Prevalence of candidate single nucleotide polymorphisms on $p53$, $IL-11$, $IL-10$, $VEGF$ and $APOE$ in patients with repeated implantation failure (RIF) and pregnancy loss (RPL)

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Prevalence of candidate single nucleotide polymorphisms on \( p53, \) \( IL-11, \) \( IL-10, \) \( VEGF \) and \( APOE \) in patients with repeated implantation failure (RIF) and pregnancy loss (RPL)

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**ABSTRACT**

Recurrent pregnancy loss (RPL; defined as the loss of three or more consecutive pregnancies) and recurrent implantation failure (RIF; when implantation is not achieved after at least three cycles of IVF) are two of the major challenges that reproductive medicine faces. Some polymorphisms have been identified as possible causes of an increased risk of these diseases. This paper studies the prevalence of the polymorphisms in \( p53, VEGF, IL-10, IL-11 \) and \( APOE \) in RIF and RPL patients that determines the risk for these pathologies. A total of 255 patients were selected (89 RPL patients, 77 RIF patients and 89 controls) and genotyped for \( p53-R72P; IL-11-1082-AG; VEGF-1154-AG; IL-10; APOE-R112C; APOE-R158C. \) Statistically significant differences were found in the prevalence of the E4 isoform (R122-R158) of the \( APOE \) gene in RPL patients (\( p < 0.05 \)), and in RIF patients, the R72P polymorphism of the \( p53 \) gene and the 1154-AG of the \( VEGF \) gene showed different distribution (\( p < 0.05 \)). Regarding the \( p53 \) and \( IL-11 \) studied polymorphisms, PP of \( p53 \) gene and GG of \( IL-11 \) are more prevalent in RPL patients without reaching statistical significance. In conclusion, our results suggest patients carrying variants in \( p53 \) and \( VEGF \) would be at risk of RIF, and those carrying variants in \( APOE \) gene would suffer RPL.

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\( p53; \) vascular endothelial growth factor; interleukin10; apolipoprotein E; recurrent pregnancy loss; recurrent implantation failure; in vitro fertilization

**INTRODUCTION**

Recurrent pregnancy loss (RPL) is defined as the loss of three or more consecutive pregnancies after the 12th week. Recently the American Society for Reproductive Medicine (ASRM) has extended this definition considering that the loss of pregnancies not visualized by ultrasound (biochemical pregnancies and/or unknown location pregnancies) should be included in the definition of recurrent pregnancy loss (Kolte et al., 2014). In couples without fertility problems pregnancy loss occurs spontaneously in 15–20% and only become recurrent in 2–3% of them (Rai & Regan, 2006). On the other hand, recurrent implantation failure (RIF) could be considered when implantation is not achieved after at least three cycles of in vitro fertilization (IVF) with at least four good quality embryos transferred to a woman under 40 years of age (Coughlan et al., 2014).

Embryo implantation requires the ability of the blastocyst to invade the endometrium and to establish its own vascular network, as well as an adequate maternal immune tolerance to the embryo. A correct trophoblastic invasion is led by an equilibrium between apoptotic and cellular generation processes (Simón, Dominguez, Remohi, & Pellicer, 2001; Smith, 2000). There are a high number of proteins involved in the invasion of the endometrium and the angiogenesis. One of these proteins is \( p53, \) a potent tumour suppressor that induces apoptosis and angiogenesis (Savion et al., 2002). Moreover, some studies reveal that \( p53 \) is involved in embryo implantation mediated by leukemia inhibition factor (LIF) (Hu, Feng, Teresky, & Levine, 2007). The \( p53 \) gene (17q13) contains 11 exons, and a single nucleotide polymorphism (SNP) in codon 72 comprising the change of a G by C resulting in a proline instead of an arginine. The \( p53 \) protein with an arginine in codon 72 is much more efficient in inducing apoptosis and angiogenesis (Pim & Banks, 2004). A decreased expression of genes involved in apoptosis and angiogenesis has been associated with RIF (Smith, 2000).

Another important protein involved in embryo implantation is vascular endothelial growth factor...
VEGF is an angiogenic factor that acts by enhancing and remodelling the vascular growth, as well as by increasing vessel permeability, which is crucial for the embryo implantation and further development of the placenta (Magdoud et al., 2012). Changes in the expression of VEGF in decidual samples in early miscarriages (Vuorela, Carpén, Tulppala, & Halmesmäki, 2000), as well as the committed fertility in VEGF knock-out mice, show the importance of VEGF (Rowe, Wulff, & Fraser, 2003) in embryo implantation and early pregnancy stages. The VEGF gene (6q21) contains 8 exons. Several SNPs have been described and associated with an abnormal VEGF expression. The SNP located in the promoter region -1154 has a higher population frequency allele (G) which, when substituted by A produces a lower VEGF expression (Watson, Webb, Bottomley, & Brenchley, 2000).

The correct embryo implantation and the development of the pregnancy also depend on the maternal tolerance to embryo and fetal tissues. A suitable cytokine production profile will stimulate fetal and placental development (Cha, Sun, & Dey, 2012). Several studies have focused on determining the role of each cytokine during embryo development, their changes during the pregnancy or the effects of an alteration in their levels on implantation and pregnancy. Interleukin 10 (IL-10) is an anti-inflammatory cytokine that plays a key role in maternal fetal tolerance. Decreased levels of IL-10 are associated with pregnancy loss (Wang, Hao, & Lin, 2011). In the IL-10 gene (1q31), the SNP in position -1082A/G controls its expression; A allele produces a decrease in the gene expression. There are other important cytokines for the progress of pregnancy such as Interleukin 11 (IL-11), an inflammatory cytokine with similar functions to LIF. IL-11 gene is located at 19q13. Different polymorphisms have been identified in the IL-11 gene that may affect its expression (Shinohara et al., 2001).

