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RESEARCH

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Analysis of genetic variants in the exon 2 and 3 of autosomal DAZLA gene among infertile South Indian men

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Abstract

Background Boule, DAZLA, and DAZ are members of the Deleted in Azoospermia family of genes, which play significant roles in gametogenesis and are important fertility factors in humans. In a few studies, deletion of the Y chromosomal DAZ cluster and single nucleotide polymorphisms in the DAZLA gene were reported to affect male fertility, although this is paradoxical because they were found in both control and fertile men. As a result, the goal of this study was to check if Y chromosomal DAZ cluster deletion and SNPs in the DAZLA gene on chromosome 3 of humans are associated to male infertility in the population. For computational analysis, different bioinformatics tools such as SIFT, PolyPhen2, Mutation Taster, FATHMM, and PROVEAN were used to analyse mutations.

Results Within the studied population, we found no association between DAZ deletion and the most prevalent DAZLA SNPs A260G (rs11710967) and A386G (rs1219183446). We also discovered two new deleterious genetic variations in exon 3 of the DAZLA gene, one nonsynonymous mutation that replaced Valine with Glutamate at the 66 codon position and the other a stop gain mutation at the 74 amino acid position. These genetic changes are found in the RRM domain of the DAZLA gene, which is confirmed by Motif scan analysis and results in a change in the DAZLA protein's secondary structure. The RRM domain is a highly conserved regulatory domain for mRNA transport and translation. Azoospermia and necrospermia infertility phenotypes were shown in infertile male samples with these genotypes.

Conclusion We can conclude that further investigation of the aforesaid new mutations in the DAZLA gene may be valuable in understanding their significance in male infertility in different populations due to the multifactorial nature of male infertility and arrays of gene expression required at every stage of spermatogenesis.

Keywords Male infertility, Single nucleotide polymorphism, DAZLA gene, DAZ deletion

Background

During spermatogenesis, male germ cells undergo mitotic and meiotic divisions in order to produce haploid spermatozoa, which necessitates strictly controlled gene expression [20]. The DAZ (deleted in azoospermia) family of genes, which includes Boule, DAZLA (Deleted in azoospermia-like autosome), and DAZ, are important fertility factors in humans [6]. DAZLA evolved in vertebrates, giving rise to DAZ in the Y chromosome during primate evolution, while Boule is regarded the most ancestral [22]. The human DAZLA gene is located on the short arm of chromosome 3 [13]. The DAZLA gene has only 11 exons and consists of two functional domains, that is, an RNA recognition motif (RRM) that helps in binding target RNA sequences and a DAZ repeat responsible for protein–protein interactions [14] and [3]. Male

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and female DAZLA knockout mice were shown to have complete meiosis arrest in germ cells during development in animal model studies [12]. In male DAZLA knockout mice, human DAZ could partially rescue the mouse DAZLA null phenotype [16]. Single nucleotide polymorphisms (SNPs) at 260 and 386 locations in exon 2 and exon 3 were found to be more common in mutation analysis of all 11 exons of the human DAZLA gene. Within the RNA Recognition motif, these SNPs cause threonine to be replaced by alanine at codons 12 (T12A) and 54 (T54A), respectively. In the Taiwanese population, SNPs at nucleotide locations 386 in the DAZL gene induce spermatogenic abnormalities such as hypospermatogenesis, maturation arrest, and Sertoli cell-only syndrome [18]. Mutations in the DAZLA gene were thought to be one of the causes of male infertility, as autosomal recessive mutations account for 60% of unexplained male infertility [7] and [15].

