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FAS-670 A/G and *FAS*-1377 G/A polymorphism in cell death pathway gene *FAS* and human male infertility

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ABSTRACT

Objective: To study the role and association of functional variations present in *FAS* gene with idiopathic male infertility. **Methods:** The case-control study comprised of two groups: 160 idiopathic infertile nonobstructive azoospermia patients and 200 fertile healthy control men. Genotyping for single-nucleotide polymorphism of *FAS*-670 A/G (rs1800682) and *FAS*-1377 G/A (rs2234767) was done by PCR-RFLP method. DNA sequencing was used to ascertain PCR-RFLP results. For *FAS*-670 A/G and *FAS*-1377 G/A functional polymorphism, allele and genotype distribution were evaluated using Chi-square test. **Results:** Allele and genotype distribution did not differ significantly between patients and controls for *FAS*-670 A/G and *FAS*-1377 G/A. **Conclusions:** Human male infertility is a complex disorder and thus other genetic or environmental factors may be contributing to the complex etiology.

1. Introduction

Human male infertility is a complex multifactorial disorder, and it affects 10%-15% couples of reproductive age worldwide[1,2]. Production of functional sperm and proper fertilization with ovum is prerequisite for normal fertility. Spermatogenesis requires an intricate interaction of the various cellular processes in different compartments of the testis like seminiferous epithelium containing spermatogenic cells, Sertoli cells and Leydig cells, macrophages, etc. Apoptosis of testicular germ cells is critical for spermatogenesis and maintains the homeostasis within the testis. A balance between growth and loss of the cells is maintained during spermatogenesis[3]. The spermatogonial apoptosis plays a major role in maintaining spermatocyte density as well as in the safeguard of Sertoli cells and fit the seminiferous tubule shape. It also helps in eliminating defective germ cells and thus in maintaining normal spermatogenesis[4]. FAS system has been implicated to be key regulator of spermatogenesis[5]. FAS (also known as CD95 or APO-1), a member of the tumor necrosis factor receptor family, interact with its natural ligand FASLG to initiate the extrinsic apoptotic pathway[6,7]. In testes, the expressions of FAS and FASLG are confined mainly to germ cells and Sertoli cells, respectively[8]. The *FAS* positive sperms are destined to undergo apoptosis via extrinsic pathway. *FAS* positive sperms which escape elimination step lead to abortive apoptosis. The failure to clear the *FAS* positive sperm may be due lack of synchronization between apoptosis and spermatogenesis[9]. Single-nucleotide polymorphisms (SNP) in the promoter regions of *FAS*, A or G at position -670 [*FAS*-670 A/G] and G or A at position -1377 [*FAS*-1377 G/A] are known to be related to the differential expression of the *FAS* gene[10,11]. The *FAS*-670 A/G polymorphisms disrupt the STAT1 transcription factor binding site, present in the enhancer region of the promoter[12]. The *FAS*-1377 G/A affects the Sp1 transcription factor binding site, present in the silencer

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region of the promoter. The disruption in the binding sites of these important transcription factors reduce promoter activity and decrease *FAS* gene expression, thus affect apoptosis process. Based on the biological and pathologic result of *FAS*, it is possible that functional genetic variations in the *FAS* gene may contribute to the clinical outcomes of male infertility. Therefore, we evaluated the effect of the 670 A/G and 1377 G/A functional variant of *FAS* in cases of idiopathic male infertility.

2. Material and methods

2.1. Subjects

Patients were recruited from the Out Patient Department of University hospital, Infertility Clinic, Institute of Medical Sciences, Banaras Hindu University and Varanasi, India. The present case-control study consists of 156 idiopathic azoospermic infertile males at the age of (32.0±4.8) years and 188 fertile men of comparable age which were taken as controls. Patients underwent a standardized clinical and laboratory evaluation. Patients married for a minimum of two years, having unprotected intercourse were considered for the present study. Three semen analyses were carried out after 3 or 4 d of sexual abstinence to ascertain their infertility status. The size, volume and consistency of testis, occurrence of varicocele, hydrocele or absence of secondary sexual characters were also recorded. Questionnaire was maintained for each patient to record details of their lifestyle, habits and family history. Informed consent was obtained from every participant of each group. Patients with obstructive azoospermia, hypogonadism, hypoandrogenism, chronic diseases, history of pelvic/spinal injuries, karyotype abnormalities and AZF microdeletions were excluded. The control group consists of healthy fertile males who have at least one child and no history of chronic illness. Approval of the University's ethical committee for research on Human material was obtained.

2.2. Genotyping of FAS-670 A/G SNP by PCR-RFLP

Genomic DNA was extracted from peripheral blood, using standard salting-out procedure. PCR amplification of *FAS*-670 A/G mutation region was done using previously described primers of the *FAS* promoter sequence[10]. The PCR conditions were 30 cycles of 55 s at 94 °C, 1 min at 58 °C, and 55 s at 72 °C. The 331 bp PCR product was digested with *Mva* I at 37 °C over night. In wild type allele, the 331 bp gives two fragments of 233 and 98 bp. If there was an 670 A/G transition the 331 bp fragment generated three fragments of 189, 98 and 44 bp (Figure 1).

