The Impact of TP53 Arg72Pro and R249S Genetic Variations on IVF-ET Outcome

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Abstract

In-vitro fertilization-embryo transfer (IVF-ET) can be mentioned as one of the most important methods that various environmental and genetic factors are involved in its success or failure. Present research aims at studying the importance of Pro72Arg and Arg249Ser of TP53 gene in success of IVF-ET. One hundred and fifty infertile women with unsuccessful IVF-ET history and 210 women with successful IVF-ET history were studied using polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) and amplification refractory mutation system (ARMS-PCR). The P53 codon 249 Ser allele was found to be over-represented in patients (p=0.0001; Odds Ratio=3.407; 95% CI; 1.81-6.38). In this study, the distribution of the genotype at codon 72 in the unsuccessful IVF women was not different from the controls (p=0.5). In conclusion, despite the P53 codon 249 Ser allele appearing to be associated with a trend of increased risk of IVF failure, P53 Pro72Arg gene polymorphism might not be a useful marker for prediction of IVF-ET outcome. However, in order to confirm the results, the studies are recommended to be conducted in larger populations.

Introduction

Infertility is the inability of a couple to achieves pregnancy over an average period of one year or 6 months (in a woman above 35 of age) years despite adequate, regular unprotected sexual intercourse (Baird, 2002). Female infertility occurs in about 37% of all infertile couples (Unuane et al, 2011). The causes of female infertility include disorders of ovulation, tubal disease and uterine or cervical factors (Hull et al., 1985).

Various Assisted Reproductive Techniques (ART) and methods have been used during the two recent decades in order to identify and treat infertility. In vitro fertilization (IVF) is one of the most important assisted reproductive techniques. Embryo implantation is a multifactorial event that depends on interplay between the blastocyst and the receptive endometrium. Despite extensive progress in ART, the pregnancy rate per initiated ART cycle and the delivery rate are still around 30% (Society for Assisted Reproductive Technology; American Society for Reproductive Medicine). Therefore, diagnosing the causes of fertilization failure in IVF is important. Failed fertilization may result from impaired spermatozoa oocyte deficiencies, or defects in the in vitro sperm or oocyte medium. The evaluation of the endometrium of repeated implantation failure (RIF) patients by gene array analysis demonstrates that the expression of various genes is altered, including those belonging to the cell cycle, Wnt signaling and cellular adhesion pathways (Salehi and Hadavi, 2012).

Studies have shown that differences seen between individuals or subpopulations of patients are often due to genetic variations, known as single nucleotide polymorphisms (SNPs) in coding regions responsible for the synthesis of hormone receptors, metabolic enzymes or transport molecules, which are specific targets for pharmaceutical drugs. Genetic variation in LHCGR, FSHR, AMH and AMHRII genes play an important role in IVF outcomes (Lindgren et al., 2016; Karagiorga, 2015).

One of the important genes in regulating the various cellular processes is P53. P53 is a tumor-suppressor gene that is located in 17p13.1 which encodes p53 protein, a transcription factor that induced the expression of genes necessary for cell cycle arrest and apoptosis, in response to DNA damage including apoptosis, cellular transcriptional control and cell cycle regulation (Hu et al., 2010; Fracisco et al., 2011; Matei et al., 2012). Mutations in the p53 gene are associated with more than 50% of human cancers, and 90% of them affect p53-DNA interactions, and result in a partial or complete loss of transactivation functions (Zubo et al., 2009).

A common polymorphism occurs at codon 72 of exon 4 (rs1042522), with two alleles encoding either arginine (CGC) or proline (CCC). The distribution of the three genotypes (Arg/Arg, Arg/Pro and Pro/Pro) depends largely on the ethnic composition of the studied population (Omor et al., 2004). The proline allele is a stronger transcription factor compared to the form with arginine; it has a lower ability in inducing cell death (Dumont et al., 2003).

A missense mutation at the third base of codon 249 of exon 7 of the p53 gene, AGG to AGT, leads to substitution of an arginine for a serine (R249S).

This substitution results in changes in P53 Protein's function, cell cycle arrest and proliferation (Mirmomeni et al., 2009). Various researched have assessed importance probability of the mentioned polymorphism in different disease.