The DAZ gene cluster is located on the Y chromosome's AZFc region and is a strong male candidate fertility gene [14]. DAZ originated from autosomal DAZLA during human evolution by transposition, amplification, and pruning, according to an evolutionary relatedness analysis [13]. DAZ has a single RNA binding domain and seven DAZ repetitions that code for a protein that is specific to the testis [15]. DAZ is made up of four genes arranged in two clusters as inverted pairs, as well as nine types of DAZ repeats with nucleotide sequence similarity of 90–96% [14] and [8]. A deletion in all four copies of DAZ is found in 5–13% of men with azoospermia and severe oligospermia, and the deletion is de novo [9] and [5]. The DAZ gene's long repeats made it vulnerable to numerous deletions [8]. Therefore, the study was aimed to analyze the association of infertility phenotype in men with SNPs A260G (rs11710967) and A386G (rs121918346) DAZL gene and Y chromosomal DAZ deletion among infertile male population of South India. Further study was done with the use of available bioinformatics tools to understand if any polymorphism is a genetic risk factor for infertility in this ethnic group.

Materials and methods

Ethical approval

The Institutional Human Ethical Committee of the University of Mysore, Mysore, India (IHEC-UOM No.143/Ph.D/2016-17) gave its approval to the study. The participants gave their informed consent and a genetic record was established with their medical, reproductive, and surgical histories.

Study subjects

Infertile men with metabolic abnormalities, infectious infections, cryptorchism, tubule defects, erectile dysfunction, and other conditions were excluded from the study. Control subjects were men with one or more children who had not undergone ART (artificial reproductive technology). Between January 2017 and February 2018, 100 controls and 200 infertile men with normal karyotypes from childless couples who had attended Fertility hospitals and clinics for male infertility difficulties were chosen. After at least 3 days of sexual abstinence, blood and sperm samples were taken. Semen analysis was carried out in accordance with the 5th edition of the WHO laboratory manual for the examination and processing of human sperm (2010).

Molecular analysis

The DNA was extracted from peripheral blood lymphocytes using GeNei Uniflex TM DNA isolation kit and quantified by nano-spectrophotometer and agarose gel electrophoresis. It was further diluted to make an optimum concentration of 25–100 ng for polymerase chain reaction (PCR).

Analysis of deletion of DAZ cluster in AZFc region of Y chromosome

To rule out deletion of Y chromosomal DAZ cluster, two STS (sequence tagged sites) markers, sY254, and sY255 (Table 1) recommended by the EAA (European Academy of Andrology) were used to run the PCR. The reaction was set up at Initial denaturation at 95 ℃ for 5 min and then 32 cycles of three steps denaturation at 95 ℃ for 1 min; annealing at 63 ℃ for 30 s and elongation at 72 ℃ for 30 s. Finally, the reaction was held at 72 ℃ for 7 min and then 4 ℃ for 8 min. The PCR product was run on 1.5% agarose gel and documented under UV illuminator.

Table 1 PCR primers for DAZ and DAZLA genes

Gene	Primer	Primer sequence (5′ to 3′)	Fragment length (bp)
DAZ	sY254	F: GGGTGTTACCAGAAGGCAAA R: GAACCGTATCTACCAAAGCAGC	380bp
	sY255	F: GTTACAGGATTCGGCGTGAT R: CTCGTCATGTGCAGCCAC	123bp
DAZLA	A260G	F: CCT GTG TAT CTA ATT ATG ATG R: CCT TAA GTT TGT AAC AGG GCC	264bp
	A386G	F: GAATGCTGAATTTTTACTCTTGAAG R: CTCTATACGTGGCTAGAGTTC	181 bp
	Exon 3	F: TGGGGGAGAAATTGTCACAT R: CCCTTTGGACACACCAGTTC3	150bp

Analysis of A260G polymorphism of DAZLA gene by RFLP (restriction fragment length polymorphism)

Polymerase Chain Reaction was set up using a set of primers (Table 1) at the following conditions: initial denaturation at 95 ℃ for 2 min, 30 cycles of denaturation at 95 ℃ for 30 s, annealing at 59 ℃ for 30 s, extension at 72 ℃ for 30 s, and final extension at 72 ℃ for 7 min. Further, it was kept for a hold at 4 ℃ for 8 min The PCR products were run on 2% agarose gel. For genotyping, the PCR product was incubated at 37 ℃ overnight with 5 units of *DdeI* restriction enzyme. The digested fragments were separated on 2.5% agarose gel and visualized under UV illuminator.

