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Genomic analysis of missense and silent mutations in CatSper 2 gene in infertile male cases

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Abstract

Background: Infertility is described as inability of a couple to conceive after a period of one year regular unprotected intercourse. About 10% - 15% of couples experience some form of infertility. Male infertility is a multifactorial disease process with a number of potential contributing causes; several factors may contribute to its etiology. Cation channels of sperm is a small family of ion channels, normally referred to as CatSper channels (CatSper1, 2, 3 & 4) which are putative six-transmembrane (6TM) spanning proteins and seem to be specific to sperm cells. CatSper 2 gene is located on chromosome 15 q13-q15 and is involved in sperm motility and hyper activation.

Materials and methods: To screen mutations in CatSper2 gene, we performed PCR followed by direct DNA sequencing in 150 infertile males and 100 healthy controls.

Results: Analysis of mutational screening of CatSper2 gene, we found a single nucleotide change (CT**G**→CT**A**) in five asthenozoospermia at exon 3 resulting in silent mutation and (C**G**T→C**C**T) in eight asthenozoospermia at exon 6 resulting in missense mutation respectively; as the change leads to amino acid sequence change from Arginine to Proline.

Conclusions: On mutational screening of CatSper2 gene, at exon 6 there was missense mutation that may lead to conformational structural change as the amino acid Arginine is hydrophilic and polar in nature in contrast to proline being hydrophobic and non-polar in nature.. The identified mutations may be a cause of asthenozoospermia in humans.

Keywords: CatSper 2 gene, Asthenozoospermia, Sperm motility, Male infertility, DNA Sequencing.

1. Introduction

Male infertility accounts for ~30-50% of all infertility cases, with unexplained infertility accounting for a further 10-20% of cases [1]. In a considerable number of infertile men, genetic aberrations are causative source of infertility. A chromosomal abnormality or a small Y chromosome deletion is sometimes detectable [2]. Therefore, recognition of such genes which are correlated with these abnormalities would have a great impact on our understanding, diagnosis and treatment of male infertility [3]. Although sperm motility is one of the most important interpreters of fertilizing ability, the mechanisms underlying motility abnormalities remain poorly understood [4]. Men with sperm motility <50% or progressive motility <25% are considered to be sub-fertile [5]. During natural fertilization, the target of a sperm is simple and elementary: to fuse with the egg and deliver its genetic information. Despite this simple goal, a sperm has to overcome many hindrances with Ca^{2+} involved in nearly every step [6]. Therefore in both invertebrate and vertebrate spermatozoa, calcium ions have continued to loom as the primary determinant of sperm cell behaviour. Ca^{2+} signalling in the sperm plays a crucial role in the regulation of events prior to fertilisation like capacitation, chemotaxis, and egg penetration [7].

Capacitation, one of the major incidents, is accompanied by various cellular alterations including increase in intracellular concentrations of Ca^{2+} and cAMP [8-10]. The low basal $[Ca^{2+}]_i$ level is maintained by Ca^{2+}

absorption by mitochondria and active Ca^{2+} extrusion by the plasma membrane Ca^{2+} pump (PMCA) [11]. In spermatozoa, numerous Ca^{2+}-permeable channels have been recognized in the plasma membrane of mammalian sperm. These comprise of voltage-gated Ca^{2+} channels (Ca_V channels), cyclic nucleotide-gated channels (CNGC), cation channels of sperm (CatSper) and the transient receptor potential (TRP) family [12-14]; but until recently their function could only be assayed in spermatocytes. On the contrary, the mechanisms controlling swimming behaviour, the entrance site of calcium ions into sperm, and the molecular identity of the calcium channels themselves all remain poorly understood [15].

Cation channels of sperm is a small family of ion channels, normally referred to as CatSper channels or CatSper, is related to the two-pore channels and distantly related to TRP (Transient receptor potential) channels. CatSper channels (CatSper1, 2, 3 & 4) are named after the first putative cation channel of sperm [16,17]. CatSpers are putative six-transmembrane (6TM) voltage-gated Ca^{2+}-permeant channels and are specific to sperm cells. CatSper1 and 2 are each essential for the hyperactivation of sperm cell motility, which is required for fertility. Sequence identities among these CatSper family members range between 22 and 27% across the ion transport domain [18]. All CatSpers are most closely related to the 6 TM voltage-gated sodium channel (Na_VBP) in bacteria, with the next closest relatives being the large mammalian Ca_V and Na_V channel classes. CatSpers have an S4 transmembrane segment with positively charged amino acids interspersed between every three amino acids. CatSper1 also contains a remarkable abundance of histidine residues in its amino terminus. CatSper channels are required for several other well characterized changes in $[Ca^{2+}]_i$ induced by stimuli such as BSA and alkaline depolarization [19]. Similar to zona-pellucida glycoproteins, acute application of BSA induces $[Ca^{2+}]_i$ increase which requires CatSper channels [19].

