Andrology-Open Access

ISSN: 2167-0250

Poongothai J, Andrology (Los Angel) 2016, 5:2
DOI: 10.4172/2167-0250.1000171

Research Article Open Access

Association Study of Single Nucleotide Polymorphisms in *KDM3A* and *LOC203413* Genes with Male Infertility

Poongothai J*, **Jeeva SE and Manonmani S**

Department of Biotechnology, PSG College of Technology, Coimbatore, India

***Corresponding author:** Poongothai J, Department of Biotechnology, PSG College of Technology, Coimbatore, India, Tel: +0422 257 2177; E-mail: poongothai_jp@yahoo.co.in

Received date: September 02, 2016; **Accepted date:** November 21, 2016; **Published date:** November 28, 2016

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Abstract

A case-control study conducted on fertile and infertile men in Coimbatore population. The study, aimed to identify and associate the following two single nucleotide polymorphisms (SNPs) rs2030259 and rs5911500 in genes KDM3A (lysine (K)-specific demethylase 3A) and LOC203413.The SNPs were identified by restriction fragment length polymorphism (RFLP) technique in 17 normospermic individuals and in 13 infertile males (9 oligospermic and 4 azoospermic) and results were analysed using fisher's exact test. Although the polymorphisms were found in both fertile and infertile men, no significant association is found for both 2 SNPs with infertility from the samples observed.

Keywords: Male infertility; SNP; Oligospermia; Azoospermia

Introduction

Infertility is a worldwide problem affects 15% of the couples [1-3]. Male infertility refers to the inability of a male to achieve pregnancy in a fertile female. Among married couples, nearly 10% of them go childless despite unprotected intercourse and nearly 50% of this problem is solely due to the male partner [2,4,5]. But nearly in 60-75% male infertile cases, the underlying cause of infertility remains undefined which are referred to as idiopathic infertility [6,7]. A recent report in India, states that nearly 50% of infertility is related to the reproductive anomalies or disorders in the male [4]. Most of the cases are likely to be of genetic origin because spermatogenesis and fertility are controlled by large number of genes and the roles of very few genes are being explored [8,9]. Other than the Y chromosomal defects the profound genetic cause of male infertility, to date only very modest information is known about non-Y-chromosomal gene defects including autosomal genes and the genes in X-chromosome. The non-Y-chromosomal genes are now gaining increased attention in their role in spermatogenesis and male fertility. Therefore the gene deletions or the SNPs in the genes involved in spermatogenesis process can be accounted for male infertility [5]. A pilot genome-wide SNP association study (GWAS) for male infertility and a follow-up study [10,11] identified numerous SNPs in many autosomal genes and in X chromosome genes. A number of reports have focused on the role of certain haplogroups, allelic variants and single nucleotide polymorphisms in male infertility [12].

The SNPs rs2030259 and rs5911500 in genes *KDM3A* (Chr. 2) and *LOC203413* (Chr. X) respectively was identified by the above study showed significant association with oligospermic and azoospermic infertility among men of European descents [13]. Thus in this present study, the association of above mentioned SNPs were evaluated for their significance with male infertility in Coimbatore district (Table 1).

	SNP rs203259 allele frequency		
	G	A	Total
Control (fertile)	11	6	17
Case (infertile)	11	2	13
Total	22	8	30

Table 1: Contingency table for SNP rs2030259 in *KDM3A* gene. The two tailed p-value equals 0.4069.

Materials and Methods

Study samples

The study sample includes 17 fertile (control) men and 13 infertile men (9 oligospermic and 4 azoospermic) of Coimbatore district, Tamil Nadu. The study was performed as two phases (proposal no. 13/017, 16/151) and were approved by Institutional Human Ethics Committee of PSG institute of medical sciences and research (IHEC, PSG IMS&R), Coimbatore.

Inclusion and exclusion Criteria

The sample size is 30. The inclusion criteria for fertile (control) men are age between 25-50 years having children. In case of infertile men, the inclusion criterion is age between 25-50 years.

Blood sample collection

After obtaining written consent from infertile patients and control men, 5 ml of blood drown using plastic disposable syringe under aseptic conditions. The collected blood samples were stored in an EDTA/tri sodium citrate coated containers at 4°C.

Genomic DNA isolation and quantification

Genomic DNA was isolated from the collected blood samples using salting out method [14]. The extracted genomic DNA was quantified using Nanodrop spectrophotometer and all the samples were diluted

Citation: Poongothai J, Jeeva SE, Manonmani S (2016) Association Study of Single Nucleotide Polymorphisms in *KDM3A* and *LOC203413* Genes with Male Infertility . Andrology (Los Angel) 5: 171. doi:10.4172/2167-0250.1000171

with TE buffer to the final concentration of 50 ng/µl, which was used for all PCR amplification.