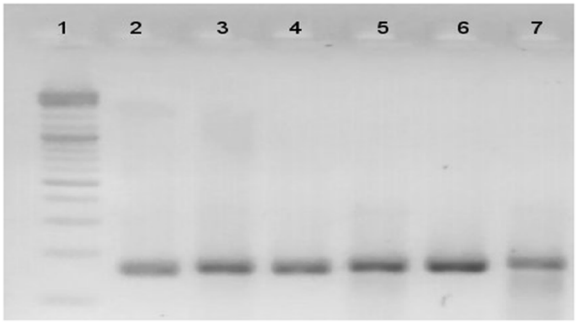

Fig. 1 Gel profile of DAZLA exon 3 gene specific fragment (150 bp). Lane 1: 100 bp DNA ladder; lane 2: control and lane 3–7: infertile samples

Analysis of A386G polymorphism of DAZLA gene by RFLP

Primers for A386G listed in Table 1 were subjected to PCR under the same conditions as mentioned above in "Analysis of A260G polymorphism of DAZLA gene by RFLP (restriction fragment length polymorphism)" section. Further, incubated the PCR product at 37 ℃ overnight with 10 units of *AluI* restriction enzyme and separated on 2.5% agarose gel.

Mutation analysis of exon 3 of DAZLA gene

With the extracted DNA from peripheral blood, PCR was performed using primers (Table 1) for 2 control and 23 infertile samples with cycling conditions of 94 ℃ for 3 min followed by 30 cycles of 94 ℃ for 1 min, 50 ℃ for 1 min,72 ℃ for 2 min and 72 ℃ for 7 min. The product was run on 1.5% agarose gel and documentation was done under UV illuminator. For Sanger Sequencing, the Sequencing mix contains Big Dye Terminator Ready Reaction Mix: 4 µl, Template (100 ng/ul): 1µl, Primer (10pmol/λ): 2 µl and Milli Q Water: 3 µl that made up a total volume of 10 µl. It is then run for PCR in conditions as follows 96 ℃ for 5 min followed by 25 cycles of 96 ℃ for 30 s, 50 ℃ for 30 s, and 60 ℃ for 1.3 min. Gene-specific fragment of 150 bp length (as shown in Fig. 1) were amplified by using high fidelity PCR polymerase, which was then run on 1.5% agarose gel. The PCR product was sequenced by Sanger Sequencing Technique bidirectionally that is forward and reverse in the ABI 3130 Genetic Analyzer using Big Dye Terminator version 3.1. Using Seaview and the MEGA graphical multiple sequence alignment editor, the nucleotide sequence data was aligned with the NCBI reference sequence (www.ncbi.nlm.nih.gov). We manually trimmed the sequence before aligning and annotating the protein sequences. Five different bioinformatics tools like SIFT, Poly-Phen2, Mutation Taster, FATHMM, and PROVEAN were used to predict the consequences of the identified variants in protein function. Furthermore, the protein's secondary structure was plotted and compared, followed by motif domain analysis using the Bioinformatics tools Garnier-EMBOSS and Motif Scan-My Hits, and 3D/tertiary structure prediction using the homology modelling tool SWISS-MODEL.

Results

The average age of the subjects was 35 years (25–50 years range) belonging to various religions and castes. The semen samples of the infertile subjects were examined for its macroscopic and microscopic properties according to WHO (2010) guidelines. Then, infertile subjects were grouped into idiopathic (normal semen profile), non-obstructive azoospermia (no sperm in semen), oligospermia (sperm count < 15 million/ml), asthenospermia (progressively motile sperm + non-progressively motile sperm < 40%), oligoasthenospermia (oligospermia + asthenospermia), and necrospermia (all dead sperms in semen). Out of 200 infertile male subjects, 129 were idiopathic, 35 azoospermic, 6 oligospermic, 17 asthenospermic, 10 oligoasthenospermic, and 3 necrospermic.

