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DAZL 260A>G and *MTHFR* 677C>T variants in sperm DNA of infertile Indian men

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DAZL (deleted in azoospermia-like) 260A>G and *MTHFR* (methylene tetrahydrofolate reductase*)* 677C>T are two important autosomal variants associated with impaired spermatogenesis. In this study, we investigated *DAZL* 260A>G and *MTHFR* 677C>T variants in sperm DNA and their frequency in oligozoospermic infertile men of Indian origin. The study on sperm DNA was performed, since it is more prone to oxidative stress-induced damage and mutation. One hundred oligozoopsermic infertile men having normal chromosomal complement with intact Y chromosome and 100 age- and ethnically-matched fertile controls were investigated for these variants in their sperm genome. Spermatozoa were separated by gradient centrifugation and DNA was isolated and analyzed for the single nucleotide polymorphisms (SNPs) by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). The results showed no significant differences in the frequency of *DAZL* AG (P = 0.58) and *MTHFR* CT (P = 0.44) between oligozoospermic infertile men and controls. However, 8% (8/100) oligozoospermic infertile men harbored both the variants and showed significantly (P<0.0001) lower sperm count (3.28 ± 1.1 vs 12.50 ± 4.09) compared to infertile men with either of the single variant. None of the fertile controls showed the presence of the both variants. In conclusion, the combined effect of both *DAZL* 260A>G and *MTHFR* 677C>T variants may have role in compromised sperm count. However, further studies are required to find the pathological role of these combined variants in male infertility.

Keywords: Male infertility, *DAZL* 260A>G, *MTHFR* 677C>T, Oligozoospermic, Single nucleotide polymorphism

Infertility affects approximately 15% of married couples[1] and in half of these cases, the pathology is traced to the male partner and the cause remains largely unknown[2]. Increasing incidence of genetic abnormalities in infertile men is a major concern[3-5]. *DAZL* (deleted in azoospermia-like) and *MTHFR* (methylene tetrahydrofolatereductase) genes show autosomal recessive inheritance pattern which plays an important role in spermatogenesis[6-8]. *DAZL* is located on chromosome 3p24 and shares 83% homology to the DAZ (deleted in azoospermia) gene and both genes encode RNA binding proteins[9]. DAZ loci on Y chromosome are believed to be derivative of the autosomal *DAZL* which arose during primate evolution[10,11]. *DAZL* protein is initially located in the nucleus of spermatogonia and then relocates to the cytoplasm during meiosis[12].

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Of all the genes, *DAZL* is believed to be the master regulator of germline gene expression, as its loss results in multiple defects, including a modest reduction in germ cell number before birth[13,14]. In addition, presence of DAZL protein in spermatids and spermatozoa has made *DAZL* an attractive candidate in the studies associated with male infertility. Methylene tetrahydrofolate- reductase is one of the key enzymes in folate metabolism encoded by *MTHFR* gene located on chromosome 1p36.3 that plays an essential role in nucleic *de novo* synthesis of thymidine[15]. Folate deficiency is known to occur frequently and the related hyperhomocysteinaemia is considered as a risk factor for various diseases, including male infertility. The most studied 677C>T (rs1801133) variant of *MTHFR* has been found to be associated with cardiovascular disease, neural defects, schizophrenia, thrombosis and also male infertility[16-19].

Studies conducted on *DAZL* and *MTHFR* variants in different populations of infertile men world-wide have revealed controversial results[6,16,20]. However, very few studies are available on these variants in

infertile men of Indian origin[20,21]. The previous studies have evaluated infertile men with azoospermia, oligozoospermia and normozoospermia in blood DNA, but both the variants have not been screened in sperm DNA which is found to be more prone to oxidative stress-induced damage and mutation than blood DNA. Sperm nuclear DNA is considered as hotspot for nucleotide variations since division of spermatogonial cell occurs at very high rate and thus there are more chances for mistakes, as the fidelity of repair enzymes may be compromised. Therefore, in the present study, we have investigated the frequency of both *DAZL* 260A>G and *MTHFR* 677C>T variants in infertile men with oligozoospermia to explore whether these variants have any influence on the phenotype.

