition gene mutation in genomic DNA. The C677T mutation creats the recognition site of the restriction enzyme *HinfI* (changing 5′..GAN***C***C…3′ to 5′..GAN***T***C…3′).

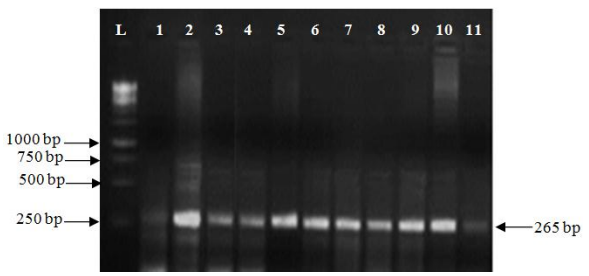

Figure 1: An ethidium bromide-stained agarose gel of PCR amplified *MTHFR* C677T SNP. Lane L: GeneRuler 50 bp DNA ladder (Fermentas # SM0371); Lane 1: negative control (dH_2O) to rule out PCR contamination; Lanes 2–7: PCR amplification products of expected base pairs sized (265 bp) in each sample.

All PCR products of SNP C677T in *MTHFR* genomic fragments were digested with *HinfI.* The presence of mentioned SNP mutation results in full enzymatic digestion of the amplified fragment, which produces two fragments with different length (94bp and 171bp). On digestion, gel electrophoresis showed 265bp fragment for the wild type allele (CC), instead of the two fragments 94bp and 171bp for the homozygous mutation allele (TT) in infertile and control subjects (Figures 2 and 3).

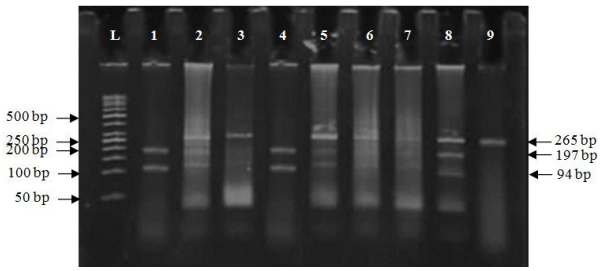

Figure 2: RFLP analysis of C677T mutation in healthy control subjects detected by 8% native poly acrylamide gel electrophoresis (PAGE) (PAGE). The 265 bp PCR product was digested with *HinfI*. Lane L: GeneRuler 50 bp DNA ladder (Fermentas # SM0371); lane 3 and 6: CC homozygous (265 bp), lane 2, 5, 6 & 8: CT heterozygous mutation (94 bp, 197 bp, 265bp); lane 1 & 4: TT homozygous mutation (94 bp, 197 bp).

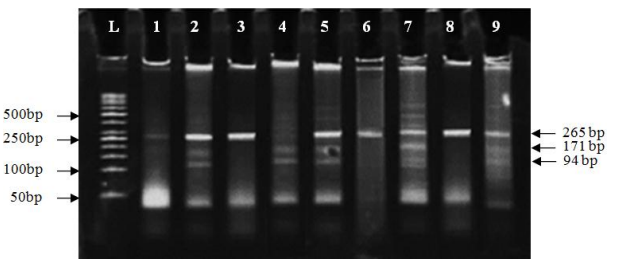

Figure 3: RFLP analysis of C677T mutation in infertile subjects detected by 8% native poly acrylamide gel electrophoresis (PAGE) (PAGE). The 265 bp PCR product was digested with *HinfI.* Lane L: GeneRuler 50 bp DNA ladder (Fermentas # SM0371); lane 1, 3 and 8: CC homozygous, lane 4: CT heterozygous mutation; lane 2, 5, 7 and 9: TT homozygous mutation.

SNP C677T in the MTHFR allele frequencies

The statistical analyses with HWE software showed that the *MTHFR* C677T allele and its genotype distributions showed highly significant Hardy-Weinberg equilibrium in infertile men and control groups (P=0.830; Chi-square 0.046) (Table 1). Overall the homozygous TT had an elevated frequency in infertile men (6.25%) compared to the control samples (1.11%). Moreover this genotype TT showed high frequencies in azoospermic group and severe-oligozoospermic group (6.67%; P=0.065; 95% CI 0.006-1.695; OR 0.15 and chi-square 3.406) and (7.4%; P=0.071; 95% CI 0.005-2.101; OR 0.14 and chi-square 3.267), respectively. However the difference between controls and infertility groups for the both genotype CT and TT were shown statistically high significant (P=0.012; chi-square 6.262; OR 1.98;

95% CI 01.117-3.522) and (P=0.058; chi-square 3.602; OR 0.169; 95% CI 0.008-1.31), respectively. Heterozygous CT genotype showed the highest frequency in normozoospermic infertile group (65.45%) than controls (44.44) and statistically also significant (P=0.066; chi-square 3.371; OR 1.95; 95% CI 0.901-4.252) (Table 2).

