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Association of *hTERT* SNP (rs2736100) with implantation failure after *in vitro* fertilization and embryo transfer (IVF-ET)

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Infertility is the failure of conception after 24 months of unprotected sexual intercourse [1]. For most infertile couples, assisted reproduction technology (ART) methods, like *in vitro* fertilization (IVF) has the highest live birth rate per their treatment cycle. However, about 75% of IVFs are not successful [2]. It has been reported that multiple factors contribute to the positive IVF outcome, including maternal age, embryo quality and the endometrial receptivity. Furthermore, research in the last decade has focused on understanding genetic factors contributing to IVF and embryo transfer (ET) outcome [3–5]. However, the exact mechanism of IVF-ET implantation failure is still largely unknown.

Telomerase or telomere terminal transferase is a ribonucleic protein that adds TTAGGG noncoding repeat sequence to the 3′ end of human chromosomes. It protects the chromosome ends from degradation and end to end fusion. Human telomerase is a ribonucleoprotein complex composed of the telomerase reverse transcriptase (hTERT) and the telomerase RNA component (TERC). *hTERT* is located at 5p15.33 [6]. There is a striking correlation between the presence of hTERT mRNA and telomerase activity. It has been documented that telomere dysfunction may lead to genomic instability. Cells with high proliferation activity like stem cells and cancer cells usually contain detectable telomerase activity. However, it is inactive in somatic cells except those with self-renewal activity [7].

A Single Nucleotide Polymorphism (SNP) is the most common type of genetic variation that may occur every 100 to 300 bases. It has been shown that *hTERT* is a polymorphic gene and among its SNPs, rs2736100 A/C within intron 2 is notable. Although its exact mechanism is unclear, multiple lines of evidence suggest that the *C* allele is associated with longer telomere length [8]. Ma et al. compared the rs2736100 and rs2736098 in patients with renal cell carcinoma. They found that there is no significant association between rs2736100/rs2736098SNPs and risk of this cancer [9]. The A allele of rs2736100 is associated with lower hTERT mRNA expression and shortened telomere length in gastric cancer tissue and cell lines. We hypothesised an effect of maternal *hTERT* rs2736100 genotype on IVF-ET outcome in infertile women.

We recruited 150 infertile women with a history of implantation failure after IVF-ET as the case group and 200 fertile women with at least 2 live births from naturally conceived pregnancies and no past history of pregnancy loss as controls. Written informed consent was obtained from all case and control subjects. This study was performed in accordance with the declaration of Helsinki and local research ethics committee approval was obtained. A rs2736100 polymorphic site has a minor allele frequency (MAF) greater than 10% (0.48). Genomic DNA was isolated from 1 ml of peripheral blood using Gpp solution kit (Gen Pajoohan, Tehran, Iran). DNA samples were dissolved in 30 µl TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA) and stored at −20 °C until use. Purity and quantity of genomic DNA were checked with Nanodrop spectrophotometer at 260 nm and 280 nm (Thermo Scientific, Mass, USA). The forward and reverse primers were designed based on relevant DNA sequences available in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/genbank) using Oligo-primer analysis software (Version 7.54, Molecular Biology Insights, USA). The primer sequences were F:5′-TCCCTGCTG ACTTAGTTCT-3′, R:5′-TGTCACTCACTGGCTAAGGAA-3′. PCR was performed with a Bio-Rad thermal cycler in 15 µl of PCR mixture containing 30 ng of genomic DNA, 0.1 M deoxyribonucleotide triphosphates (dNPTs), 10× PCR buffer (50 mM KCl, 10 mM Tris HCl, and 0.1% Triton X-100), 1.5 mM $MgCl_2$, 0.5 units of Taq polymerase, and 2 pmol of each primer. Following an initial denaturation step (5 min at 95 °C), samples were subjected to 30 rounds of PCR at 95 °C for 30 s, 53 °C for 40 s, and 72 °C for 40 s with a final extension time of 2 min at 72 °C. A no template control was set as an endogenous control. A 375 bp PCR amplicon was amplified and digested with *SfcI*. The products were verified on 2% agarose gel containing safe stain

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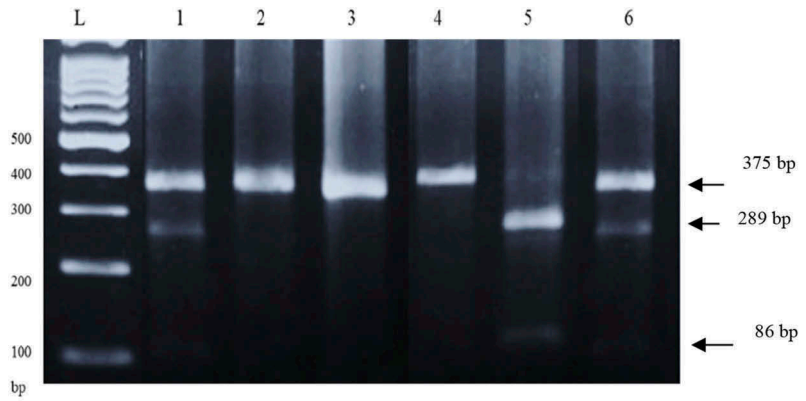

L: DNA ladder, 2, 3, 4: AA homozygote, 1, 6: AC heterozygote and 5: CC homozygote.