DAZ deletion

For DAZ deletion analysis, DNA samples from fertile males with one or more children were used as positive controls and female DNA samples as negative controls. The absence of bands in the PCR amplified products of both STSs markers in the sample with DAZ deletion (shown in Fig. 2) was confirmed after three rounds of PCR. Among the 300 patients investigated in the study, one idiopathic case showed a deletion in the Y chromosomal DAZ cluster. The pedigree study verified that there was no family history of infertility or consanguinity in this case.

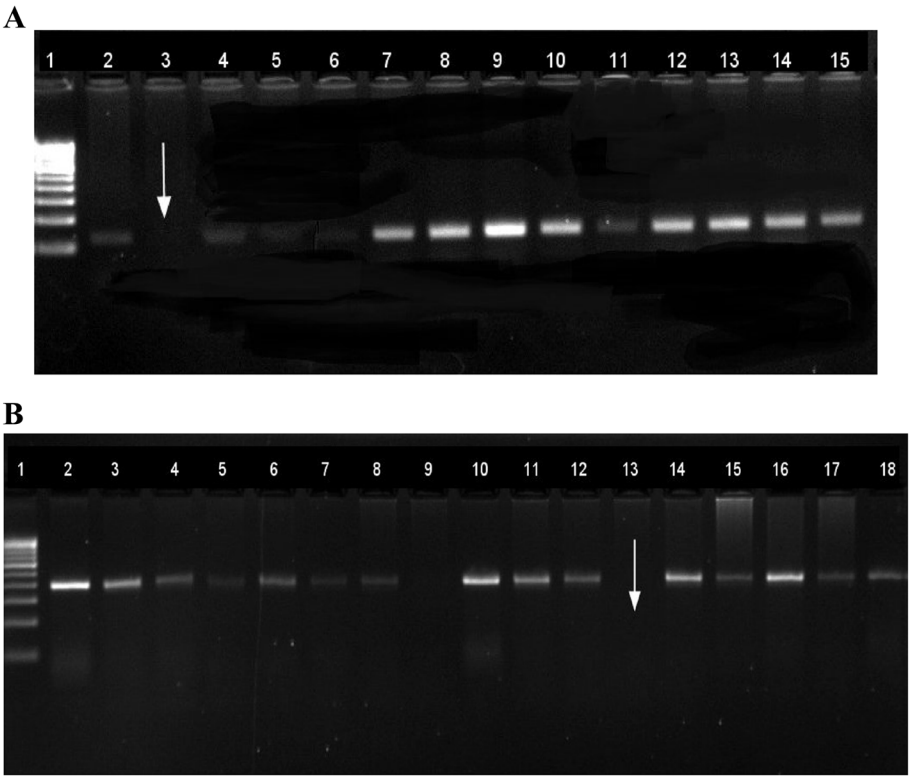

Fig. 2 **A** PCR amplicons for sY255(123bp) Lane 1: 100 bp ladder; 2–15: infertile samples. **B** PCR product sY254 (380 bp) Lane 1: 100 bp ladder; 2:+ve control; 9:-ve control; 3–8 and 10–18: infertile samples

DAZLA polymorphism

The restriction enzyme DdeI does not cut the allele 260 A, which results in a single 264 bp band, however the 260 G allele results in two bands of 67 bp and 197 bp fragments after restriction digestion. The heterozygous A260G allele results in three distinct bands: 264 bp, 197 bp, and 67 bp. We observed that all of the samples were homozygous for the A260A allele type (shown in Fig. 3). In the case of the A386G polymorphism, Alu1 cleaved the 181 bp PCR product into two 115 bp and 66 bp products for the AA homozygous allele and three 115 bp, 53 bp, and 13 bp fragments for the AG alleles. Figure 4 shows the gel profile of the A386G specific fragment and the restriction digestion product.