TP53 is selectively activated in endometrial tissues at the implantation stage. Estrogen levels also increase at the implantation stage, which in turn activates the estrogen signaling pathway in endometrial tissues through ERα. Selective activation of p53 and ERα coordinately regulates LIF expression at the implantation stage to induce uterine decasualization and ensure embryonic implantation.

Considering the role of P53 protein in fertilization and embryo implantation, this research was conducted in order to study the TP53 Arg72Pro and R249S genetic variations and IVF- ET outcome in a population of Guilan province, Iran.

Materials and Methods

Normally ovulating infertile women (n=150), with at least a 3 year history of infertility and unsuccessful IVF history were recruited for this study. Patients were recruited from Alzahra Hospital, IVF/ET unit, Rasht, Iran. Infertility was defined as unsuccessful attempts to become pregnant after at least 24 consecutive months of unprotected intercourse. Cases with a history of inadequate ovulatory response, ovarian surgery, endometriosis, endocrine diseases, radiotherapy and chemotherapy were excluded. An ongoing pregnancy indicates pregnancies continuing over 12 gestational weeks. Two hundred and ten control subjects who had successful IVF history were also enrolled into study. Recruited subjects are selected among Guilac ethnicity and all of them have no genetic relationship with each other. Informed consent was obtained from all healthy controls and patients before their enrolment. The study groups were subjected to a structured questionnaire (regarding demographic, medical, lifestyle and reproductive information). Whole blood samples, collected using EDTA as an anticoagulant, were taken for DNA extraction and genetic analyses. The protocol followed was consistent with the World Medical Association Declaration of Helsinki (Ethical Principles for Medical Research Involving Human Subjects).

Genotyping
Genotyping was performed without the knowledge of the case/control status of the study subjects. The total DNA was isolated from a 250μl blood sample using the GPP Solution kit according to the manufacture's recommendation. The extracted DNA was eluted in 50 μl of distilled water and stored at -200C until polymerase chain reaction (PCR) was run. The purity of DNA was checked on 0.8% agarose gel electrophoresis, incorporated with ethidium bromide. The electrophoresis was carried out with 1X Tris-acetic acid-ethylene diamine tetra acetic acid (EDTA) buffer (pH 7.4) at a constant voltage (110 V) for one hour. The bands in the gel were visualized and quantified under ultraviolet illumination. Amplifications were carried out using primers designed by Oligo primer analysis software (Version 7.54, Molecular Biology Insights Inc., and Cascade, CO, USA).

Primers for PCR were obtained from MWG-Biotech (Ebersberg, Germany). PCR was carried out in a thermo-cycler (2400; Perkin- Elmer, Norwalk, CT). Assay results were interpreted by two independent investigators who were blinded to case-control status; 20% of the samples were repeated for quality control to ensure that no coding errors occurred.

Determinations of TP53 genetic variants (Arg72Pro and Ser249Arg) were executed   by cleaved amplification polymorphism sequence-tagged site (polymerase chain reaction– restriction   fragment   length   polymorphism, PCR–RFLP)   and   amplification   refractory mutation system (ARMS-PCR), respectively. The PCR-RFLP employed used one sets of primer, which span the 380-bp fragment described previously (Salehi and Hadavi, 2012). The Arg allele contains a BstUI restriction site, yielding two fragments of 210 and 170 bp. The Pro allele was not cut, and had a single band of 380 bp. The heterozygous genotype (Arg/Pro) gave three bands (380, 210, and 170 bp) (Figure 1). The p53249   primers   (forward: 5'-TTGGGCCTGTGTTATCTCC-3'; reverse: 5'GTGTGATGATGGTGAGGATGTGC-3') for allele R and (forward: 5'-TGCATGGGCGGCATGAACCGGTGT-3';   reverse: 5'-ACGCACCTCAAAGCTGTTCCGTC-3' for allele S were used to amplify a 119 bp fragment and 439 bp, respectively. ARMS-PCR was performed with 20–30 ng extracted genomic DNA, and DNA amplification with 2 units of Taq DNA polymerase, 10 mM deoxyribonucleoside triphosphates   and 13.5 pmol each primer. Cycle conditions were as follows: initial denaturation step of 94ºC for 5 min; 35 cycles of 94°C for 30s, 54°C for 30s, 68°C for 60s and final extension step of 72°C for 2 min. The PCR product were separated on 2% agarose gel and visualized by ethidium bromide staining. All the samples with ambiguous results were repeated to ensure quality control purposes.