DISCUSSION

Folates play essential roles in the synthesis of nucleic acids and in epigenetic regulation of gene expression by remethylation of homocysteine into methioninine[14]. Low level of folate leads to hyperhomocysteinemia and hypomethylation status could negatively impact on spermatogenesis by resulting in oxidative stress. Oxidative stress is well known to cause damage to sperm olasma membrane and mitochondrial and nuclear DNA[1]. The folate metabolism are necessary for male spermatogenesis as DNA hypomethylation disrupting gene expression or by inducing uracil misincorporation during DNA synthesis leading to errors in DNA repair, strand breakage and chromosomal anomalies[12]. MTHFR gene encodes key regulatory enzyme involved in folate metabolism, and genetic variants of this gene may affect some men to reduced sperm counts[13,18].

However, in stratified analysis by ethnicity, the variant genotype of the MTHFR C677T polymorphism was significantly associated with the risk of male infertility in Asian population. A et al.[14] noted that mutant homozygote (TT) as well as carrier with allele (TT+CT) in infertile patients was significantly higher than those in controls, suggesting that there was an association of SNP C677T in the MTHFR gene with male infertility. Lee et al. 2006, suggested that the MTHFR 677TT were significantly associated with the azoospermia group.

The present study was consistent with these studies. Overall the homozygous TT allele variation was common in azoospermic group and severe-oligozoospermic group while CT and TT were shown statistically high significant frequencies in all infertile groups and heterozygous CT genotype was the highest frequency in normozoospermic infertile group (65.45%) than controls (44.44). It is biologically believable that MTHFR polymorphisms play important role as a risk factor of male infertility.mutant allel and genotype at C677T were strongly associated with male infertility in our population.

This is in contrast with the reports where the C677T variant was not associated with infertility A study of 77 subfertile men of Dutch ancestory found no significant difference in the frequency of the CC/CT/TT genotypes[15]. Another study comprising of 93 infertile and 105 fertile and/ or normospermic individuals of Italian ancestry did not find any association between the C677T allele and male infertility[16.]

The association between MHRT mutations was observed in the current population thatC677T genotype in heterozygous is more susceptible that heterozygous A1298C variants. Recently reported that MTHFR C677T polymorphism is capable of causing male infertility susceptibility in Asians, but not in Caucasians[17] . These conflicting results from studies on various populations ahow that the role of C677T in susceptibility to male infertility may depend on environmental factors such as levels of dietary folate uptake[18]. The prevalence of the C677T variant is known to be low (6.6%) in African (Gambia, Kenya, Madagascar and Central African Republic) populations where as in Arabs, Berbers Moroccan, Indian individuals the frequency of the homozygote TT has been estimated at 6% and the T allele to 26.4%.

The frequencies of the genotypyes observed in the current study, TT with a frequency of 6.25% and 1.11% and a T allelelic frequency of 20.63% and 23.33% in the infertile and control groups respectively, supports these reports[19].

It is concluded that the *MTHFR* variants C677T act as risk factors in male sterility counts in the Pakistani population. A significant proportion of men with azoospermia, severe oligozoospermia, oligospermia and noromzoospermic infertile have been reported to have methylation anomalies in their mature sperm[20'21].

Table 1: Hardy-Weinberg distribution of *MTHFR* C677T genotypes in infertile and control population.

	Allele X	Allele Y	Observed Frequency	%	HW - Expected Frequency	%	Chi-squre	p-value
CC	310	0	155	911.76	155.57	61.73	0.002	
CT	86	86	86	505.88	84.86	33.67	0.015	
TT	0	22	11	64.71	11.57	4.59	0.028	
Total	396	108	17	100.00	252.00	100.00	0.046	**0.8307**
	0.79	**0.21**	34				(p-value) Chisq w 1 df	

The role of *MTHFR* status, dietary folate and methylation profiles and their correlation in human sperm also facts further investigation. Further studies are necessary to determine the influence of the environment, especially the consumption of dietary folate, on sperm counts, morphology and DNA anomalies of men with different MTHFR variants.