PCR amplification and restriction analysis

The SNP region of respective genes was retrieved from NCBI SNP database and the primers for the SNP region were designed using Primer3 Input (version 4. 0. 0) tool. The primers 5'-GGACAGTGAGTGAGGTCTTGA-3' and 5'-GAACGGAGGAAAGGAGGAAG-3' (Integrated DNA Technologies) were used to amplify the SNP rs2030259 in *KDM3A* gene and the primer sets 5'-GCTGCTGGTTATTCAGGGTGCAA-3' and 5'-GAAGACATACATGGGCCAGCA-3' (Integrated DNA Technologies) were used to amplify the SNP rs5911500 in *LOC203413* gene.

The presence of unique restriction site in SNP region was identified using NEB cutter V2.0 tool and the restriction enzymes were identified for the investigation of SNPs by restriction fragment length polymorphism [15-17].

Using specific primer sets, each SNP regions were amplified and the respective restriction enzymes, AanI (PsiI) (ER2061, Fermentas, Thermo Scientific) for the SNP rs2030259 and TaqI (FD0674, Fermentas FastDigest™, Thermo Scientific) for the SNP rs5911500 were used to perform restriction analysis according to the enzyme manufactures protocol.

In short, the restriction mixture contains 10 µl (0.1-0.5 µg of DNA) of PCR product, 18 µl nuclease free water, 2 µl 10X restriction buffer, 1-2 µl specific restriction enzyme and the mixture were gently spin down for few seconds and incubated at 37˚C (AanI) and 65˚C (TaqI) for 1 hour. Then the restricted products were resolved in 2% agarose gel at 70 V.

Results

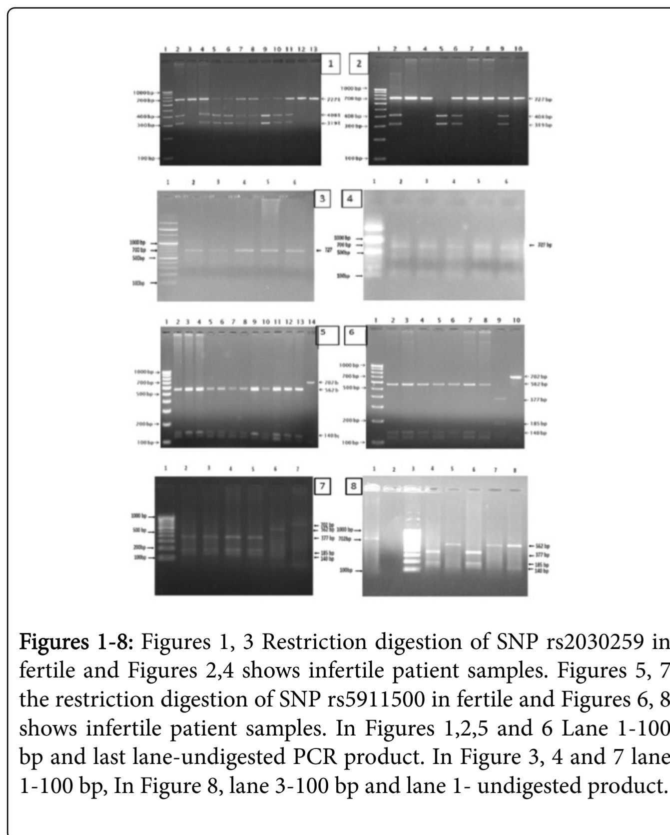
For all the samples (17 fertile and 13 infertile), genomic DNA were isolated and both the SNP regions were amplified using specific primer sets.

Following the PCR amplification of the SNP regions, restriction analysis were performed and the restricted products were resolved using 2% agarose gel electrophoresis and visualized by gel documentation system.

The restriction digested samples for both SNPs resolved in agarose gel and the documented image follows. The figure 1, 3 shows that among the 17 fertile samples, eight samples (Figure 1, in lane 3, 12, 13 and Figure 3, in lane 2,3,4,5,6) were wild type (G/G), 4 samples (Figure 1,in lane 5, 6, 9, 10) were homozygous (A/A) mutants and 5 were heterozygous (G/A) mutants (Figure 1, samples in lane 2, 4, 7, 8, 11) for the SNP rs2030259.