In CatSper knockout sperm, hyperactivated motility does not develop during capacitation, however other biochemical modification such as protein tyrosine phosphorylation and the Acrosome reaction appear to be intact [20-22]. These inferences conclude that there are different requirements for Ca^{2+} during sperm capacitation, with the change in motility requiring a CatSper-associated Ca^{2+} influx and the other cellular changes using CatSper-independent mechanisms [19,23]. There are evidently CatSper-independent Ca^{2+} entry pathways such as the ATP-induced increases in $[Ca^{2+}]_i$ are intact in CatSper1 knockout sperm [19]. Four different proteins have been identified: CatSper-1 [**17**], -2 [**20**], -3 and -4 [18,24]. With the elapsing time, sperm motility decreases if any of the four CatSper proteins are eliminated. Thus, CatSper channels may be involved in regulating basal motility [20,25].

Among these, CatSper2, gene is located on chromosome 15 q13-q15.Through targeted disruption of each of the CatSper proteins, it has been shown that all four are necessary for hyperactivation [25,26]. The importance of CatSper proteins in male fertility has been clearly established: disruption of the CatSper genes leads to complete male infertility in both humans and mice. Mutation in CatSper1 and 2 are associated with asthenoteratozoospermia and male infertility [27,28]. Since the discovery of CatSper channel, several mutations and deletions in CatSper1 and CatSper2 subunits have been shown to be associated with cases of male infertility in humans [29].

In view of association of human male infertility with mutation of CatSper1 and CatSper2 genes [28,30] which were only detected in individual families; our aim in this study was to find any correlation between male infertility and CatSper2 gene in our population. To accomplish this, we screened different exonic regions of the CatSper 2 gene.

2. Materials and Methods

2.1 Patient Selection

A sum of 250 semen samples were collected [according to World Health Organization(WHO) criteria (WHO, 2010)] from Department of Gynaecology, KGMU, Lucknow and Department of Gynaecology, ELMC&H, Lucknow each in an autoclaved container in aseptic conditions. The Institutional Review Board and Ethics Committee of KGMU, Lucknow, approved this study. Before enrolment in the study each subject's written informed consent was obtained in response to a fully written and verbal explanation of the nature of study. The potential participants, each with infertility persisting longer than 1 year, were examined before the study was conducted. The main inclusion criterion was disease free cases [31-33]. Moreover, subjects having diabetes, hypertension, arthritis, tuberculosis, endocrinal disorders or human immunodeficiency virus infection or those taking any drugs and smokers and other conditions known to influence sperm physiology and male fertility were excluded. Additionally, medical histories of patients and their female partners were recorded. The age of the subjects ranged between 22– 45 years. The study included 150 subjects of control group and 150 subjects of study group consisting of three subgroups: Oligozoospermia (n = 50), Asthenozoospermia (n = 50), Normozoospermia (n = 50). The control group comprised of age-matched healthy men who had previously initiated at least one pregnancy and exhibited a normal semen profile defined as in by WHO [5].

2.2Extraction of semen DNA

DNAs were extracted from semen samples collected in an autoclaved container in aseptic conditions. DNA extraction was done by using a commercially available Semen extraction kit (Bangalore Genei, India) and the DNA was stored at -20°C.

2.3 PCR Amplification of genomic DNA

PCR was performed in a gradient thermocycler (ABI, USA) using 0.2ml PCR tubes. The final volume of PCR reaction mixture was 25µl containing 30ng genomic DNA, 20 picomole of forward and reverse primers, 1X*Taq Buffer*, 10mM of dNTP mix and 1 unit of *Taq* polymerase. Amplification was carried out for 42 cycles using different primers for exon 3 **F5'-CCTTTTGCTTGCCCACTGTGAC-3'** as a forward and **R5'-GGAAATGCAAATGGAGGGGTGA-3'** as a reverse primer. For Exon 6 **F5'-TTGCGGTGTTTTTGTTTGTTTTG-3'** as a forward and **R5'-GGCCTAAAAGTGAAAGACGTGGAT-3'** as a reverse primer.

