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 oligozoospermic 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 couples¹ and in half of these cases, the pathology is traced to the male partner and the cause remains largely unknown². Increasing incidence of genetic abnormalities in infertile men is a major concern³.⁵. DAZL (deleted in azoospermia-like) and MTHFR (methylene tetrahydrofolate reductase) genes show autosomal recessive inheritance pattern which plays an important role in spermatogenesis⁶.⁸. DAZL is located on chromosome 3p24 and shares 83% homology to the DAZ (deleted in azoospermia) gene and both genes encode RNA binding proteins⁹. DAZ loci on Y chromosome are believed to be derivative of the autosomal DAZL which arose during primate evolution¹⁰.¹¹. DAZL protein is initially located in the nucleus of spermatogonia and then relocates to the cytoplasm during meiosis¹².

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 birth¹³.¹⁴. In addition, presence of DAZL protein in spermatids and spermatozoa has made DAZL an attractive candidate in the studies associated with male infertility. Methylene tetrahydrofolic-acid 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 thymidine¹⁵. 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 infertility¹⁶.¹⁷. Studies conducted on DAZL and MTHFR variants in different populations of infertile men world-wide have revealed controversial results⁶,¹⁶,²⁰. However, very few studies are available on these variants in
infertile men of Indian origin\textsuperscript{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 \textit{DAZL} 260A>G and \textit{MTHFR} 677C>T variants in infertile men with oligozoospermia to explore whether these variants have any influence on the phenotype.

\section*{Materials and Methods}

\subsection*{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.

\subsection*{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\textsuperscript{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.

\subsection*{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.

\subsection*{PCR-RFLP (Restriction fragment length polymorphism) analysis}

PCR-RFLP analysis was performed for genotyping \textit{DAZL} 260A>G and \textit{MTHFR} 677C>T variants using the following set of forward and reverse primers: \textit{DAZL} (forward): 5’ CCTGTGTATCTAATTATGATG 3’; \textit{DAZL} (reverse): 5’ CCTTAAGTTTGTAACAGGGCC 3’; \textit{MTHFR} (forward): 5’ TGAAGGAGAAGGTGTCTG 3’; \textit{MTHFR} (reverse): 5’ AGGACCGGTGC CGTGAGAGTG 3’.

PCR was carried out in a volume of 25 µl containing 30 ng sperm DNA, 1.5 mM MgCl\textsubscript{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 (\textit{DAZL}, 56°C for 1 min \textit{MTHFR}, 68°C for 1 min) and 72°C for 1 min with a 5 min 72°C final extension. The amplified 264 bp \textit{DAZL} and 198 bp \textit{MTHFR} products were digested by \textit{Ddel} (5’....C\textsubscript{8}GTG....3’) and \textit{HinfI} (5’....G\textsubscript{5}ANTC....3’) restriction enzymes, respectively (Promega). Digested products were electrophoresed on a 3\% agarose gel (for \textit{DAZL}) and native polyacrylamide gel electrophoresis (for
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 oligozoospermic 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 infertility16,23,24. 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

<table>
<thead>
<tr>
<th>Category</th>
<th>Volume (ml)</th>
<th>Sperm count (× 10⁹/ml)</th>
<th>Forward sperm motility (%)</th>
<th>Abnormal spermmorphology (%)</th>
<th>Age (Yrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infertile men</td>
<td>3.21 ± 1.02</td>
<td>11.59 ± 4.75*</td>
<td>26.10 ± 21.21*</td>
<td>30.86 ± 26.12</td>
<td>32.85 ± 3.15</td>
</tr>
<tr>
<td>Controls</td>
<td>3.54 ± 1.45</td>
<td>107.45 ± 95.40</td>
<td>66.07 ± 12.92</td>
<td>25.57 ± 9.65</td>
<td>33.15 ± 4.16</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Variants</th>
<th>Allelic frequency (%)</th>
<th>P value</th>
<th>Genotypic frequency (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTHFR 677C&gt;T</td>
<td>Infertile (n = 100)</td>
<td>Control (n = 100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C: 0.93</td>
<td>C: 0.905</td>
<td>0.4673</td>
<td>CC-0.86</td>
<td>CC-0.81</td>
</tr>
<tr>
<td>T: 0.07</td>
<td>T: 0.095</td>
<td></td>
<td>CT-0.14</td>
<td>CT-0.19</td>
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<td></td>
<td></td>
<td></td>
<td>TT-0.00</td>
<td>TT-0.00</td>
</tr>
<tr>
<td>DAZL 260A&gt;G</td>
<td>Infertile (n = 100)</td>
<td>Control (n = 100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A: 0.895</td>
<td>A: 0.915</td>
<td>0.6095</td>
<td>AA-0.79</td>
<td>AA-0.83</td>
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<tr>
<td>G: 0.105</td>
<td>G: 0.085</td>
<td></td>
<td>AG-0.21</td>
<td>AG-0.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GG-0.00</td>
<td>GG-0.00</td>
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Significance of allelic and genotypic frequency between infertile and control men was calculated by Fisher’s exact test
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. However, very few studies are available on the DAZL 260A>G and MTHFR 677C>T variants on male infertility. 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. 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. 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. 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. Similarly, in Taiwanese population 260A>G (p.Thr12Ala) is not associated with male infertility. Our study also demonstrated no significant association of DAZL 260A>G variant in oligozoospermic infertile men.

Another variant MTHFR 677C>T has been shown to be associated with male infertility in Indian population, however, another study on the same population has shown no association between MTHFR 677C>T and male infertility. MTHFR gene knock-out model is reported to result in abnormal spermatogenesis and infertility in mice, providing its strong association in male fertility. This common MTHFR variant 677C>T changing alanine to valine (p.Ala222Val) results in thermolabile enzyme with reduced activity. Its polymorphic distribution varies greatly in different populations and is reported to be associated with variety of diseases, where its activity is essential. A previous study 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 oligozoospermic 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. Secondly, 677C>T mutation in MTHFR gene might result in hyperhomocysteinemia in low folate status 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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References