Role of *TP53* Gene Polymorphism in Male Infertility

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ABSTRACT

Introduction: The *TP53* gene is located on chromosome 17p13.1. *P53* gene considered as the guardian of the genome belongs to a three-membered gene family *p53*, *p63*, and *p73*.¹ *p53* plays a vital role in human cancer and whose mutation was observed in nearly 50% of cancer all around the world. In the present study, genotype distribution was assessed in 147 infertile males, fertile males, and the associations of *TP53* Arg72Pro polymorphism with hormonal and seminal parameters were investigated.

Materials and methods: A total of 147 infertility men and 150 fertile men were taken for the study. Polymerase chain reaction (PCR) was carried out to amplify the exon 4 of the human *p53* CODON 72 genotyping was performed by restriction fragments length polymorphism (RFLP).

Results: In summary, the AA genotype and the A allele are significantly associated with azoospermic men when compared to fertile men.

Conclusion: The study found preliminary evidence demonstrated that the *TP53* gene Arg72 Pro polymorphism contributes significant association to male infertility.

Aim and objective: To analyze the association of *p53* gene polymorphism in male infertility cases in the study population.

Keywords: Azoospermia, Male infertility, *p53* gene polymorphism, Polymorphism, *TP53* gene.

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INTRODUCTION

Infertility affects 15% of married couples.² During the process of spermatogenesis, spermatocytes with synaptic errors are eliminated by apoptosis are found to be *p53* dependent.³ The *p53* tumor suppressor protein is expressed in the testis and *p53* plays a vital role in meiotic stages confined to the pachytene primary spermatocytes.⁴ Sjoblom and Lahdetie (1996) observed the expression of *p53* in normal and gamma-irradiated rat testis which shows the role for *p53* in meiotic recombination and repair. Depletion of *p53* from germ cells results in an increased incidence of unrepaired DNA breaks in pachytene spermatocytes and increased numbers of caspase-3 positive (apoptotic) germ cells.⁵ Paul et al. demonstrated that deletion of *Ercc1* (excision repair cross-complementing gene 1), *Msh2* (MutS homolog 2, involved in mismatch repair pathway), and *p53* can have differential but overlapping effects on germ cell function and sperm production in which gene mutations can have an impact on male fertility.⁶ The following are some of the apoptotic-related gene products influenced by *p53* that induces apoptosis by intrinsic or extrinsic pathways. PIDD (*p53*-induced protein with death domain), PERP (*p53* apoptosis effector related to PMP-22), Apaf-1 (apoptotic protease-69 activating factor-1), Scotin, a novel *p53*-inducible proapoptotic protein located in the endoplasmic reticulum and the nuclear membrane, and p53AIP1 (*p53*-regulated apoptosis-inducing protein (1)). A cohort study done by Eisenberg et al., to evaluate the cancer incidence in infertile men with azoospermia and infertile men without azoospermia with a mean age of 35.7 years revealed that infertile men with azoospermia had 2.2-fold cancer risk compared to infertile men without azoospermia.⁷ Men with azoospermia have an increased risk of subsequently developing cancer, suggesting a possible common etiology between azoospermia and cancer development.

MATERIALS AND METHODS

Inclusion Criteria

- Cases with idiopathic causes for male infertility with a female partner found normal after a gynecological examination.

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- Cases and controls with no recent history of illness, fever, or drug intake.

Exclusion Criteria

- Cases whose semen samples contained $>1 \times 10^6$ million leukocytes and with high viscosity.
- Cases having varicocele, hydrocele, cryptorchidism, bilateral absence of vas deferens, and any other congenital abnormalities.

p53 Gene Polymorphism

Human genomic DNA was isolated from the lymphocytes using modified Miller's protocol.⁸ Agarose of 0.7% was found to be appropriate for electrophoresis of human genomic DNA. Electrophoresis was carried out at 50 V for 2 hours. The gel was viewed under a UV-transilluminator (UVitec, Lark innovative Inc.), and photographs were taken using Nikon cool pix 4500, digital camera.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) was carried out to amplify the exon 4 of the human *p53* CODON72 gene which has the C72G polymorphism.

Genotyping was performed by restriction fragments length polymorphism (RFLP) analysis. Polymerase chain reaction amplicons were digested with *Bst*U1 enzyme. In the homozygous mutant, *Bst*U1 enzyme recognizes 5'CGCG3' site and digested into two fragments (175 and 132 bp). In homozygous normal, *Bst*U1 will not recognize any site (no digestion), and produce a single fragment (307 bp). In the heterozygous mutant, *Bst*U1 produces three fragments (307, 175, and 132 bp).