The Figure 2 shows that among 13 patient samples, nine were wild type (G/G) (Figure 2, in lane 3, 4, 7, 8 and Figure 4, in lane 2,3,4,5,6) and three (Figure 2, in lane 2, 6, 9) were heterozygous (G/A) mutants and the sample in Figure 2, lane 5 alone was homozygous (A/A) mutant in patient samples for the SNP rs2030259.

Analysis for SNP rs5911500 shows,13 wild types totally, in figure 5, all the 12 fertile samples were wild type (C)



Figures 1-8: Figures 1, 3 Restriction digestion of SNP rs2030259 in fertile and Figures 2,4 shows infertile patient samples. Figures 5, 7 the restriction digestion of SNP rs5911500 in fertile and Figures 6, 8 shows infertile patient samples. In Figures 1,2,5 and 6 Lane 1-100 bp and last lane-undigested PCR product. In Figure 3, 4 and 7 lane 1-100 bp, In Figure 8, lane 3-100 bp and lane 1- undigested product.

Figure 7 shows 4 mutant types (lane 2,3,4,5) and 1 wild type (lane 6) for the polymorphism (C/T). In case of patient samples for the SNP rs5911500 the figure 6 shows that among the 8 patient samples, seven (samples in lane 2-8) were wild type (C) and the one (sample in lane 9) was mutant (T). Figure 8 shows, 3 wild type (lane 5, 7, 8) and 2 mutant types (lane 4, 6) for the polymorphism (C/T) (Table 2).

	SNP rs5911500 allele frequency		
	C	T	
Control (fertile)	15	2	17
case (infertile)	11	2	13
Total	26	4	30

Table 2: Contingency table for SNP rs5911500 in *LOC203413* gene.

Statistical analysis

The study compares the differences in frequencies of an allele between infertile and fertile population, either fisher's exact test or X^2 (chi square) test can be performed. Since the sample size is small (30), fisher's exact test was performed to evaluate the statistical significance of the SNPs with infertility.

Discussion

Although the causes for infertility were widely studied, the etiology across different populations still varies and remains poorly understood. In the current study, autosomal and X-chromosome SNPs rs2030259 and rs5911500 SNPs associated with infertility were

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evaluated for the association with male infertility. This study found no significant association of SNPs with infertility, which is in contradiction with results of Aston et al. and Plaseski et al. [4,16]. No greater difference was found in the allelic frequency between the fertile and infertile group and it may due to uneven sample size (Table 3).

Sample size	rs2030259					rs5911500			
	G/G (WT)	A/A (HG)	G/A (HT)	Allele frequency		C/C (WT)	C/T (MT)	Allele frequency	
				G	A	C		T	
Fertile (12)	8	4	5	0.62	0.38	13	4	0.88	0.12
Infertile (8)	9	1	3	0.81	0.19	10	3	0.88	0.12
WT-Wild type, HG-Homozygous, HT-heterozygous, MT-mutant									

Table 3: Prevalence of SNPs in fertile and infertile samples.

This disagreement may be due to difference in the population studied or the SNPs were population specific and due to smaller and uneven sample size. As there is no significant association of SNPs with infertility, the other possible explanation for this discrepancy is that these SNPs may not be responsible for the infertility, but more likely its linkage with other SNPs in the same genes or in other genes might have an effect on spermatogenesis and it is to be evaluated. The two tailed P-Value equals 1. The p-values should be less than 0.05 to be statistically significant. Since we got the P values higher than 0.05, there is no statistically significant association between the SNPs and male infertility. From many association studies it can be speculated that male infertility is similar in nature to the majority of complex diseases studied to date, that the disease have multigenic cause and no single SNP is responsible for an appreciable proportion of disease [11]. Hindorff et al. [12] identified genetic variants associated with different diseases through genome-wide studies and it has been understood that thousands of cases and controls will be required to capture an appreciable proportion of the loci responsible for the heritable component of many common complex diseases [18]. Therefore concurrent analysis of the entire genome by comprehensive Genome Wide Association Studies (GWAS), replication assessments of GWAS may reveal the evidence required for association of several novel candidate genes and their abnormalities that may play a role in infertility. Also such studies with larger independent samples of control and diseased will be needed to pinpoint accurate pathogenic DNA variants/mutations in candidate loci and to determine whether these associations are spurious or reflect population-specific variants. Further research in pathology of infertility will not only lead to a more accurate understanding of the genetic risk factors but may also yield new findings and for the development of new diagnostic and predictive testing protocols and clues for more precise therapeutic strategies that could be useful to populations suffering from infertility. This present study has to be enriched with more number of samples with wide ethnic people for significant association and for more appropriate validation of SNPs with infertility.

Limitations

Family studies could not be performed.

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