Original Article

Association of p53 polymorphism with ICSI/IVF failure and recurrent pregnancy loss

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Background: The p53 tumour suppressor gene is a well-known factor regulating apoptosis in a wide variety of cells. Alterations in the p53 gene are among the most common genetic changes in human cancers. Several polymorphisms of the p53 tumour suppressor gene have been associated with recurrent pregnancy loss (RPL).

Aims: To evaluate the association of polymorphisms p53 codon 72 with the response to *in vitro* fertilisation (IVF) treatment and occurrence of repeated miscarriages.

Methods: The homozygous and heterozygous genotypes and allelic frequencies of Arg and Pro p53 at codon 72 were identified by using polymerase chain reaction–restriction fragment length polymorphism technique in 70 infertile women with more than two IVF failures. Each comparison was made with 97 women experiencing RPL and 32 fertile women each with at least two healthy children as the control group.

Results: The frequency of homozygous Pro/Pro genotypes was found significantly higher among the women with RPL than the other two groups ($P = 0.041$). Whereas, Arg/Arg genotype was significantly different in the recurrent implantation failure (RIF) group ($P = 0.005$).

Conclusion: It is concluded that p53 codon 72 polymorphism may serve as a susceptible factor affecting the chances of RPL and RIF.

Key words: IVF failure, p53 tumour suppressor gene polymorphism, recurrent pregnancy loss.

Introduction

The maintenance of human pregnancy depends on several factors. Endocrine dysfunction, chromosomal aberrations and uterine abnormalities are present in 50% of women with recurrent miscarriage.[1] Several factors have been considered for cases with idiopathic recurrent miscarriage.

Angiogenesis is the formation of new blood vessels from pre-existing blood vessels, which has a critical role during implantation and embryonic development. Normal pregnancy needs adequate fetal placental circulation. It has been reported that decreased placental vascular development is associated with early embryonic mortality.[2] Abnormal apoptosis may cause pregnancy loss. The occurrence of apoptosis in normal human placenta has been documented with a low incidence of apoptosis in the first trimester and increased apoptotic activity as gestation progresses.[3]

It is evident that the normal angiogenesis and apoptosis may play an important role in maintaining normal pregnancy. It has been already shown that the low expression of apoptosis as well as angiogenesis-related genes are associated with recurrent pregnancy loss (RPL).[4] Gene polymorphisms have also been proposed as susceptibility factors, increasing the chances of miscarriage, in otherwise healthy women.[5,6]

As well as pregnancy loss, recurrent implantation failure (RIF), in assisted reproductive techniques (ART) can be the result of a problem with the embryo or with the environment in which the embryo is implanted. In addition, chromosomal abnormalities result in most of implantation failures.[7] The process of implantation involves the ability of the implanting blastocyst to establish its own blood supply.[3,8] A suitable trophoblastic invasion results from the balanced effects of growth factors and apoptosis. A number of proteins such as p53 involved in trophoblastic invasion and angiogenesis have been identified.[9]

The p53 tumour suppressor gene encodes a multifunctional transcription factor. It is a well-known factor regulating cell apoptosis. Alterations of P53 are one of the most common genetic changes in human cancers. Beside its role as a tumour suppressor gene, p53 plays a critical role in regulating angiogenesis.[10,11] Recently, it has been reported that p53 is a potential mediator of pregnancy with oestrogen and progesterone activities.[12] Dysfunction of p53 may causes increase of cytoplasmic p53. It consecutively may initiate immunity to abnormally expressed p53, which is revealed by autoantibodies in the blood. Alterations in the p53

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gene product may be caused by polymorphic sites within the gene. A common sequence polymorphism is located within the proline-rich domain of p53 encoding either proline or arginine at position 72. In a recent study, an increase in the frequency of p53 codon 72 involving proline polymorphism has been shown among women experiencing RPL. This proposes that proline polymorphism may increase the chances of miscarriage.[13] Based on this, we hypothesised that the polymorphism of p53 gene is associated with RIF and RPL