The digested PCR amplified products were run on 2% agarose gel. 800 mg of agarose was melted in 40 mL of 1× TBE buffer. 8.0 µL of the sample was added with 2 µL of loading dye which was sufficient for the analysis of the digested products. Electrophoresis was carried out at 50 V for 2 hours. The restricted samples in the gel that is run along with the marker were viewed under UV-transilluminator and photographed. Then, the genotypes are noted.

PCR Amplification

The DNA samples were processed for studying the presence of Arg72Pro polymorphism in the *p53* gene. The samples were amplified using the primers TD7 and TD8 and the temperature was standardized to 66.3°C, an intense specific band of the expected size 307 bp with the absence of non-specific bands was observed (Fig. 1).

RFLP Analysis

The restriction digestion using the *Bst*U1 (*Bacillus stearothermophilus*) enzyme was carried out for all the amplicons. The agarose gel suitable for the restriction digestion using the *Bst*U1 enzyme was the analysis of the digested products was standardized to 2% agarose. The expected size of the digested products was observed. In the homozygous mutant, *Bst*U1 enzyme recognizes 5' CG/CG 3' site and produces two fragments (175 and 132 bp) (Fig. 2). In normal, *Bst*U1 enzyme will not recognize any site. In heterozygous mutant type *Bst*U1 enzyme produces three fragments (307, 175, and 132 bp) (Fig. 2). The frequency of the different genotypes, CC, CG, and GG has been compared between infertile men and fertile men (Table 1).

RESULTS

The *TP53* Arg72Pro polymorphism

Genotype and Allele Frequencies

The Arg72Pro polymorphism of the *TP53* gene was denoted as A and P, respectively. Tables 2 and 3 show the distributions of genotypes of

TP53 Arg72Pro polymorphism. The *TP53* genotype distribution was in agreement with Hardy–Weinberg equilibrium within the infertile men (azoospermic, oligospermic, oligospermic, teratozoospermia men) and the control group (fertile men) ($p < 0.05$). Among the genotypes, AP was most common (52.7% infertile and 48.3% in infertile male patients). The AA genotype frequency was found to be higher in infertile male patients (36.7%) when compared to controls (30.7%), even though no significant differences were observed between the groups. No significant differences were observed with the TT genotype between infertile men (15.0%) when compared to controls (16.7%) (Table 2).

Table 3 shows the distribution of genotypes between the control and different subgroups of infertile men (patient groups). The AP genotype was found to be more common between the groups. The frequency of the AA genotype was found to be significantly higher in azoospermic men (59.1%) when compared to controls ($p = 0.002$) (Table 4) and no significant differences were observed when compared to oligospermic (32.4%), severe oligospermic (30.2%), oligo/astheno/teratozoospermic men (15.4%) groups. The PP genotype was found to be lower in azoospermic men (9.1%) when compared to other groups showing a significance ($p = 0.030$) (Table 4).

Overall significant difference was observed between azoospermic men and the control group ($\chi^2 = 11.80$, d.f. = 2, $p = 0.003$). The allele frequencies showed a significant difference between azoospermic men ($p = 0.002$) and the control group. However, there were no significant differences between the control and other subgroups of infertile men (Table 3).

Odds Ratios

As a significant association was obtained while comparing azoospermic men with fertile men, odds ratios were computed for comparison of various genotypes models between these groups (Table 4). The relative risk was observed to be twice as high in subjects with A allele carriers when compared to P allele carriers. Three of the five genotype comparisons viz., AA vs PP (OR 3.53, 95% CI 1.11–11.27; $p = 0.033$), AA vs AP (OR 3.19, 95% CI 1.51–6.72; $p = 0.002$), and AA vs AP + PP (OR 3.27, 95% CI 1.63–6.54; $p = 0.001$) showed significant association among azoospermic men. The odds ratio for A vs P allele comparison attained statistical significance ($p = 0.004$).

None of the genotype comparisons showed significant association in azoospermic men (Table 5).

DISCUSSION

Several studies on animals have shown that *p53* is evolutionarily conserved. The *p53* protein is reported transiently activated in

Fig. 1: Agarose gel picture showing the PCR amplification of *TP53* gene

Fig. 2: Agarose gel picture showing the RFLP analysis of *TP53* gene

Table 1: Primer sequence of *TP53* gene

S. no.	Primer name	Primer sequence	Primer length	GC%	Tm °C	Product size
1	(Forward primer)	5' CACCCATCTACAGTCCCCCTGC 3'	23 mer	60.9	60	307 bp
2	(Reverse primer)	5' CTCAGGGCAACTGACGGTGCAAG 3'	23 mer	60.9	60	307 bp

Table 2: Genotype and allele frequencies of Arg72Pro polymorphism (rs1042522) at *TP53* gene: all subjects

Subjects	Genotype frequency			χ^2 HW	Allele frequency	
	AA	AP	PP		A	P
Fertile men						
All n	46	79	25	0.830	0.570	0.430
%	30.7	52.7	16.7			
Infertile men						
All n	54	71	22	0.029	0.609	0.391
%	36.7	48.3	15.0			

AA, wild-type homozygote; AP, heterozygote; PP, mutant homozygote

$p(\chi^2_{2df})$ independence of genotype frequencies by the groups compared (fertile vs infertile men) $\chi^2 = 1.23$, $p = 0.541$.