2.4 CatSper 2 gene analysis by Sequencing

Amplified fragments of all samples were characterized by automated sequencing. The PCR product of each sample was first purified and then submitted in 25 µl quantity with 20 picomoles of appropriate primer. The sequencing was performed by automated direct DNA sequencing technique, which incorporates fluorescently labelled di-deoxy-nucleotides during cycle sequencing and separates the resulting products by capillary electrophoresis for detection on an ABI 3730XL DNA Analyser (Applied Biosystems, USA). Multiple alignment and sequence analysis were done using BLAST (Basic Local Alignment Search Tool), BioEdit, FinchTV, and Auto Assembler Software (Applied Biosystems, USA). Mutations were reconfirmed by sequencing amplicons in both directions and in independent second samples.

3. Results

Our study included 150 infertile male cases and 100 normal fertile male controls. The mean age of onset for infertile male cases and normal fertile male controls was 32.5 ± 1.30.

We evaluated different exonic regions of CatSper2 gene. A sum of 13 point mutations for CatSper 2 gene at exons 3 and 6 were found in this investigation in 13 individual cases of Asthenozoospermia (Table 1). Among this, five silent mutations were detected at exon 3 and eight missense mutations were detected at exon 6.

3.1 Mutational analysis of exon 3 of CatSper 2 gene:

We found a single nucleotide change (CT**G**→CT**A**) at exon 3 in five asthenozoospermia cases. These are silent mutations as there is only change of nucleotide and no change in amino acid Leu27Leu. (Figure 1and 3[A]).

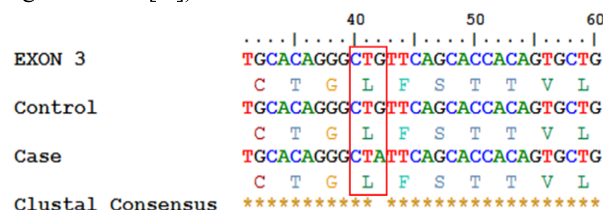

Figure 1: Nucleotide and Amino acid sequence of exon 3 showing silent mutation perceived in five asthenozoospermia cases.

3.2 Mutational analysis of exon 6 of CatSper 2 gene

We found a single nucleotide change (C**G**T→C**C**T) at exon 6 in eight asthenozoospermia cases. This is a missense mutation as the changes leads to amino acid sequence change from arginine to proline (Figure 2 and 3[B]).

Figure 2: Nucleotide and Amino acid sequence of exon 6 showing missense mutation perceived in eight asthenozoospermia cases.

At codon **205** (C**G**T) will produce arginine while mutated codon **205** (C**C**T) will produce proline. This results in the conformational change in the structure of mutant protein when matched up to wild type protein. The conformation of the polypeptide change can be predicted by Ramachandran plot which has three separate regions. First one is the region that has the psi & phi values that generate parallel and antiparallel β sheets. The second one has phi & psi values that generate right handed alpha helix. The third one has phi & psi values that generate left handed alpha helix.

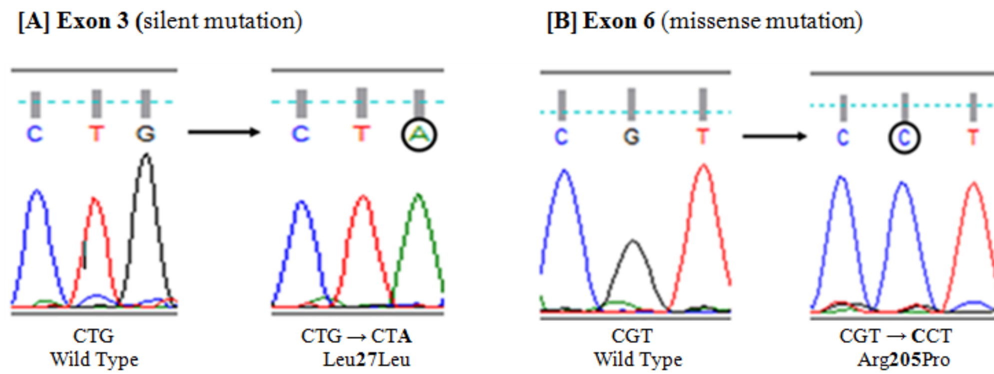

Figure 3: Nucleotide sequencing chromatogram of CatSper2 gene diplaying silent and missense mutations:
[A] Exon 3 nucleotide change from G→A results in no amino-acid change Leu27Leu.
[B]Exon 6 nucleotide change from G→C results in the amino-acid substitution Arg205Pro.

Table 1: Summary of CatSper 2 gene mutations at exon 3 and 6 in infertile male cases

No. of cases with mutation	Exons	Substitution	Mutations (our study)	Point mutation	Category of infertile male cases	References
5 cases	3	CTG→CTA	Leu**27**Leu	Silent	Asthenozoospermia	Not reported
8 cases	6	CGT→CCT	Arg**205**Pro	Missense	Asthenozoospermia	Not reported

4. Discussion

To the best of our knowledge, this study is the first done from in and around the city of Lucknow, Uttar Pradesh, northern India. In order to search for possible mutational outcomes in infertile cases we screened different exonic regions of CatSper 2 gene. Analysis of mutational screening of CatSper 2 gene revealed mutations at codon **27** of exon 3 and codon **205** of exon 6 in Asthenozoospermia patients. Five silent mutations, in exon 3 and eight missense mutations in exon 6 of CatSper 2 gene were identified.