Statistical Analysis

The statistical significance of the differences between groups was calculated by the $\chi 2$-test. Statistical significance was assumed when p values were < 0.05.The odds ratios (OR) were calculated and are given with the 95% confidence intervals (CI). All statistical analyses were conducted by the MedCalc statistical software (version 12.1, Mariakerke, Belgium).

Results

The mean ages of the cases were 31±6.2 (23-40 years) and of the samples from the controls were 28.5±7.8 (20-38 years). There was no age significant differences between the patients and control subjects (P>0.0005).

Prevalence and ORs of the TP53 Arg 72 Pro polymorphism of cases and controls are given in Table 1. As shown in Table 1, prevalence rates of TP53 Arg72Pro in cases (56%) did not differ significantly from that in the controls (56%).

The heterozygous genotype was lower, though not significantly (p= 0.4) in cases compared to the control group, with an (OR 1.2; 95% CI; 0.7-2.03). The proportion of individuals homozygous for the TP53 Arg72Pro variant (Arg/Arg, Arg/Pro and Pro/Pro) was also significantly less common in cases (80%), than controls (76%) and the (OR 1.25; 95% CI; 0.75-2.08).Therefore, the P53 Pro/Pro genotype (OR 1.18; 95% CI; 069-2.02) was not associated with IVF-ET outcome. The allele frequency of TP53 Arg72Pro was not statistically different (P=0.63).

Genotype distributions for polymorphism of TP53 R249S were 42% RR, 8% RS, 50% SS among controls, and 10% RR, 20% RS, 70% SS among cases with (Table 1), and the difference was statistically significant (p<0.05). Individuals carrying the SS genotype were 95 fold at a higher risk of IVF-ET failure (OR= 2.48; 95% CI; 1.39 -5.25 p=0.002). There was a significant difference in the distribution of allele frequencies between the two groups (p<0.0001). The prevalence of S allele was higher in patients than controls. The variant S allele (Ser) of TP53 increased risk IVF-ET failure (OR=3.407; 95% CI; 1.81-6.38; p<0.0001).

Discussion

To our knowledge, the current study is the first focusing on association between p53 polymorphisms with IVF-ET outcome in subjects with infertility. Our findings suggest that individuals homozygous (SS) for TP53 R249S have higher risk of IVF-ET failure. This association may be predictive of embryo implantation. However, our results showed no significant differences in the allele distribution of the TP53 Arg72Pro polymorphism between two groups. Also, the combined genotype of P53 Ser249Arg (RR+SS) and (RR+SR) respectively (OR= 5.88; 95% CI; 3.11 to 11.11, P< 0.0001) and (OR= 6.49, 95% CI,; 3.56 to 11.8 P<0.0001) showed a significant association in IVF-ET outcome. The allele Ser was considered as a high risk factor with success of IVF-ET (OR= 3.407; 95% CI; 1.37 to 4.34, P< 0.0001).

R249 is essential for stabilizing the hairpin conformation of the L3 loop. R249S mutation leads to structural changes in L2 and L3 loops (Wong et al., 1999). It is showed experimentally that structural change occurs on the L3 loop due to this mutation and DNA binding affinity is significantly reduced (Rauf et al., 2010). A transfer from C to G is occurred in codon 72 of exon 4 of the gene which results in change of proline amino acid into arginine. Proline allele is a stronger transcription activator compared to arginine allele but it is a weaker apoptosis inducer. The lower level of apoptosis in P53 P72 allele carriers might cause misguided growth of cells or tissues leading to miscarriage (Paskalin et al., 2012). Previous studies in mice and humans have demonstrated that the p53 family proteins play important roles in regulation of reproduction, including maintaining primordial and primary follicular pool size, germ cell genomic integrity, ovulatory ability, pre-implantation development, and embryonic implantation. Oligonucleotide array experiments showed that the TP53 link to the promoter regions and regulate approximately of 300 different genes through its DNA binding