$p(\chi^2_{1df})$ men $\chi^2 = 0.93$, $p = 0.336$

Table 3: Genotype and allele frequencies of Arg72Pro polymorphism (rs1042522) at *TP53* gene: fertile vs subgroups of infertile men

Subjects	Genotype frequency			χ^2 HW	Allele frequency	
	AA	AP	PP		A	P
Fertile men						
All n	46	79	25	0.830	0.570	0.430
%	30.7	52.7	16.7			
Infertile men (subgroups)						
All n	54	71	22	0.029	0.609	0.391
%	36.7	48.3	15.0			
Azoospermic n	26 ^a	14 ^a	4 ^a	1.010	0.750 ^e	0.250 ^e
%	59.1	31.8	9.1			
Oligospermic n	11 ^b	15 ^b	8 ^b	0.417	0.544	0.456
%	32.4	44.1	23.5			
Severe oligospermic n	13 ^c	25 ^c	5 ^c	1.798	0.593	0.407
%	30.2	58.1	11.6			
Oligo/astheno/teratozoospermic N	4 ^d	17 ^d	5 ^d	2.493	0.481	0.519
%	15.4	65.4	19.2			

AA, wild-type homozygote; AP, heterozygote; PP, mutant homozygote

^a $\chi^2 = 11.80$, $df = 2$, $p = 0.003$ for genotype frequencies in control vs azoospermic men

^b $\chi^2 = 1.15$, $df = 2$, $p = 0.563$ for genotype frequencies in control vs oligospermic men

^c $\chi^2 = 0.73$, $df = 2$, $p = 0.693$ for genotype frequencies in control vs severe oligospermic men

^d $\chi^2 = 2.56$, $df = 2$, $p = 0.278$ for genotype frequencies in control vs oligo/astheno/teratozoospermic men

^e $\chi^2 = 9.27$, $df = 1$, $p = 0.002$ for allele frequency in control vs azoospermic men

early spermatocytes peaking between the leptotene and zygotene stages.⁹ A very recent and additional function of *p53* in reproduction has been reported in mice showing that *p53* regulates blastocyst implantation and maternal reproduction through regulation of leukemia inhibitory factor (LIF).¹⁰ Leukemia inhibitory factor, a pleiotropic cytokine is said to be transiently expressed in the uterus on the onset of implantation. Studies have shown that LIF^{-/-} mice to be defective in reproduction and failure of implantation and that *p53*^{-/-} mouse had impaired implantation because of decrease LIF levels in the uterine. Reversal and enhanced implantation of impaired reproduction by exogenous injections of LIF into *p53*^{-/-} female mice indicated that LIF is a target gene of *p53* in reproduction.

Table 4: Genotype and allele frequencies of Arg72Pro polymorphism (rs1042522) at *TP53* gene: fertile vs azoospermic men

Subjects	Fertile men	Azoospermic men	p value
Genotypes	150	44	
Arg/Arg	46	26	0.002
	30.7%	59.1%	
Arg/Pro	79	14	0.867
	52.7%	31.8%	
Pro/Pro	25	4	0.030
	16.7%	9.1%	

OR of Arg allele is 2.26 (95% CI, 1.33–3.86)

Table 5: Odds ratios (OR) and 95% confidence intervals (CI) of azoospermic men according to *TP53* (rs1042522) genotype

Genotypic combinations	p value (χ^2_{2df})	OR	95% C.I.		p value [#] (χ^2_{2df})	OR [#]	95% C.I.	
			Lower	Upper			Lower	Upper
Co-dominant model								
AP vs PP	0.867	1.108	0.334	3.673	0.966	1.026	0.307	3.434
AA vs PP	0.033	3.533	1.108	11.267	0.048	3.256	1.011	10.485
AA vs AP	0.002	3.19	1.514	6.715	0.003	3.15	1.490	6.675
Dominant model								
AA vs AP + PP	0.001	3.266	1.631	6.537	0.001	3.191	1.586	6.421
Recessive model								
AA + AP vs PP	0.223	2.0	0.657	6.092	0.286	1.843	0.599	5.668

OR, odds ratio; χ^2 , Chi-square; CI, confidence interval; p, probability; #, age adjusted

The calculation was performed following a recessive genotype model for Arg/Arg vs Arg/Pro and Pro/Pro

The calculation was performed for Arg/Arg and Arg/Pro vs Pro/Pro

Although these factors are said to play a role in male infertility with spermatogenesis failure, several recent studies have focused on single-nucleotide polymorphisms (SNPs) and their association with human infertility.