Figure 1. Agarose gel electrophoresis for detection of *hTERT* polymorphism.

(Figure 1). The CC homozygote showed two bands of 289 bp and 86 bp, the heterozygote showed three bands of 289 bp, 86 bp and 375 bp and the AA homozygote showed single band of 375 bp. For quality control, genotyping of 5% of samples were randomly repeated, yielding 100% similarity.

Hardy–Weinberg equilibrium was determined to evaluate deviation between observed and expected genotype frequencies for both cases and controls using the Oege online server (http://www.oege.org/software/Hardy–Weinberg.html). Three allele groups were considered for rs2736100 (CC, CT, and TT). The statistical significance of differences between patients and controls was calculated by the χ^2 test. Odds ratio (OR) and 95% confidence interval (CI) were used to describe the strength of association between *hTERT* rs2736100 polymorphism and IVF-ET outcome. Statistical analyses were conducted using MedCalc statistical software (Version 17.9.7, Mariakerke, Belgium). A value of $P < 0.05$ was considered statistically significant.

The mean age of the control group was 31 ± 11.7 years and the mean age of the cases was 32.5 ± 8 years ($p = 0.69$). Genotype and allelic distributions in the cases and controls were compared to analyse the possible risk factors of IVF-ET implantation failure. All samples were successfully genotyped. The observed genotype frequencies were in the Hardy–Weinberg equilibrium ($\chi^2 = 0.22$, $P = 0.63$). So, there was no population stratification and no sampling bias.

Table 1 shows the genotype and allele distributions of the *hTERT* polymorphism in cases and controls. Cases were more likely to be positive for the codominant AC and CC genotypes, the dominant AC +CC genotype and the C allele.

In this case–control study of 150 infertile women cases and 200 fertile women controls, we observed that there is a significant association between *hTERT* rs2736100 polymorphism and IVF-ET outcome. Our

Table 1. Genotype and allele frequencies of *hTERT* rs2736100 polymorphism in cases and controls.

	Cases	Controls	OR	
Genetic models	*N* (%)	*n* (%)	(95% CI)	*P*-value
Codominant				
AA	28 (18.6)	68 (34)	1.00	
AC	88 (58.7)	100 (50)	2.13 (1.26–3.61)	0.004
CC	34 (22.7)	32 (16)	2.58 (1.34–4.95)	0.004
Dominant				
AA	28 (18.6)	68 (34)	1.00	
AC+CC	122 (81.3)	132 (66)	2.24 (1.35–3.71)	0.007
Recessive				
AA+AC	116 (77.3)	168 (84)	1.00	
CC	34 (22.7)	32 (16)	1.53 (0.89–2.63)	0.11
Overdominant				
AA+CC	62 (41.3)	100 (50)	1.00	
AC	88 (58.7)	100 (50)	1.41(0.92–2.17)	0.108
Alleles				
A	144 (48)	236 (59)	1.00	
C	156 (52)	164 (41)	1.55 (1.15–2.10)	0.003

data suggested that individuals with the CC genotype might be associated with an increased risk of IVF-ET implantation failure. We found no significant difference in the risk associated with the recessive and overdominant genetic models when reference was AA+AC and CC+AA genotype, respectively. In this study, the frequency of C allele in controls (0.41) is similar to other reports of a Chinese population (0.41) and Polish population (0.45), but lower than that reported in Armenia (0.6) and a little higher than that for Japanese women (0.39), according to ensemble database.

So far, there is no report concerning the relationship of *hTERT* rs2736100 polymorphism with IVF-ET outcome. Recently a case–control study examined four *hTERT* polymorphism, rs2853672, rs2853669, rs2735940 and rs2736108, in IVF outcome using TaqMan real-time quantitative PCR. They showed that the TTTG (rs2853672/rs2853669/rs2735940/rs2736108) haplotype was more likely to lead to more than three good-quality embryos in patients aged ≤35 years [10].

A few studies have been published in which particular genetic variations are associated with risk of infertility and

implantation failure [11]. The C allele of *VEGF* 936 C/T SNP increases the relative risk of IVF-ET/ICSI failure in a Chinese population [5]. We previously reported that the effect of the *GSTM1* null allele and *LIF* 3951 C/T polymorphism in relationship with in IVF-ET outcome [3,4].

It is accepted that folliculogenesis is accompanied by significant proliferation of granulosa cells. As granulosa cells undergo successive mitosis during follicular growth, telomere length progressively shortens because of the DNA end replication problem. Telomerase acts as a reverse transcriptase in the elongation of telomeres. The decreasing telomerase activity in the granulosa cells leads to increased rate of apoptosis and number of the atretic follicles [12]. Liu and colleagues suggested that short telomeres in the oocytes contribute to anomalous fertilization of gametes and abnormal cleavage of embryos [13].

We note some limitations that must be considered. First, only one polymorphic site has been investigated which may not represent the entire gene. Second, potential selection bias might have occurred because of the hospital-based case–control study. Third, we did not include women whose IVF was successful, and fourth, numerous factors such as age, life style and genetic factors act individually and together to influence risk of implantation failure.

This work represents an advance in biomedical science because it indicates that *hTERT* rs2736100 CC genotype may be a risk factor for implantation failure.

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Disclosure statement

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