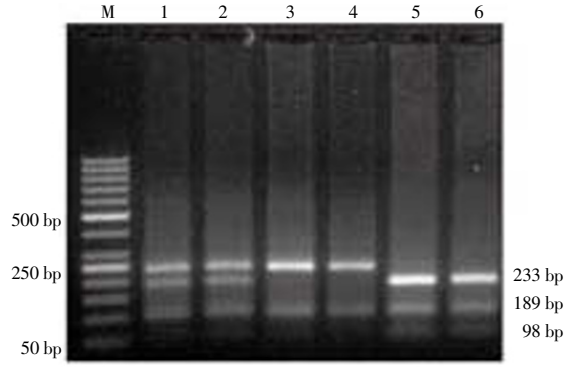

Figure 1. *FAS*-670 A/G mutation detected by PCR-RFLP. A 331 bp PCR product was digested with *Mva* I. The allele A is cut by the enzyme and gives 233 and 98 bp, whereas the G allele yields 189, 93, and 44 bp products. Lane 1 shows the marker (50 bp ladder); Lanes 2 and 3, AG heterozygous; Lanes 4 amd 5: AA homozygous, Lanes 6 and 7: GG homozygous genotype.

2.3. Genotyping of FAS-1377 G/A SNP by PCR-RFLP

Genomic DNA was extracted from peripheral blood, using standard salting-out procedure. PCR amplification of *FAS*-1377 G/A mutation region was done using previously described primers of the FAS promoter sequence[11]. The PCR conditions were 30 cycles of 55 s at 94 °C, 1 min at 62 °C, and 55 s at 72 °C.The 122 bp PCR product was digested with *Bst*U I at 37 °C over night. In wild type allele, the 122 bp gives two fragments of 108 and 14 bp. If there was 1377 G/A transition, the 122 bp fragment will be obtained (Figure 2).

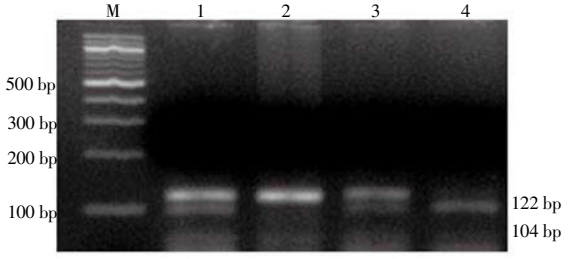

Figure 2. *FAS*-1377 G/A mutation detected by PCR-RFLP. A 122 bp PCR product was digested with *Bst*U I. In wild type allele G, the 122 bp gives two fragments of 108 and 14 bp. If there was 1377 G/A transition, the 122 bp fragment will be obtained. Lane 1 shows marker (100 bp ladder); Lanes 1 and 3, GA heterozygous; Lane 2, GG homozygous; Lane 4, AA homozygous genotype.

2.4. Sequencing

DNA sequencing was used to ascertain PCR-RFLP results. Automated sequencing was performed according to the manufacturer's instructions using the BIG Dye Terminator kit (Applied Biosystem). Forward primer was used for cycle sequencing. The sequence was aligned and analyzed using AB DNA Sequencing Analysis software (version 5.2).

2.5. Statistical analysis

Allele and genotype distribution between groups were evaluated using Chi-square test or Fisher Exact test. The difference in frequencies between the case and control groups was analyzed for statistical significance at the 95% confidence interval using χ^2 test. The allele frequency of *FAS* genotype was in Hardy-Weinberg equilibrium. Odds ratios were calculated and reported within the 95% confidence limits. Linkage disequilibrium was evaluated using (Haploview software version 4.1). Sensitivity and specificity was calculated using Graph Pad Prism5. A *P*-value <0.05 was considered as significant in all the analyses.

3. Results

A total of 344 individuals (156 infertile patients and 188 fertile controls) were examined. Patients underwent a standardized evaluation consisting of a questionnaire, physical examination, semen analyses and hormonal tests. The power of the pilot study is statistically significant for *FAS*-670 A/G . PCR-RFLP analysis was performed to assess the genotypes of 670 A/G and 1377 G/A polymorphism in the promoter region of *FAS* gene in infertile cases and controls. Recessive model was used to compare genotypic frequencies for 670 A/G and 1377 G/A polymorphism in the FAS gene between the cases and controls (Table 1). *FAS*-670 A/G and *FAS*-1377 G/A mutation distribution were in Hardy-Weinberg equilibrium. The value of minor allele frequency, sensitivity and specificity are summarized in Table 2. The allele frequencies of the 670 A/G polymorphism between the infertile males and healthy controls were comparable (18.5% and 18.1% respectively.) For *FAS*-1377 polymorphism, A allele frequency in infertile patients was 20.0% and in controls 17.6 %. In the current study, there was no substantial difference in the distribution of both alleles of the *FAS*-670 and *FAS*-1377 polymorphisms between patients and controls.