Materials and Methods

Study population

One hundred oligozoospermic infertile men (sperm count <20 million/ml) seeking treatment and 100 age-matched fertile controls were included in the study. Fertile controls were defined as men who had fathered a child in the last 2 yrs and had sperm concentration of 107.45 ± 95.40 million/ml. All the subjects in the study were north Indian population belonging to group of Indo-Aryans. After approval from the Institute Review Board, the semen samples were collected from the patients with their informed consent. After thorough clinical examination and questionnaire evaluation, infertile oligozoospermic men with normal chromosomal complement (46; XY) and intact Y chromosome (absence of Yq microdeletion) with no abnormal andrological findings were included in the study.

Semen analysis

Semen samples were obtained by masturbation after four days of sexual abstinence. Samples were collected in a sterile plastic container and after liquefaction at 37°C, standard semen analysis was performed as per WHO guidelines[22] , whereas sperm morphology was evaluated by fixing the semen smear with 90% ethanol and stained with Giemsa. Sperm were classified based on head, midpiece and tail defects. Classification system, which designates sperm as normal (oval), amorphous, tapered, duplicated or immature, strict criteria to identify normal sperm was followed and sperm cell having "normal" morphology was included in the study. In both the cases (infertile and control), the sample having leukocyte concentration >1 million/ml were not included in the study.

Sperm DNA isolation

After semen analysis, the sample was layered in isolate (Irvine Scientific Co., Santa Ana, CA) sperm separation medium and centrifuged at 3000 rpm for 10 min to separate sperm cells from contaminants like immature germ cells, leukocytes, epithelial cells and debris. The pellet was then treated with osmotic solution to lyse cells other than spermatozoa left. After confirming the absence of other cells by examining the smear under microscope, sperm cells were washed twice with sperm washing media (IrvineScientific Co., Santa Ana, CA). The washed cells were subjected to DNA isolation by the following method. The sperm pellet was incubated at 55°C overnight with the sperm lysis medium (60 mM DTT, 4% SDS and 350 µg/ml proteinase K made in lysis buffer) and after complete digestion, sperm DNA was precipitated by adding equal amount of chilled isopropanol. The pellet was then washed with 70% ethanol, dried at 37°C and dissolved in tris-EDTA (TE) buffer.

PCR-RFLP (Restriction fragment length polymorphism) analysis

PCR-RFLP analysis was performed for genotyping *DAZL* 260A>G and *MTHFR* 677C>T variants using the following set of forward and reverse primers: *DAZL* (forward): 5' CCTGTGTATCTAATTATGATG 3'; *DAZL* (reverse): 5' CCTTAAGTTTGTAACAGGGCC 3'; *MTHFR* (forward): 5' TGAAGGAGAAGGTGTCTG CGGGA 3'; *MTHFR* (reverse): 5' AGGACGGTGCG GTGAGAGTG 3'.

PCR was carried out in a volume of 25 µl containing 30 ng sperm DNA, 1.5 mM $MgCl_2$, 200 mM each deoxynucleotide triphosphate, 2 mM of each primers, 0.5 U Taq DNA polymerase and 10X reaction buffer. The PCR conditions were as follows; initial denaturation at 94°C for 3 min followed by 35 cycles of 94°C for 30 s, annealing temperature (*DAZL*, 56°C for 1 min *MTHFR,* 68°C for 1 min) and 72°C for 1 min with a 5 min 72°C final extension. The amplified 264 bp *DAZL* and 198 bp *MTHFR* products were digested by *DdeI* (5′....C↓TNAG....3′) and *HinfI* (5′....G↓ANTC....3′) restriction enzymes, respectively (Promega). Digested products were electrophoresed on a 3% agarose gel (for *DAZL*) and native polyacrylamide gel electrophoresis (for

MTHFR) to identify the variants. Upon digestion, 197 and 67 bp were produced from amplified *DAZL* product while amplicons of *MTHFR* (198 bp) produced 175 and 23 bp product.

Statistical analysis

Genotype and allelic frequency was calculated by Fisher's exact test. The difference in the sperm count between the groups was calculated by student's 't' test. $P<0.0001$ was considered as significant. GraphPad software was used to calculate all the statistical analysis.

Results and Discussion

When the semen parameters were compared between cases and controls a significant difference in sperm count and motility was observed (Table 1). No significant difference was observed in the frequency of *DAZL* AG and *MTHFR* CT genotype between oligozoospermic infertile men and controls (Table 2). None of the samples showed homozygous variant of either *DAZL* 260A>G or MTHFR 677C>T in these studied population (Figs 1 and 2).