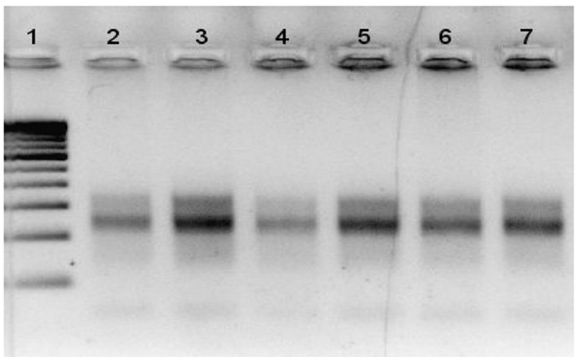

Fig. 3 264 bp PCR product restriction digestion by Alu1 AA homozygous (264 bp). Lane 1: 100 bp ladder; 2-positive control; 3–7: infertile samples

Mutation analysis

Since a polymorphism in exon 3 of the DAZL gene was linked to infertility in a previous study, mutation analysis in exon 3 was carried out with the help of a bioinformatics tool. The variations in the nucleotide and protein sequences shown in Table 2 were discovered by mutation analysis. Four non-synonymous single nucleotide variations (nsSNVs), one non-sense mutation, and two synonymous SNVs were identified. To predict the impact of these variations on biological function, we used five different web server tools: SIFT (Sorting Intolerant From Tolerant), Poly-Phen2 (polymorphism phenotyping v2), Mutation Taster, FATHMM (Functional analysis through hidden Markov models v2.3), and PROVEAN (Protein Variation Effect Analyzer).

As indicated in Table 3, the pathogenicity score was predicted for all of the variants found in the sequence

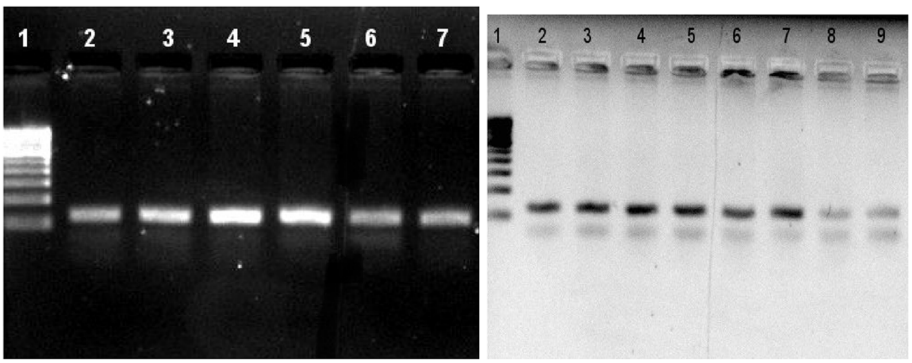

Fig. 4 **A** Gene-specific fragment of A386G (181 bp) Lane 1:100 bp; Lane 2: control; Lane 3–7 infertile samples. **B** 181 bp RFLP product digested by Alu1 115 bp and 66 bp (AA homozygous) Lane 1:100 bp; Lane 2:control; Lane 3–9: infertile samples

Table 2 Results of the mutation analysis of exon 3 of DAZLA gene in chromosome 3

Sample ID	Physical location	Nucleotide variation	Residue change	Amino acid change	Type of variation	Phenotype
94	Chr3:16639671	c.165G>C	GAG to GAC	p.E55D	Nonsynonymous SNV	Oligoasthenospermia
119	Chr3:16639671	c.165G>C	GAG to GAC	p.E55D	Nonsynonymous SNV	Azoospermia
109	Chr3:16639651	c.185G>C	AGA to ACA	p.R62T	Nonsynonymous SNV	Oligoasthenospermia
73	Chr3:16639643 Chr3:16639639	c.193T>A c.197T>A	TCA to ACA GTG to GAG	p.S65T p.V66E	Nonsynonymous SNV Nonsynonymous SNV	Azoospermia
128	Chr3: 16639618 _16639616	c.218_220insG	GAT to TGA	p.D74X	Nonsense mutation	Necrospermia
95	Chr3:16639605	c.231T>A	GGT to GGA	p.G77G	Synonymous SNV	Idiopathic
103	Chr3:16639605	c.231T>G	GGT to GGG	p.G77G	Synonymous SNV	Idiopathic