Tumor suppressor protein *TP53* (*p53*), is an important gene and is said to play a pivotal role in modulating cell growth, division, and apoptosis.¹¹ It is located on chromosome 17p13 and encodes a 53-kDa nuclear phosphoprotein. Around 13 variants have been reported that harbor in *TP53* with the most important one being the G → C transversion at the second position of the codon 72 in exon 4 that encodes a proline (P) or arginine (R), resulting in differences in structure and function.¹²

The *p53* protein is said to play a crucial role in maintaining genomic stability in somatic cells and thereby preventing tumor formation.¹³ The *p53* P72 allele is weaker in inducing apoptosis and in suppressing cellular transformation when compared to the R72 allele. Similarly, individuals with the *p53* P72 allele have been reported to be at an increased risk for cancer compared to the *p53* R72 allele (Murphy, 2006) and that the P72 allele was found to be enriched in patients with recurrent implantation failure *in vitro* fertilization (IVF) clinical setup.¹⁴ Recently, a meta-analysis showed that the P72 allele to be associated with the risk of endometriosis in Asians leading to infertility in females¹⁵ and also with type 2 diabetes in an Italian population¹⁶

Also, several other SNPs have been identified in genes at critical nodes in the *p53* pathways that include *p53*, *Mdm2*, *Mdm4*, and *Hausp* influencing cancer susceptibility.^{10,17–19} Thus, the possibility of these genes involving the *p53* pathway undergoing selection might also involve in influencing male infertility.

In the present study, we examined the genotype distribution in 147 infertile male patients and 150 controls. As known, the frequency distributions of genetic polymorphisms often vary across diverse ethnic backgrounds and geographic locations, yielding conflicting results. The frequency of the AA genotype was found to be 30.7% in our population which is near equal to the Chinese population²⁰ and different from Iranians.²¹

No significant associations were observed between patients and controls which was similar to other studies on male infertility.^{9,21} But when the infertile men were divided into different groups, we observe a significant association of the *p53* Arg allele to be associated with male infertility. A recent meta-analysis by Chan et al. also did not observe any significant association of the

p53 polymorphism Arg allele with azoospermic males.²² Similar studies that were conducted on infertile men did not find any association with azoospermic men^{20,21} as all the above three studies were not subgrouped and thus no clear association of *TP53* codon 72 polymorphism with idiopathic infertility.

In our study, when subgroups of subfertile men were analyzed, azoospermic men were found to be significantly associated with male infertility. The Arg allele (59.1%) was found to be significantly associated ($\chi^2 = 9.27$, $df = 1$, $p = 0.002$). A similar association was observed in men with idiopathic infertility showing an increased frequency of the Arg allele (62.5%) in a Chinese population.²³

When odds ratios were computed between azoospermic men and controls. Out of all five genotypic combination, the co-dominant models AA vs PP (OR = 3.53; CI: 1.11–11.27; $p = 0.03$), AA vs PA (OR = 3.19; CI: 1.51–6.72; $p = 0.002$), and the dominant model AA vs AP + PP (OR = 3.27; CI: 1.63–6.54; $p = 0.001$) showed significant association when compared with controls. A similar kind of association was observed in the azoospermic group in Chinese males (OR = 1.988; CI: 1.26–3.14; $p = 0.003$).

We did not observe any significant difference between the oligospermic, severe oligozoospermia, oligo/astheno/teratozoospermic group, and controls in the rs1042522 frequency. Thus, this clearly shows that the Arg allele to have a dominant effect in South Indian male infertility and especially on a subgroup of azoospermic males. Our present work also did not reveal any significant association with any other subject characteristics and other clinical variables.

CONCLUSION

Our study showed that the Arg allele frequency of *TP53* to be associated with azoospermic males and male infertility. A recent study has shown a relationship between *p53* codon 72 Arg/Arg genotype and increased apoptosis that contributes to spermatozoa motility and male fertility in men with varicocele. Male infertility is a heterogeneous disorder, with multiple environmental factors and numerous genetic factors contributing to impaired spermatogenesis warranting a better insight into the biological mechanisms of *p53* with male infertility in humans. Thus study showed that the *TP53* gene Arg72Pro polymorphism contributes significant association to male infertility in the South Indian population.

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