However, 8% (8/100) of oligozoopsermic infertile men harbored both the variants (P = 0.0068), (OR: 18.470, 95% CI: 1.051 to 324.73) which was not observed in the control population (Fig. 3). Oligozoospermic infertile men with the both the variants showed significantly lower (P<0.001) sperm count (3.2 ± 0.8 million/ml) compared to infertile men with either of the variants (11.4 ± 6.1 million/ml). Similarly, the sperm with abnormal morphology in infertile men having both variants was found to be significantly higher than having either of the variants (Fig. 4).

DAZL 260A>G and *MTHFR* 677C>T are two important autosomal variants associated with male infertility[16,23,24]. Screening of these variants has not been reported previously in sperm DNA. Sperm are produced continuously throughout reproductive life, so the number of cell divisions and chromosome replications that have occurred increases with age. The germline mutations rate in human males is generally much higher than in females, mainly because there are many more germ-cell divisions. The sperm nuclear DNA is considered as hotspot for nucleotide variations. There are more chances for

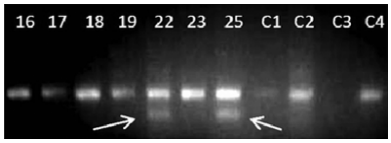

Fig. 1—Gel picture showing RFLP pattern of DAZL 260A>G by DdeI enzyme on 3% agarose [The arrow shows heterozygous pattern of case 22 and 25]

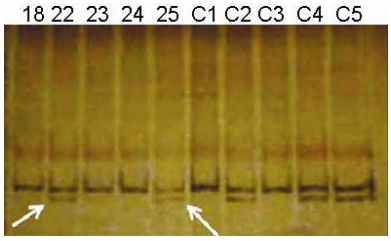

Fig. 2—Gel picture showing RFLP pattern of MTHFR 677C>T variants by HinfI enzyme on 12% PAGE [The arrow show heterozygous pattern of case 22 and 25]

Table 1—Semen parameters and age of oligozoospermic infertile men and controls

Category	Volume (ml)	Sperm count ($\times 10^6$/ml)	Forward sperm motility (%)	Abnormal spermmorphology (%)	Age (Yrs)
Infertile men	3.21 ± 1.02	11.59 ± 4.75*	26.10 ± 21.21*	30.86 ± 26.12	32.85 ± 3.15
Controls	3.54 ± 1.45	107.45 ± 95.40	66.07 ± 12.92	25.57 ± 9.65	33.15 ± 4.16

Significance was calculated by Welch-test. *P<0.0001 was considered as significant

Table 2—Genotype and allelic frequency of MTHFR C677T and DAZL A260G variants in infertile and control men

Variants ↓	Allelic frequency (%)		P value	Genotypic frequency (%)		P value
	Infertile (n = 100)	Control (n = 100)		Infertile (n = 100)	Control (n = 100)	
MTHFR 677C>T	C: 0.93 T: 0.07	C: 0.905 T: 0.095	0.4673	CC-0.86 CT-0.14 TT-0.00	CC-0.81 CT-0.19 TT-0.00	0.4461
DAZL 260A>G	A:0.895 G:0.105	A:0.915 G:0.085	0.6095	AA-0.79 AG-0.21 GG-0.00	AA-0.83 AG-0.17 GG-0.00	0.5891

Significance of allelic and genotypic frequency between infertile and control men was calculated by Fisher's exact test

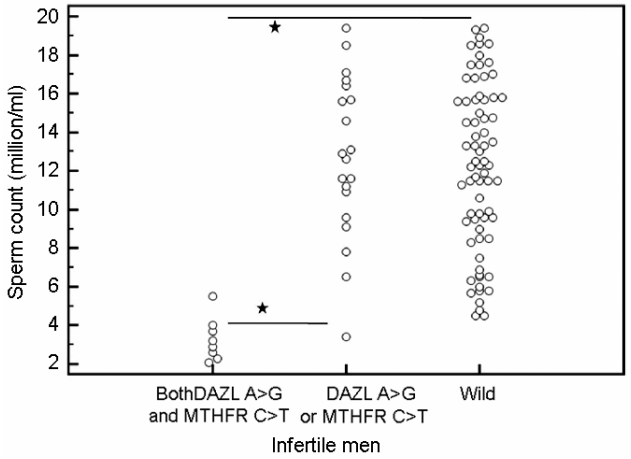

Fig. 3—Dot plot shows comparison of sperm count of infertile men with and without MTHFR 677C>T and DAZL 260A>G variants [*P<0.001 by Kruskal-Wallis test, followed by Post-hoc analysis]

