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Original Research Article

An intronic polymorphism of the gene Plakophilin-3 is associated with IVF failure

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ABSTRACT

Background: Implantation failure is determined when transferred embryos fail to implant following in vitro fertilization (IVF). In recent years, many studies suggest that implantation failure could be related to several genetic factors. In the current study, authors aimed to investigate the association of *PKP3* rs10902158 (G>A) polymorphisms with the risk of implantation failure after ICSI treatment.
Methods: 97 women, who underwent ICSI treatment owing to male factor infertility, were prospectively recruited in this cross-sectional study. Genomic DNA was prepared from peripheral blood samples in order to analyze the polymorphism (rs10902158) at the *PKP3* gene by PCR-RFLP. The Results were presented as a genotype (GG, GA, and AA), and their relationship to IVF outcome was analyzed.
Results: The patients were divided into two groups according to clinical pregnancy: the pregnant group included 51 patients (53%) and the non-pregnant group included 46 patients (47%). The clinical pregnancy outcome was significantly different between genotypes, which was 0%, 45.8% and 58.8% in the patients having the genotype AA, GA and GG respectively (p-value = 0.03).
Conclusions: The presence of the allele A of the *PKP3* SNP rs10902158 is associated with a reduced clinical pregnancy outcome in the patients undergoing ICSI treatment and may be helpful predictor for implantation failure.

Keywords: Implantation failure, In-vitro fertilization, Plakophilin-3, SNP

INTRODUCTION

Implantation failure was one of the most common causes of unsuccessful pregnancy in women undergoing in vitro fertilization (IVF) or intra- cytoplasmic sperm injection (ICSI) which are the most common assisted reproductive technology (ART) treatments. Despite advances that have been made since the introduction of these techniques, implantation of the embryo is still the most important rate-limiting step: even high-quality embryos frequently fail to implant resulting in an implantation rate of approximately 25-30% per transferred embryo.[1] Implantation failure (IF) refers to when an implanted embryo fails to result in the development of an intrauterine gestational sac following embryo transfer (ET), as determined by ultrasonography.[2] Several causes of IF have been reported, including embryo, uterine and immunological factors, as well as thrombophilic

conditions, however, the genetic mechanisms underlying IF remain not completely understood.[2]

Embryo implantation is a multifactorial event that depends on the interaction of the blastocyst with the receptive endometrium and consists of molecular signaling by the embryo, followed by apposition and attachment to the endometrium.[3] Following the formation of the fetal-maternal interface, the essential second step involves the invasion of the embryo through the endometrium.[4]

Recently, several genetic-association studies have revealed associations between the IF risk and certain genetic polymorphisms, including tumor suppressor protein p53, cyclooxygenase-2 (COX-2), methylenetetrahydrofolate reductase (MTHFR), plasminogen activator inhibitor-1, vascular endothelial growth factor, and leukocyte antigen-G.[3-9]

The architecture and integrity of epithelial cells is mediated by several types of cell-cell junctions, including adherent junctions, desmosomes, tight junctions, and gap junctions.[10]

Desmosomes are adhering junctions that are involved in cell-cell adhesion, differentiation, and signal transduction.[11,12] They are responsible for strong intercellular adhesion and are as such indispensable in tissues that undergo mechanical stress.[13] Desmosomal adhesion was also shown to be involved in epithelial morphogenesis to an extent comparable to the role of adherent junctions.[14] They are composed of 3 zones: extracellular core region, outer and inner dense plaque. Desmosomal cadherins (desmogleins, desmocollins) extend into extracellular core and outer dense plaque, while their cytoplasmic tails connect to plakoglobin, Plakophilins and desmoplakin. Desmoplakin associates to keratin intermediate filaments within inner dense plaque to establish connection between intermediate filaments and plasma membrane.[15,16]

Mature desmosomes are characterized by being calcium independent, also known as "hyperadhesion" of desmosomes.[17-19]

Loss of tissue integrity due to failure of desmosomal adhesive function is considered a major underlying cause of inherited, auto- immune, and bacterial toxin–mediated disorders.[20-22]

Plakophilins are structural proteins and therefore part of cytoplasmic plaques of desmosomes. They are considered to be important in connecting other desmosomal proteins, but also for attachment of intermediate filaments to desmosomes, which makes them important for stability and adhesion of cells and tissues.[23] They are members of the p120ctn family of armadillo- related proteins important for intercellular interactions and cytoskeletal maintenance.[10,24] Armadillo proteins are characterized by a series of repeated motifs of about 45 amino acids called arm repeats. Members of the p120ctn family share a characteristic organization of their arm repeat domain which suggests an ancient evolutionary relationship.[25] They can also exert signaling functions by generating and transducing signals that affect transcription, post-transcription regulation and translation.[24-26]