Table 3 Pathogenecity scores of the identified variations

Amino acid variation	SIFT	PolyPhen-2	Mutation Taster	FATHMM	PROVEAN
E55D	Tolerated	Possibly damaging	Benign	Tolerated	Nuetral
R62T	Tolerated	Possibly damaging	Benign	Tolerated	Deletorious
S65T	Tolerated	Benign	Benign	Tolerated	Nuetral
V66E	Tolerated	Probably damaging	Disease causing	Tolerated	Deletorious
D74X	–	–	Disease causing	–	Deletorious

data. The variant was considered detrimental if it was predicted to be a deleterious or disease-causing variation by at least two or three bioinformatics algorithms. Out of the seven mutations found, we found two deleterious mutations: a non-synonymous SNV (V66E) that replaces the amino acid valine (V) with glutamic acid (E) at the 66 codon position, and a non-sense mutation (D74X) caused by an insertion of G between c.218 and 220 positions of the nucleotide sequence, resulting in a stop gain mutation (Fig. 5).

In the V66E protein, turns were replaced by coils in a 2D structural prediction. In addition, loss of sheets, turns, and coils was observed in the mutated V66E variation, as well as a truncated protein in the case of the D74X mutation. The normal amino acid sequence of exon 3 was found within the RNA recognition motif (RRM) domain of the DAZL gene through using Motif scan software My Hits. As a result, these two detrimental mutations in the RRM domain, nsSNP V66E, and nonsense mutation D74X, may affect protein stability

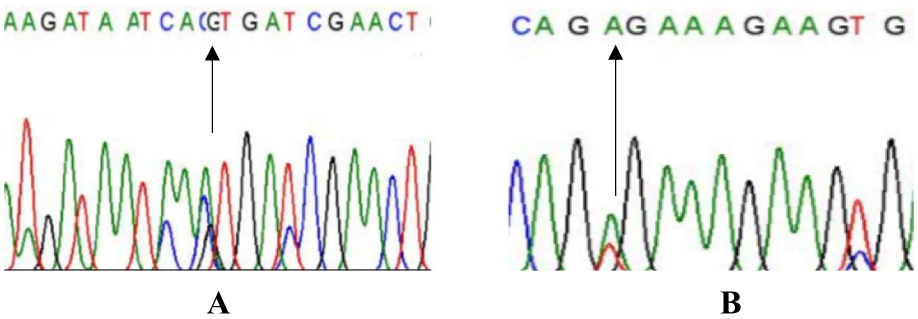

Fig. 5 **A** Sequence image of c.218_220insG (D74X). **B** Sequence image OF c.197T>A (V66E). **C** Normal. **D** Mutated (p.V66E)