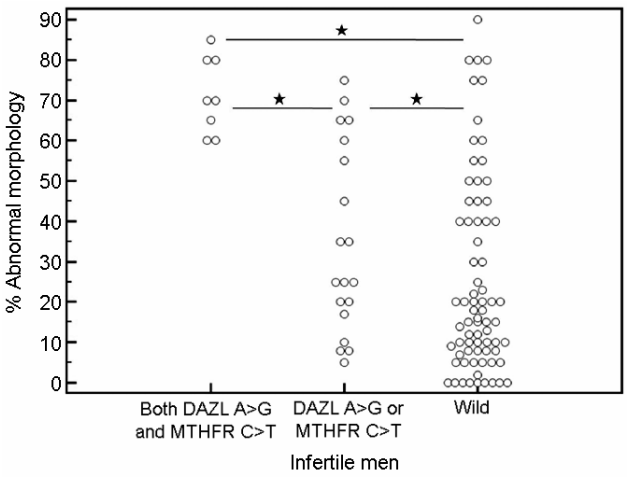

Fig. 4—Dot plot shows comparison of percent abnormal sperm morphology of infertile men with and without MTHFR 677C>T and DAZL 260A>G variants [* P<0.05 by Kruskal-Wallis test followed by Post-hoc analysis]

mistakes as the fidelity of repair enzymes may be compromised. There is evidence showing that DAZL protein may function at multiple stages during spermatogenesis and the activity of *MTHFR* is much higher in testis than in other major organs, suggesting their important role in spermatogenesis[7,25]. However, very few studies are available on the *DAZL* 260A>G and *MTHFR* 677C>T variants on male infertility[20,21].

DAZL 260A>G has been reported in infertile men belonging to various ethnic and geographical regions. The homozygous 260G results in severe spermatogenic failure as primordial germ cells or spermatogonia carrying homozygous 260G are eliminated by selective mechanism[26]. Previous study in Indian population has reported two polymorphic sites (260A>G/437A>G) in the *DAZL* gene in people of different ethnic/linguistic origins and found that they are not associated with male infertility[21]. Similarly, in Taiwanese population 260A>G (p.Thr12Ala) is not associated with male infertility[8]. Our study also demonstrated no significant association of *DAZL* 260A>G variant in oligozospermic infertile men.

Another variant *MTHFR* 677C>T has been shown to be associated with male infertility in Indian population[20] , however, another study on the same population has shown no association between *MTHFR* 677C>T and male infertility[27,28]. *MTHFR* gene knock-out model is reported to result in abnormal spermatogenesis and infertility in mice, providing its strong association in male fertility[29]. This common *MTHFR* variant 677C>T changing alanine to valine (p.Ala222Val) results in thermolabile enzyme with reduced activity[30]. Its polymorphic distribution varies greatly in different populations and is reported to be associated with variety of diseases, where its activity is essential[31].

A previous study[20] from India has revealed a significant association of homozygous 677C>T (4%, 6/151) variant in infertile population, which was not observed in our study. This might be due to inclusion of only oligozoopsermic infertile men in our study. However, the role of folic acid diet consumed by the subject is not known in both the studies, as folic acid diet can compensate for the altered pathogenesis to a certain degree. The susceptibility to male infertility related to SNP 677C>T is not yet clear, as the phenotypic effect of the mutation is modulated by exogenous factors such as folate supplementation, providing an important example of the gene-environment interaction in phenotype development.

In human, *MTHFR* 677 allele T can reduce *MTHFR* activity and subjects with homozygous TT are associated with decreased global genomic methylation as compared with CC genotype[32]. Secondly, 677C>T mutation in *MTHFR* gene might result in hyperhomocysteinemia in low folate status[33] and a high level of homocysteine can induce auto-oxidation that might cause DNA damage and thus may lead to arrest of spermatogenesis. Interestingly, 8% of the cases in the studied population contained both the *DAZL* AG and *MTHFR* CT heterozygous variants, showing enhanced

risk for male fertility. These men had significantly lower sperm counts compared to the population with either 260A>G or 677C>T variants.

In conclusion, the study demonstrated the additive effect of *DAZL* and *MTHFR* variants in infertile male Indian population. However, a large cohort and functional study is required to further evaluate the additive effect and association of these variants on male infertility.

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