Plakophilin-3 (PKP3) belongs to the Plakophilin family. It is described as beta-catenin and armadillo protein of the desmosomal plaque, which is synthesized in simple and stratified epithelia, present in cytoplasm, membrane and nucleus.[27,28] However, it also has important non-junctional functions, such as regulating protein synthesis, growth control, and transcription.[29-31] There is a growing interest about Plakophilin-3 functions and functional interactions at the cellular and molecular levels, particularly, in cancer initiation and progression.[10,23]

The importance of cell-cell junction proteins in placentation and implantation biology is known.[32] Although many studies indicate that alterations of intercellular adhesion may be associated with implantation failure, no study has investigated if the dysregulation or genetic polymorphisms of Plakophilins are associated with IF.[32-34]

Our objective is to evaluate the association between the SNP rs10902158 of the *PKP3* gene and the risk of IF after ICSI treatment. To our knowledge, no studies based on Plakophilin-3 polymorphism or expression and its correlation to fetal implantation have yet been published.

METHODS

A total of 97 women, who underwent ICSI treatment owing to male factor infertility, were prospectively recruited in this cross-sectional study from 1st October 2017 to 1st April 2018, at Assisted Reproduction Unit at Orient Hospital, Syria. All Participants gave informed consent, according to the protocol approved by the local Ethics Committee at Damascus University and Health Ministry.

Inclusion criteria

- Patients age less than 38 years, with good ovarian reserve (normal basal FSH, AMH), having normal gynecological, hormonal and anatomical state, and at least two good quality embryos were easily transferred.

Exclusion criteria

- Women with known immunological disease (APS, LAC, ACL, ATA) or thrombophilia (protein S or C deficiency, Factor V Leiden, hyperhomocysteinemia etc.), Women with pelvic pathology: Fibroids, Adenomyosis, Endometriosis, Polyps, Septum,

Hydrosalpinx) and when endometrium thickness was <7 before ultrasound guided embryo transfer (ET).

All patients were on long protocol using GnRH agonist. Ovulation induction was by HMG or rFSH and the dose was adjusted according to response and when 3 leading follicles reached 17-18 mm, 10000 IU of HCG was given, and oocytes retrieval was scheduled 35 hours later.

Ultrasound guided embryo transfer (ET) of 2-3 embryo was performed in cleavage stage (day 3). Luteal phase support was achieved with vaginal micronized progesterone gel (9%) once daily. Clinical pregnancy (presence of gestational sac in ultrasound) was done after 3 weeks post ET. The patients were divided into two groups according to clinical pregnancy: the pregnant group and the non-pregnant group.

Molecular genotyping

DNA extraction

Blood samples were obtained on the day of oocytes retrieval prior to the procedure. The leukocyte genome was extracted using the Genomic DNA Isolation Kit (GeneDireX, Malaysia) according to the manufacturer's instructions and was stored at -20 C^0 until use.

PCR amplification for PCR-RFLP

The genomic region of the PKP3 gene (intron 1) that contains the SNP rs10902158: G>A, was amplified by PCR with the following primers: forward primer, 5'-TGCAACAGGGCATACTGGTC-3'; and reverse primer, 5'-CCTGGGCTCACCTGTGTC-3'. Primers were synthesized by Eurofins Genomics (Ebersberg, Germany). PCR amplification was carried out using 1X OnePCR reaction mixture (GeneDireX, Malaysia) and 150 nM of each primer. The PCR amplification was performed in a GeneAmp PCR System 9700 (Applied Biosystems, USA). Samples were incubated for 5 min at 94ºC to denature the target DNA followed by 45 cycles at 94ºC for 30 s, 59ºC for 50 s and 72ºC for 30 s and finally for 7 min extension at 72 °C. An amplicon of 650 bp was expected.

Restriction digest of the amplified DNA

Aliquots of 18 µL of each 650 bp amplified product were digested with the restriction enzyme Sau96I (Thermo Fisher Scientific, USA) as recommended by the manufacturer.

Electrophoresis

Electrophoresis of digested fragments was carried out on 2.5% agarose gel stained with ethidium bromide, and presence of fragments was visualized by UV transilluminator. Fragment sizes were assessed against a 100 bp DNA ladder (GeneDireX, Malaysia).

Statistical analysis

For the analysis of the data, Pearson chi-square and continuity correction chi-square tests have been used. For all analyses, a value of less than 0.05 was considered statistically significant. SPSS Software (version 25) was used for the analysis.

RESULTS

Infertility investigation

To confirm the male factor infertility, a routine infertility investigation was done in all women (serum FSH, LH, PRL, E2, TSH, AMH concentrations) and it detected no sign of perturbation. In addition, the statistical analysis by Pearson Chi-square test between the previous parameters and the genotype of the *PKP3* SNP rs10902158 was showed no significant statistical difference. There was also no significant difference in age between patients according to the genotype (Table 1).

Table 1: Characteristics of patients according to the genotype (the values represent mean±SD).