Methods

Patients

In order to study the effect of p53 gene polymorphism on RPL and RIF, a total of 167 women referring to Research and Clinical Center for Infertility in Yazd were screened: 97 women with a history of recurrent miscarriage and 70 women with a history of RIF after in vitro fertilisation (IVF) or intracytoplasmic sperm injection. RPL was defined as two or more consecutive spontaneous abortions, and RIF was considered as a cumulative of four to six cleaved embryos transferred, which is shown by human chorionic gonadotrophin (HCG) serum concentrations less than 5 mIU/mL 14 days after embryo transfer. Both male and female partners in the two study groups were evaluated. Karyotype for detection of chromosomal abnormalities was done on both male and female partners in RPL group. Genital tract anomalies were evaluated in all the women by transvaginal sonography and hysterosalpingography. Immunological risk factors such as antiphospholipid antibodies, antinuclear antibodies and thrombotic factors were ruled out by blood sample analysis in all the women. In addition, semen analysis with normal results was performed in the male patients.

The control group ($n = 32$) consisted of volunteers premenopausal women with at least two live births and no history of abortion. Written consent was taken from the participating women. Since there has not been adequate study about p53 SNP in Iran and the exact minor allele frequency is not identified, the control group in this project was used to determine the allele frequency of the cases. The previous studies in Iran were not population-based, therefore the control group was matched with the cases in basal characteristics.

This prospective study was approved by the ethics committee of the Research and Clinical Center for Infertility at Yazd University of Medical Sciences.

Genetic study

Each patient was subjected to blood sampling through peripheral venous tapping into an EDTA-containing tube. After collection, the samples were stored in a freezer (–20°C) until DNA extraction. DNA was extracted from peripheral blood by the standard salting out technique. The analysis of the P53 genotype at codon 72 was performed as described by Pietrowski *et al.*[6] with some modification. We used the primers GCCAGAGGCTGCTCCCCC and CGTGCAAGTCACAGACTT for amplification of the Pro codon and primers TCCCCCTTGCCGTCCCAA and CTGGTGCAGGGGCCACGC for amplification of the Arg codon.

Polymerase chain reaction conditions compromised an initial denaturing step at 94°C for 5 min, followed by 45 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 45 s, with a final extension at 72 °C for 5 min. Reaction products were fractioned on a 2% agarose gel and visualised after ethidium bromide staining with a digital camera system (Gel Digidoc II, Kiagen, Iran).

Statistical analysis

The Statistical Package for Social Sciences 15.0 software (SPSS Inc., Chicago, IL, USA) was used to analyse the data of all the patients. The differences in the frequencies of the p53 alleles in the studies and control group were analysed using chi-squared test. A P-value of ≤ 0.05 was considered statistically significant. Odds ratio (OR) was calculated by multivariate nominal logistic regression.

Results

The characteristics of the women experiencing RIF, the women with RPL, and the control group are presented in Table 1.

The p53 Arg/Pro allele frequencies and genotype of the aforementioned groups are given in Table 2.

The frequency of Arg/Arg genotype was 42.9%, 23.7% and 12.5% in RIF, RPL and control group, respectively ($P = 0.005$, OR 7.5, 5.7, respectively). Arg/Pro genotype was accrued in 42.3% of the women with RPL and in 40%

Table 1 Characteristics of women with recurrent pregnancy loss, recurrent implantation failure and control group

Variables	RPL ($n = 97$)	RIF ($n = 70$)	Control ($n = 32$)
Age (years)	28.00 ± 3.70	32.30 ± 4.80	29.20 ± 5.20
No. of pregnancy losses	3.67 ± 1.10	–	–
No. of live births	0.18 ± 0.42	–	–
No. of previous IVF/ICSI cycle	–	2.53 ± 1.09	–

RIF, recurrent implantation failure; RPL, recurrent pregnancy loss.

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Table 2 Genotype and allele frequencies of P53 codon 72 Arg and Pro polymorphism among women with recurrent pregnancy loss (RPL), recurrent implantation failure (RIF) and control group

	RPL (n = 97, 194 alleles)	RIF (n = 70, 140 alleles)	Control (n = 32, 64 alleles)	P-value	Power test
Genotypes					
Pro/Pro	33 (34%)	12 (17.1%)	7 (21.9%)	0.041*	0.88
Arg/Pro	41 (42.3%)	28 (40%)	21 (65.6%)	0.038	0.96
Arg/Arg	23 (23.7%)	30 (42.9%)	4 (12.5%)	0.005*	0.99
Alleles					
Arg	86 (44.3%)	88 (62.8%)	29 (45.4%)	0.002***	0.92
Pro	108 (55.7%)	52 (37.2%)	35 (54.6%)	0.002****	0.95

*Values in parentheses are percentage.

of the RIF group compared with 65.6% of the control women (P = 0.412, OR 1.9, 1.3 respectively). The homozygous of Pro/Pro genotype were found 17.1% in the women with RIF, 34% in woman experiencing RPL and 21.9% in the control group (P = 0.041, OR 4.7, 1.7 respectively). The allele frequency in these groups is in Hardy–Weinberg disequilibrium. It is probably due to high consanguinity in Iranian population.