In the missense mutation, the change leads to amino acid sequence change from Arginine to Proline. The amino acid Arginine is hydrophilic and polar in nature in contrast to proline being hydrophobic and non-polar in nature. This may lead to conformational structural change in the protein product. This may affect the normal influx and efflux of calcium ions which in turn hinders acrosome reaction during fertilization which leads to increased infertility rate risk in related asthenozoospermia cases. It is noteworthy that this mutation is only found in asthenozoospermia cases and not in oligozoospermia or normozoospermia cases. No frame shift mutation or truncation was detected in any of the samples.

Likewise the anomalous spermatozoa in asthenozoospermic males with CatSper 2 gene anomaly, suggests that in addition to a role in hyperactivated motility, CatSper proteins are also important for the normal development of spermatozoa [16, 28, 29]. This is further supported by the fact that CatSper2 gene is expressed in meiotic cells as well as spermatozoa [28].

Since the clinical examination of fertility in male patients is commonly limited to routine semen analysis which is evaluated by examining the ejaculate for different abnormalities like semen volume, pH, sperm number, morphology and motility. These measures are rather rudimentary and therefore many of the etiologies go undiagnosed.

CatSper is a very good candidate gene for male infertility because of the phenotype expressed in a mouse knockout model included male infertility, poor sperm motility, the inability to fertilize intact eggs, as well as the loss of cAMP-induced Ca^+ influx [34]. Basically in mice, genetic disruption of any one of the four sperm-specific CatSper channels (CatSper 1, 2, 3 &4) leads to male infertility by impairing sperm motility [24,25]. In recent studies, on humans, CatSper 1 mutation has been correlated with nonsyndromic male infertility (NSMI) [27] whereas loss of CatSper 2 gene along with other gene deletion in the vicinity leads to deafness-infertility syndrome (DIS) [26,28,30]. It is known fact that the CatSper proteins are a family of four sperm-specific alkalinization- activated cation channels (CatSper 1, 2, 3, 4) that are highly conserved in humans and mice [17,18]. The mutant CatSper 1 proteins surmised for the NSMI families lack all six transmembrane domains and the P-loop [27]. Thus, even if a truncated protein is made, it is presumed that CatSper 1 channel activity would be impeded in homozygous carriers [29]. Likewise, in infertile men with deletion in CatSper 2 gene along with DIS (Deafness Infertility Syndrome); there may be loss of the CatSper 2 protein. Moreover, CatSper proteins are also known to be expressed during the meiotic stages of spermatogenesis [16]. As stated by Hildebrand *et al.*[29] that two studies have shown a correlation between CatSper 1, 2, 3 expression level and sperm motility in human

patients, but these constitute the most comprehensive studies reported [35,36]. Therefore the deficit of information on the subject of heritable forms in men results in difficulty in identifying families with male infertility [37]. Recent findings show that disruption of the CatSper 1 or CatSper 2 genes segregate male infertility in several human families [26].

The foremost depiction of CatSper2 gene mutations along with Syndromic male infertility (SMI) was discovered in a French family in some individuals who had a multifaceted phenotype comprising of infertility, deafness and congenital dyserythropoietic anemia type 1 (CDA1) [28]. Similar (though not identical) deletions have also been reported in three unrelated Iranian families who segregate DIS [30]. At the genomic level, the deletions in these families were at 15q15.3 encompasses a complete loss of STRC and CatSper 2 gene. Mutation of STRC causes human nonsyndromic hearing loss at the DFNB16 locus [38] and CatSper 2 male mice are infertile [16], implicating the disruption of both genes in the pathogenesis of DIS (Deafness Infertility Syndrome) in these human families.

Comprehensively in this report we defined new primer sets for screening of exonic region of CatSper2 gene which could be used not only in normal population but also in infertile cases. A significant contribution of CatSper gene presence or absence in males could provide us a suitable male infertility screening test. Although the author also realised further multicentric studies with large sample size should be carried out to strengthen the obtained results.

Overall, our data of this gene could serve as a resource for studies of complex genetic diseases that may be associated with CatSper, individual responses to drug therapy and non- hormonal contraceptives. Also they could be useful in linkage analysis and association study.

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Conflict of Interest

The authors declare no conflict of interest.

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