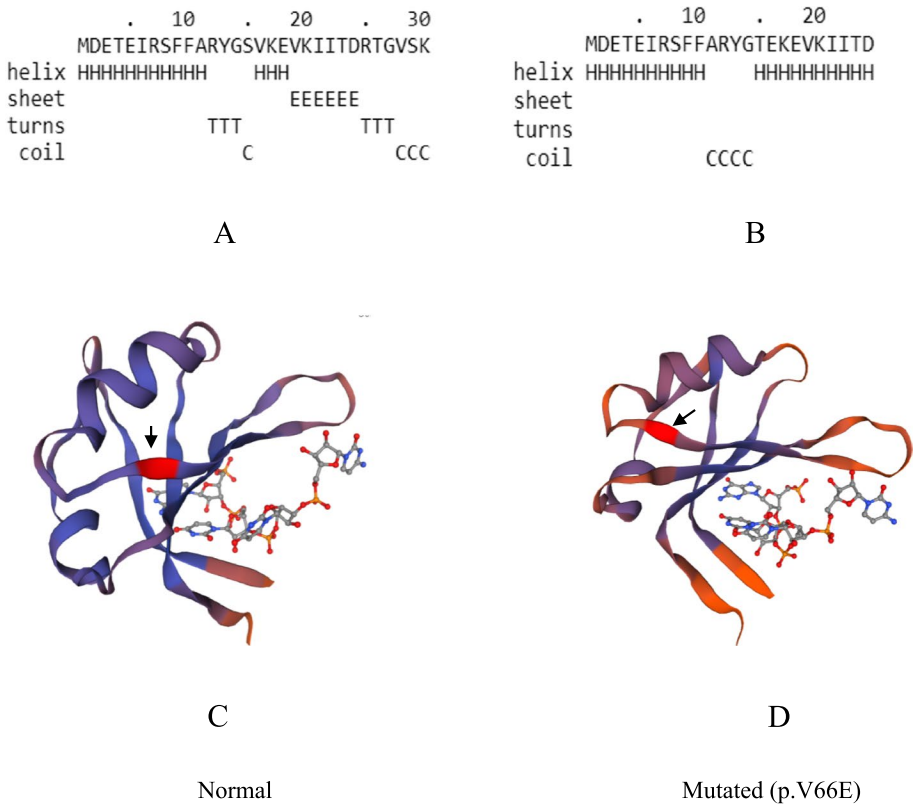

Fig. 6 **A**, **B** Predicted 2D structure of normal and mutated p.V66E. **C**, **D** Predicted 3D structure of normal and mutated p.V66E

and function. The secondary structure and tertiary structure of the normal and mutant V66E proteins are shown in Fig. 6.

Discussion

DAZLA is an autosomal homologue of the DAZ gene, and it may be one of the genes crucial in the pathogenesis of male infertility [17, 24]. The autosomal DAZL/DAZLA gene was transferred to the Y chromosome after the Old World primate lineage split from the New World monkey lineage about 30–40 million years ago. As a result of this translocation, the DAZ gene cluster on the Y chromosome was formed [14]. The DAZLA and DAZ sequences in human males exhibit a high degree of similarity and are over 90% identical, and the fact that sperm are still produced even when the DAZ genes are completely deleted suggests that DAZ and DAZL may have comparable functions [6]. In the 5′ and 3′ UTRs, the nucleotide sequence similarity of human DAZLA and DAZ transcripts was 75 and 87%, respectively [11]. DAZLA expression is restricted to the testis and so plays a role in spermatogenesis [21]. During

spermatogenesis, it regulates the production, transport, and localization of target mRNAs and proteins that are capable of generating mature male germ cells [6]. Furthermore, it has been shown that the DAZ gene cluster is deleted in 5–13% of men with azoospermia or severe oligozoospermia. So far, no point mutations, specific DAZ arrangements, or intragenic deletions have been documented,however, significant DAZ deletions have been linked to spermatogenic failure [15] and [9]. The deletion of DAZ genes is thought to have had no effect on sperm maturation in humans, but did reduce the number of sperm [4]. A study from Taiwan identified two novel DAZLA gene polymorphisms, A260G or T12A (rs11710967) and A386G or T54A (rs121918346), and SNP A386G was associated with spermatogenic failure [18]. The results of studies investigating into the association between these DAZLA polymorphisms and male infertility in various populations are mixed. Therefore, we looked for a full deletion of the DAZ cluster of genes on the Y chromosome, as well as two important single nucleotide polymorphisms (SNPs) in the DAZLA gene on human chromosome 3. Furthermore, a mutation study of DAZLA exon 3 was carried out in order to identify and annotate any genetic variants. We didn't find any of the aforementioned polymorphisms in the DAZLA gene in either the control group or the case group. Additionally, only one instance of infertility had completely deleted DAZ genes, but interestingly, the patient's sperm parameters complied with WHO requirements at normal reference levels. Furthermore, DAZ genes cluster deletion has been linked to male infertility in Caucasian and Asian ethnic groups, according to a recently meta-analysis study [10]. However, our findings conflict with a previous study that found that around 5–13% of azoospermic and oligospermic males had all four copies of DAZ deleted, compared to a lower incidence in our study. This might be the result of a lesser sample size compared to the meta-analysis study's higher sample sizes of 2820 cases and 1589 controls. A systematic review and meta-analysis study reported that despite the A386G polymorphism contributing to numerous spermatogenic abnormalities in oligospermic and non-obstructive azoospermic Taiwanese males, the T54A or A386G polymorphism in exon 3 had no link with male oligozoospermia or azoospermia in Caucasian and other Asian ethnicities. Although the A260G or T12A polymorphism was found in lower frequency in both control and infertile men, it was found in lower frequencies in both groups [5]. Our study supports the findings in different populations with different ethnic backgrounds lacking the prevalence of A386G polymorphism among German, Italian, Chinese, Northern, Central, and Eastern Indian, Tamil, and Japanese infertile men [1, 2, 11, 19, 23, 25].