REFERENCES

1. Dhillon VS, Shahid M and Husain SA. Associations of MTHFR DNMT3b 4977 bp deletion in mtDNA and GSTM1 deletion, and aberrant CpG island hypermethylation of GSTM1 in non-obstructive infertility in Indian men. *Mol. Hum. Reprod.*, 2007; 13: 213–222.
2. Pieri Pde C, Pereira DH, Glina S, Hallak J, MacElreavey K and Moreira-Filho MA. A cost-effective screening test for detecting AZF microdeletions on the human Y chromosome. Genet. Test, 2002; 6: 185–194.
3. Ferlin A, Arredi C and Foresta A. Genetic causes of male infertility. *Reprod. Toxicol*., 2006; 22: 133–141.
4. Lei H, Oh M, Okano R, Jutterman KA, Goss Jaenisch and Li E. *De novo* DNA cytosine methyltransferase activities in mouse embryonic stem cells. *Development*, 1996; 122: 3195–3205.
5. Stevenson RE, Schwartz CE, Du YZ and Adams MJJ. Differences in methylene-tetrahydrofolate reductase genotype frequencies between whites and blacks. Am. J. Hum. Genet., 1997; 60: 229–230.
6. Bailey LB and Gregory J. Polymorphism of methylenetetrahydrofolate reductase and other enzymes: metabolic significance, risks, and impact on folate requirements. *J. Nutr.*, 1999; 129: 919–922.
7. Friso S, Choi SW, Girelli D, Mason JB, Dolnikowski GG, Bagley OO, Jacques P, Rosenberg FH, Corrocher R and Selhub J. A common mutation in the 5,10-methylenetetrahydrofolate reductase gene affects genomic DNA methylation through an interaction with folate status. *Proc. Nat. Acad. Sci.*, 2002*;* 99: 5606–5611.
8. Stern LL, Mason JB, Selhub J and Choi SW. Genomic DNA hypomethylation, a characteristic of most cancers, is present in peripheral leukocytes of individuals who are homozygous for the C677T polymorphism in the methylentetrahydrofolate reductase gene. *Cancer Epiedmiol. Biomark. Prev*., 2000; 9: 849–853.
9. Kirke PN, Mills JL, Molloy AM, Brody LC, O'Leary VB and Daly L. Impact of the MTHFR C677T polymorphism on risk of neural tube defects: case-control study. *Brit. Med. J*., 2004; 328: 1535–1536.
10. Benn PA and Perle MA. Chromosome staining and banding techniques. In: Rooney DE, Czepulkowski BH, eds. Human Cytogenetics: A Practical Approach. Vol I. Constitutional Analysis. 1992; Oxford, United Kingdom: IRL Press Ltd 91–118.
11. Sambrook J, Fritsch EF and Maniatis T. Commonly used techniques in molecular cloning. In: molecular cloning: a laboratory manual, 2nded, Vol. 3. Cold Spring Harbor, 1989; NY: Cold Spring Harbor Laboratory Press Appendix E3.
12. Chan D, Cushnie DW, Neaga OR, Lawrance AK and Rozen R. Strainspecific defects in testicular development and sperm epigenetic patterns in 5,10-methylenetetrahydrofolate reductase-deficient mice. *Endocrinology*, 2010; 151: 3363–3373.
13. Paracchini V, Garte S and Taioli E. MTHFR C677T polymorphism, GSTM1 deletion and male infertility: a possible suggestion of a gene-gene interaction? *Biomarkers*, 2006; 11: 53–60.
14. Yang Y, Zhang SZ, Li N and Zhang W. Single nucleotide polymorphism C677T in the methylenetetrahydrofolate reductase gene might be a genetic risk factor for infertility for Chinese men with azoospermia or severe oligozoospermia. *Asian J. Androl.*, 2007; 9: 57–62.
15. Ebisch IM, van Heerde WL, Thomas CM, van der Put N and Wong WY. C677T methylenetetrahydrofolate reductase polymorphism interferes with the effects of folic acid and zinc sulfate on sperm concentration. *Fertil. Steril.*, 2003*;* 80: 1190–1194.
16. Lee HC, Jeong YM, Lee SH, Cha KY and Song SH. Association study of four polymorphisms in three folate-related enzyme genes with non-obstructive male infertility. *Hum. Reprod*., 2006 21: 3162–3170.
17. Wu W, Shen O, Qin Y, Lu J and Niu X. Methylenetetrahydrofolate reductase C677T polymorphism and the risk of male infertility: a meta-analysis. *Int. J. Androl.*, 2011; doi: 10.1111/j.1365-; 2605..01147.x. [Epub ahead of print].
18. Ebisch IM, Thomas CM, Peters WH, Braat DD and Steegers-Theunissen RP. The importance of folate, zinc and antioxidants in the pathogenesis and prevention of subfertility. *Hum. Reprod. Update*, 2007; 13: 163–174.
19. Paluku They-They T, Hamzi K, Mazabraud A and Nadifi S. Frequency of C677T polymorphism of methylene tetrahyrofolate reductase (MTHFR) gene among berber and arabic Moroccan populations. *Antropology*, 2009; 20: 11–17.
20. Kobayashi H, Sato A, Otsu E, Hiura H and Tomatsu C. Aberrant DNA methylation of imprinted loci in sperm from oligospermic patients. Hum. Mol. Genet., 2007; 16: 2542–2551.
21. Marques CJ, Costa P, Vaz B, Carvalho F and Fernandes S. Abnormal methylation of imprinted genes in human sperm is associated with oligozoospermia. *Mol. Hum. Reprod*., 2008; 14: 67–74.