domain (D'Orazi, 2012). LIF is an important player in implantation in humans. The regulation of LIF by p53 is conserved from mice to humans (Hu et al., 2007). LIF-deficient female mice have a defect in reproduction due to the complete lack of uterine decidualization at the implantation stage that leads to the failure of blastocyst implantation. The levels of LIF protein increase at the time of expected implantation in fertile women.It has been suggested that ER (estrogen receptor) and p53 cooperate in the regulation of blastocyst implantation through the induction of LIF and CGB7 (Sohr and Engeland, 2011). Leukemia inhibitory factor (LIF) is a cytokine that has been the focus of many studies (Aghajanova et al., 2009; Allegra et al., 2009; Rashid et al., 2011). In humans, p53 protein containing Arg at codon 72 produces twofold higher levels of LIF transcript and more LIF protein compared with the Pro form of the p53 protein in cultured human cells (Hu et al., 2006). In IVF clinics, the Pro form of the p53 single nucleotide polymorphism (SNP) has been found at higher frequencies in women who had difficulties with implantation of fertilized eggs than in control populations (Kay et al. 2006; Kang et al. 2009).

The rapid development of IVF-ET has increased the curative rate of infertility about 20%-35% and the delivery rate up to 18.6% (Cao, 2002; Lian and Wang, 2010). Environmental and genetic factors are some of the elements influencing IVF-ET's success. Several epidemiological studies have evaluated the connection of P53 variants and risk of different types of disease (Sadeghi et al. 2013; Marine et al. 2006).

The present study has some limitations. First, this study was a single-center investigation on a relatively small scale. Thus, replication studies with large independent cohorts are warranted. Second, we have analyzed two functional TP53 polymorphisms and in future, exploring other exonic and intronic genetic variations in the TP53gene would also be interesting.

In conclusion, our study implicates that there is no significant relationship between R72P polymorphism of P53 gene and success of in-vitro fertilization and embryo transfer. But T allele, in S249R polymorphism in P53, considered as a risk factor for IVF-ET failure. Hence, our result should be treated with caution until we will be replicated by larger and ethnically diverse populations. Further longitudinal studies will be needed to resolve the impact of the p53 polymorphisms on the IVF-ET outcome.

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Table (1): Distribution of TP53 Codon 72 and 249 Genotypes in Cases and Controls