Further, SNP databases and ClinVar databases (https://www.ncbi.nlm.nih.gov/clinvar/) were searched for the seven genetic variants discovered by mutation analysis. Only variations in physical location, chr3:16639651 (rs1367937384) and chr3:16639617 (rs1432731991), were detected in the dbSNP database (https://www.ncbi.nlm.nih.gov/snp/), but not in the NCBI ClinVar database. All of the other variations discovered in our research have yet to be documented in any of the databases mentioned above. Azoospermia and Necrospermia were the infertility phenotypes related to the nsSNP V66E and nonsense mutation D74X. The deleterious nsSNP V66E at codon position 66 replaced the non-polar amino acid Valine with the basic amino acid Glutamic acid, while D74X reduced the coding sequence, affecting the protein's secondary structure and function. Both genetic variations are found in the DAZLA gene's RNA Recognition Motif domain, which is crucial for target RNA binding during translation activation.

In the studied population, functional DAZL A/G polymorphisms at 260 and 386, as well as DAZ cluster deletion, had no connection with male infertility. However, two deleterious mutations in the exon 3 of DAZLA gene that replace Valine with Glutamate at position 66 and a stop gain mutation at position 74 are particularly concerning because they are located within the RRM domain. Finally, we postulated that different geographic regions and their environments, as well as ethnic background, may have an impact on certain SNPs that cause spermatogenic failures, and that different populations are more likely to have different genetic risk factors for male infertility.

Abbreviations

DAZ	Deleted in Azoospermia
DAZLA	Deleted in azoospermia-like autosome
RRM	RNA recognition motif
SNPs	Single nucleotide polymorphisms
RFLP	Restriction fragment length polymorphism
ART	Artificial Reproductive Technology
WHO	World Health Organization
PCR	Polymerase chain reaction
STS	Sequence tagged sites
EAA	European Academy of Andrology
nsSNV	Non-synonymous single nucleotide variations
SIFT	Sorting intolerant from tolerant
Poly-Phen2	Polymorphism phenotyping v2
FATHMM	Functional analysis through hidden Markov models v2.3
PROVEAN	Protein Variation Effect Analyzer

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Authors' contributions
Conceptualization, methodology, formal analysis and investigation, original draft preparation; funding acquisition: Puja Devi Nongthombam. Review and editing; supervision: Dr S. S. Malini. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The questionnaire and methodology for this study was approved by the Institutional Human Ethical Committee of the University of Mysore (IHEC-UOM No.143/Ph.D/2016-2017). Written informed consent was obtained from the subjects after which samples were collected (blood and semen).

Consent for publication
The authors affirm that human research participants provided informed consent for publication and no identifying information of all participants is included in this article.

Competing interests
The authors have no conflicts of interest to declare that are relevant to the content of this article.

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