Parameter	Genotypes			*P*-value*
	AA (n=5)	GA (n=24)	GG (n=68)	
Age (years)	31.8±3.27	29.92±4.09	30.13±4.51	0.82
FSH (mIU/ml)	7.41±3.68	6.42±1.65	6.99±1.86	0.26
LH (mIU/ml)	1.99±0.65	1.66±1.08	2.01±1.03	0.95
PRL (ng/ml)	20.45±2.54	22.18±16.13	15.61±9.27	0.72
E2 (pg/ml)	51.52±72.32	22.4±13.09	24.01±13.51	0.69
TSH (µIU/ml)	1.59±0.36	2.70±0.90	2.46±1.24	0.81
AMH (ng/ml)	3.15±1.57	3.27±2.47	3.76±2.27	0.84

*Pearson Chi-square.

The patients were divided into two groups according to clinical pregnancy: the pregnant group included 51 patients (53%) and the non-pregnant group included 46 patients (47%).

The relation between IVF outcome and the PKP3 SNP rs10902158.

The clinical pregnancy outcome was significantly different between the genotypes, where the women carrying the A allele have an obvious reduction in clinical pregnancy outcome than women with the G allele with a p-value of 0.03 as calculated by Pearson Chi-square test (Table 2).

Table 2: The clinical pregnancy outcome according to the genotype.

IVF outcome	Genotypes			
	AA	**GA**	**GG**	
Non-pregnant (n)	5	13	28	
Pregnant (n)	0	11	40	
Clinical pregnancy rate (%)	0	45.8	58.8	*P*=0.03*

*Pearson Chi-square.

Table 3: The IVF outcome parameters according to the genotype (the values represent mean±SD).

The IVF outcome parameter	Genotypes			*P*-value*
	AA (n=5)	**GA (n=24)**	**GG (n=68)**	
Oocyte (n)	10.40±8.99	13.75±5.68	12.99±6.7	0.04
MII (n)	6.20±5.36	7.96±3.90	8.57±5.20	0.08
Fertilization (n)	6.00±5.24	6.04±3.71	5.84±4.20	0.24
Embryos (n)	5.40±4.56	5.92±3.54	5.82±4.20	0.05

*Pearson Chi-square.

While the number of oocytes and embryos (p=0.04 and p=0.05, respectively) were significantly affected by the presence of the A allele, the number of MII oocytes and the number of fertilization were not affected as shown in (Table 3).

DISCUSSION

The techniques of assisted reproduction have developed to circumvent human infertility. However, a high rate of implantation failure was recorded in these techniques.[2] Among the many causes evaluated, the single nucleotide polymorphism (SNP) in different genes was growly described.[5-7] The SNP may affect gene expression, RNA stability, and protein function and therefore may cause disease.[35]

Desmosomal cell adhesion is indispensable for folliculogenesis and normal embryonic development.[32] PKP3 is a novel important integrator of desmosomes junction and it has important non-junctional functions.[24,25] The intronic SNP rs10902158 of the *PKP3* gene have recently related to the risk of death in HIV disease and tuberculosis.[36,37] In this study, we examined the potential association of this SNP and the risk of IF after ICSI treatment.

Our results showed that no influence of the *PKP3* SNP rs10902158 on the hormonal characteristics of patients indicating that PKP3 do not have an effect on hormones that influence or predict the IVF outcome.

Interestingly, our data found that all women who carried the genotype AA had implantation failure (IF). The carrying of allele A was also significantly associated with decrease in oocyte numbers, embryo numbers, and clinical pregnancy rate (CRP). These findings suggest that a functional variant in the *PKP3* gene may influence the folliculogenesis and the early stage of embryo development.

A recent study demonstrated that a polymorphism in the first intron affected the transcription activity of the gene.[38] Similarly, this may be suggested for the *PKP3* gene where the site of the SNP rs10902158 is in the first intron. Therefore the allele A of this SNP could influence intron removal or affect the enhancer activity and hence PKP3 protein quantity, so destabilizing the desmosomes integrity.

In addition, the defect of PKP3 production may also alter the molecular mechanisms of implantation. Because PKP3 regulate the inflammation, one of the possible mechanisms is the influence of PKP3 on the inflammatory reaction that is mediated by the embryo implantation.[39,40]

According to this hypothesis, a polymorphism in COX-2, a key enzyme in inflammation, was associated with increased risk of implantation failure.[6]

CONCLUSION

The present study is the first study, which indicates that the presence of the allele A of the *PKP3* SNP rs10902158 is associated with a reduced clinical pregnancy outcome in the patients undergoing ICSI treatment.

This variant may also constitute a helpful biomarker to predict the implantation failure. More studies are needed for both parents to demonstrate this hypothesis and to elucidate the precise molecular mechanism of PKP3 in folliculogenesis and embryo development.

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