Significant differences in Pro allele frequency of p53 were detected in RPL group compared to the other groups. Chi-squared value was 0.002. Arg allele frequency was significantly higher in RIF patients than in the control and RPL groups with an allelic value of 0.002. The frequency of Arg allele was 62.8%, 44.7% and 45.4% in RIF, RPL and the control group, respectively. The frequency of Pro allele in RPL patients was 55.3%, in RIF, 37.2% compared with 54.6% in the control group.

Discussion

Recurrent implantation failure after ART techniques and spontaneous RPL are important clinical problems, the potential causes of which are poorly defined. Recent reports have suggested that causes of RIF include overexpression or underexpression of genes that encode proteins necessary for successful implantation.[14,15]

Based on the above observation, the present study investigated the relationship between RPL, RIF and p53 codon 72 gene polymorphism. Significant differences in genotypic frequencies were found among women experiencing RPL compared with RIF and the control group. The results indicated that women with homozygote genotype of Pro have a significantly higher risk of RPL than women with the Arg/Arg genotype. Co-dominant segregation of these alleles was observed in a previous study.[16] This is in line with Pietrowski *et al.* study, which reported a significant association between the genotype of Pro/Po and the occurrence of RPL.[6] No significant differences were found between genotyping frequencies among RPL patients and controls in Coulam *et al.* study.[17] There is evidence that Pro 72 induces a higher level of G1 arrest than Arg.[18] It is known that a balance in proliferation and apoptosis is necessary to ensure normal embryonic development during pregnancy. It is proposed that changes of Prolin region of p53 can affect the regulation protein involved in cell apoptosis.[13] p53 plays a significant role in maternal reproduction, through leukaemia inhibitory factor (LIF). LIF is the cytokine produced and secreted by the endometrial glands of uterus. If the epithelial cells lining the uterus are exposed to LIF, implantation occurs. In the absence of p53, insufficient LIF is produced, the uterus does not become adequately receptive and fewer blastocysts are implanted.[19–21] The fetal growth is dependent on a good exchange between mother and fetus, therefore cell arrest in blood vessels and cytotrophoblasts can result in imbalances of cell differentiation leading to abortion.

Another explanation may be the power of Pro allele at inducing apoptosis, which is significantly lower than Arg allele.[22] Because placental development is a dynamic process of cell proliferation and degeneration, the lower level of apoptosis in these carriers might lead to misguided growth of cells.[13] This event has been demonstrated in mice with induced pregnancy loss displaying a high level of p53 expression that decreased as apoptosis was completed.[23] However, more controlled studies are needed about role p53 pro polymorphism in RIF.

Our findings showed an increased risk of RIF in women with Arg/Arg genotype, whereas Kay *et al.* study reported a significant increase in Arg/Arg genotype frequency among women with RPL.[24] It can be demonstrated with the function of p53 acting in various types of stress including DNA damage,[25] which is present in implantation site. Recently, it has been shown that pro allele of p53 codon 72 has a much higher potential for preferentially induce DNA-repair target promoters[26] and that deficiency of maternal stores of these DNA repair factors can affect preimplantation development.[27] Therefore, it might be expected that Arg/Arg carriers are more associated with increasing implantation failure than with Pro/Pro carriers.

In Pietrowski study, a significant difference was found when comparing allelic frequencies among RPL patients and the controls.[13] On the contrary, Coulam *et al.*[17] reported an insignificant association between the Pro allele and the occurrence of RPL.

In conclusion, the findings generated from this study showed that p53 codon 72 polymorphism would be expected

to play a major role to bring about RPL. Further investigation is needed to determine the mechanisms of both RPL and RIF, and whether the p53 codon 72 polymorphism is a risk factor.

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