|	Case (n=150) n (%)	Controls (n=210) n (%)	ORs (95%CI)	P value
P53 P72R				
Pro allele	(48)	(52)	1(Ref)	
Arg Allele	(52)	(48)	0.85(0.48-1.84)	0.56
P/P	30 (20)	50 (24)	1.18 (0.69-2.02)	0.63
P/R	84 (56)	118 (56)	1.2 (0.7-2.03)	0.4
R/R	36(24)	42 (20)	1.44 (0.73-2.86)	0.27
R/R + P/R	120 (80)	160 (76)	1.25 (0.75-2.08)	0.4
P53 R249S				
Arg allele	(80)	(54)	1(Ref)	
Ser allele	(20)	(46)	3.407 (1.81-6.38)	<0.0001
R/R	15 (10)	88(42)	1 (Ref)	
S/R	30 (20)	17 (8)	0.56 (0.3-1.08)	0.08
S/S	105 (70)	105 (50)	5.86 (3.18-10.8)	<0.0001
R/R + S/R	135 (90)	122 (58)	6.49 (3.56-11.8)	<0.0001
R/R + S/S	120 (92)	193 (92)	5.88 (3.11-11.11)	<0.0001
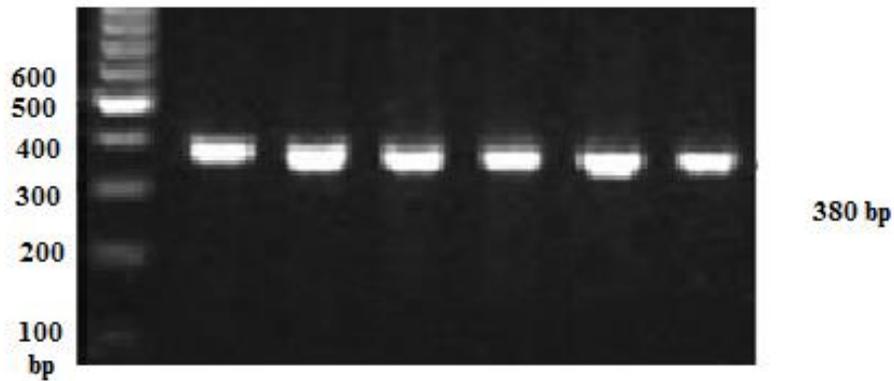
Figure 1(A): Agarose gel electrophoresis of the P53 gene PCR amplification products. Fragments of 380 bp indicate Pro allele of P53. The Pro was not cut and has a single band of 380 bp.
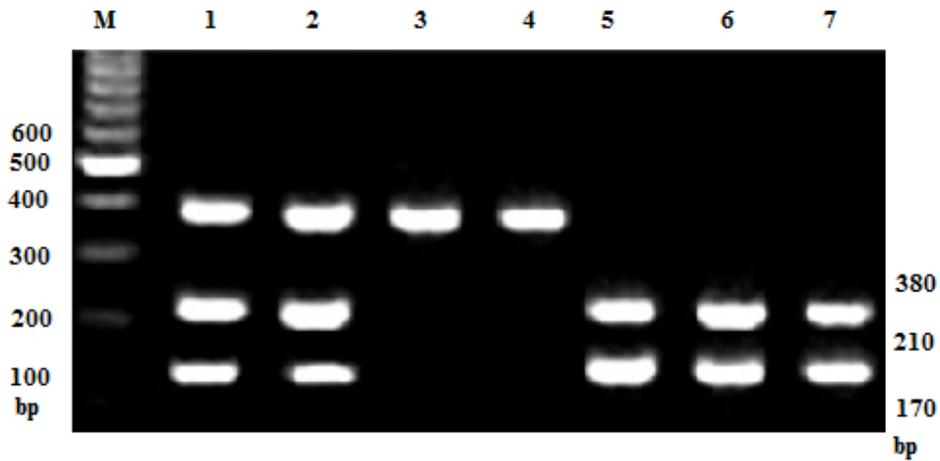

Figure 1(B): Agarose gel electrophoresis of PCR fragments after BstU1 digestion. M: 100 bp DNA marker; lines 1, 2: Arg/Pro genotype produced three fragments (380, 210 and 170 bp); line 3, 4: Pro/Pro genotype failed to be cleaved by BstU1; lines 5, 6, 7: Aeg/Arg genotype produced two fragments (210 and 170 bp).
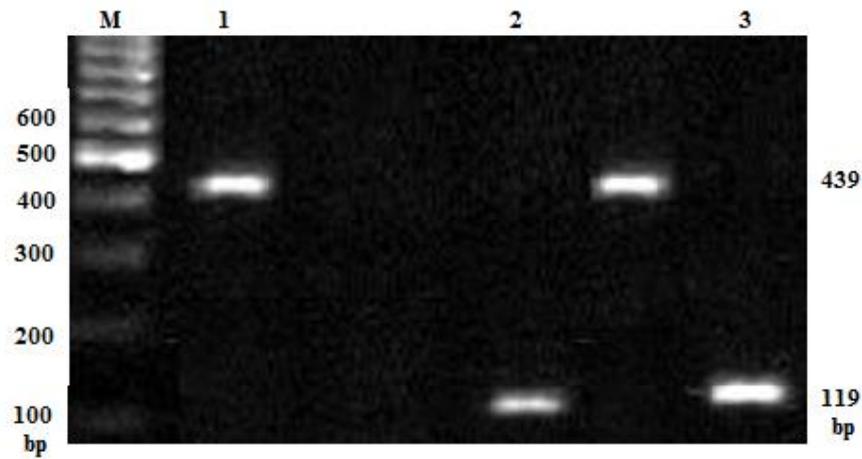
Figure 1(C): Agarose gelelectrophoresis stained by ethidium bromide after tetra-primer amplification refractory mutation system PCR. M: 100 bp DNA marker; lines 1: fragments showing Ser allele homozygous, lines 2: fragments indicating Arg allele homozygous; lines
3: fragments presenting Ser and Arg for heterozygous.

1= S/S
2=R/R
3=R/S