Table 1
Distribution of genotypes and allele frequencies of A670G SNP and G1377A SNP of *FAS* in the study population.

Polymorphism	*FAS*-670 A/G					*FAS*-1377 G/A				
	Case (*n*=156)	Control (*n*=188)	OR	95% CI	*P* value	Case (*n*=155)	Control (*n*=187)	OR	95% CI	*P* value
AA	74 (47.3%)	64 (34.04%)				38 (24.5%)	43 (22.9%)			
AG/GA[a]	53 (33.9%)	90 (47.8%)	1.03	0.597 9-1.789 0	0.90[b]	86 (55.5%)	111 (59.3%)	1.67	0.676 5-2.012 4	0.58[b]
GG	29 (18.5%)	34 (18.1%)				31 (20.0%)	33 (17.6%)			

[a]AG is specifically for *FAS*-670 A/G, while GA for *FAS*-1377 G/A. [b]Recessive genetic model: C/C+C/T versus T/T. OR: Odds ratio; 95% CI: 95% confidence interval.

Table 2
Information of single nucleotide polymorphism and single marker association.

Ref No. of SNP	Position	Minor allele frequency	Alleles	Sensitivity	Specificity
rs1800682	90 749 963	0.39	A:G	0.526 7	0.477 4
rs2234767	90 749 256	0.48	G:A	0.579 8	0.355 8

4. Discussion

Apoptosis occurs in the testis as trivial physiological mechanism to limit the number of germ cells in the seminiferous tubule. Sertoli cells which tightly regulate germ cell proliferation and differentiation are implicated in the control of germ cell apoptosis. FAS, a transmembrane receptor protein, transmit an apoptotic signal within cells when bound by FASLG[13]. A cell death signal starts with FASLG on the sertoli cells, which binds to the FAS receptor on the spermatogenic cell surface and activates caspases in the target spermatogonia and spermatocytes[14]. In humans, altered meiotic and postmeiotic germ cell maturation might be associated with an up regulation of *FAS* gene expression, triggering apoptotic elimination of defective germ cells[15]. FAS/FASLG expression in the human testis is developmentally regulated and may be responsible for the quality of the sperm[16]. Also, in testis, the FAS system has also been implicated in maintaining immune privilege[17]. In the present study, functional polymorphism in cell death pathway gene (*FAS*) was genotyped to study its role in human male infertility. There was no significant change in the frequencies of allele between cases and controls. A low linkage disequilibrium was detected between SNP 670 A/G SNP and 1377 G/A SNP in controls (|D/|=0.06, *r*=0.003, |D/| confidence bound=0.00-0.18). Prior to the present study, two studies have evaluated the association of *FAS* variants (670 A/G & 1377 G/A) with human male infertility. In study of Wang *et al*., they have analyzed the frequency of FAS variants (670 A/G and 1377 G/A) & *FASL* 844 C/T in Han Chinese men[18]. Their results suggest that *FASLG* 844 C/T SNP may be a genetic predisposing factor of idiopathic azoospermia or severe oligozoospermia among Han Chinese men[18]. In contrast to this study, Ji *et al*. have found that functional polymorphisms in the promoter of the *FAS*-670 A/G and *CASP*8 (-6526N ins/del) genes were significantly associated with sperm apoptosis and semen quality in Chinese population[19]. Individuals carrying *FAS*-670 G/G genotype had a low apoptosis rate associating with poor sperm motility and decreased sperm concentration compared with the AA genotype, and they did not found any association with *FAS*-1377 G/A polymorphism. The altered apoptotic signaling may have outcome like impaired spermatogenesis resulting in subfertility/infertility. FAS exist as membrane-

bound and soluble forms with opposite roles on triggering apoptosis. They originate from the same gene by alternative splicing. Membrane FAS receptors trigger apoptosis, whereas, on the other hand soluble FAS (sFAS), which lacks transmembrane domain, binds to FAS ligand antagonizing FAS-FAS ligand apoptotic pathway[20,21]. Lime *et al*. showed protective effect of G allele, which may reduce sFAS levels preventing the apoptotic inhibition caused by the soluble form in prostate cancer[22]. The tested polymorphism in the *FAS* promoters was selected, because they functionally affect STAT1 and Sp1 transcription factor-binding sites resulting in decreased FAS expression. *FAS* gene plays a key role in regulation of apoptotic cell death and altered form of this signaling pathway may be one of the factors associated with human male infertility. To our best knowledge, this is the first study to assess the potential influence of the *FAS*-670 A/G and *FAS*-1377 G/A variant on the risk of male infertility in an Indian population. In summary, our study underscores the significance of genetic variants in the regulatory regions of FAS in infertile patients.

Conflict of interest statement

We declare that we have no conflict of interest.

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