Finally, other proteins that are not major players might also affect the correct implantation and development of the pregnancy. For example, apolipoprotein E (APOE) is involved in the transport of lipoproteins, lipid soluble vitamins and cholesterol into the lymphatic system and subsequently into the bloodstream (Mahley, Weisgraber, & Huang, 2009). A deficient protein could produce lipid accumulation in the bloodstream increasing thrombotic risk. The ApoE (19q13) gene consists of four exons. It has three polymorphic isoforms: ApoE E2 (cys 112, cys 158), ApoE E3 (cys 112, arg 158) and ApoE E4 (arg 112, arg 158) that produce six different genotypes: E2/E2, E2/E3, E2/E4, E3/E3, E3/E4 and E4/E4. Different genotypes have been associated with reproductive problems mainly RPL (Li, Chen, Wu, & Li, 2014).

In a previous study, it was shown that there is an association between p53 R72P polymorphism and RIF and RPL patients (Lledo et al., 2014). We considered to extend the study by obtaining the complete genotype from the same patients for all the possible genetic variants associated with RIF and RPL. The aim of this paper is to study the prevalence of the polymorphisms previously described in p53, VEGF, IL-10, IL-11 and APOE in RIF and RPL patients that determines the risk for these pathologies.

Materials and methods

Study population

RPL and RIF patients were selected according to current definitions, previously discarding any other cause for RPL and RIF in the couple by using ultrasound, hysteroscopy, karyotype, thrombophilia or sperm FISH. All the subjects included in the study gave their informed consent to collection of peripheral blood samples suitable for molecular analysis and this was approved by the Instituto Bernabeu Institutional Review Board (Ref: RL01). Oocyte donors, with no history of RIF or RPL, were used as controls. The DNA samples were obtained from a swab and/or blood. To evaluate the prevalence of SNPs in p53, VEGF, IL-10, IL-11 and APOE genes and their effect in RIF and RPL patients, we designed a retrospective case control study. Two hundred and fifty-five women were selected and genotyped: 89 RPL patients, 77 RIF patients and 89 controls without fertility problems.

Genotyping

Taqman® Life Technologies allelic discrimination real time PCR was used for genotyping (rs1042522 p53 R72P; rs1800896 IL-11 -1082AG; rs1570360 VEGF -1154 AG; rs11668344 IL-10; rs429358 APOE R112C; rs7412 APOE R158C) following the instructions of the manufacturer.

Statistical analysis

The genotyping results were analyzed using SPSS v20.0. To determine significant differences between groups, Pearson’s Chi-square was used accepting $p < 0.05$ for significant values and the odds ratio were controlled with binary logistic regression. RIF, RPL and controls were considered as separate groups in order to compare them.
Results

The allelic frequencies of the different genetic variants studied are shown in Figure 1. Statistically significant differences were found in the prevalence of the R72P polymorphism of *p53* gene and the E4 isoform of *APOE* gene (*p* < 0.05) in RIF and RPL patients. Regarding the studied polymorphism in *VEGF*, *IL-10* and *IL-11* polymorphism showed no differences in the distribution of genotypes.
To investigate the influence of the different allele of each polymorphism we analyzed the pattern of inheritance (dominant vs recessive) for RPL and RIF patients. For RPL patients the E4 variant of the APOE gene is associated with an increased risk of recurrent miscarriage odd ratio 2.648 (CI 95% 1.238–5.667).

Moreover, the recessive model of p53 polymorphism (RR/RP vs PP) and the dominant model of IL-11 (AA/AG vs GG) showed that the PP genotype for the p53 gene and the GG for the IL-11 are increased in RPL patients without reaching statistical significance (Table 1).

For RIF patients, the dominant model of the p53 polymorphism (RR vs RP/PP) and VEGF (GG vs GA/AA) show a different distribution which is associated with an increased risk of implantation failure. The odds ratio of 2.778 (CI 95% 1.477–5.208) is obtained for the p53 polymorphism and 1.842 (CI 95% 1.002–3.422) for VEGF in RIF patients Table 2.

Discussion

Repeated miscarriage and implantation failure are two of the major challenges that reproductive medicine faces due to the multiple factors that can cause them. This requires a multidisciplinary approach for the diagnosis and treatment of these couples. There is a percentage of RPL and RIF patients for which the diagnosis and prognosis from current knowledge is limited. Therefore, the identification of genetic variants that predispose to RIF and RPL opens new areas of research to improve the reproductive success of these patients.

In this paper, we have studied the prevalence of certain genetic polymorphisms in RPL and RIF patients with the aim of identifying potential risk factors. To our knowledge, it is the first study where a genetic profile including different genetic variants for RIF and RPL have been evaluated in the same patient. Our results reveal that the R72P polymorphism in the p53 gene, the GG in the IL-11 and the E4 isoform in the APOE gene are more prevalent in RPL patients.

Regarding RIF patients, in addition to the p53 polymorphism, we observed a different distribution in the -1154 polymorphism -AA/AG of VEGF gene compared with controls. These results are relevant for the diagnosis of RPL and RIF patients, but they also show that although these two pathologies share some common aspects, there are other totally different factors that make them be treated as separate entities.

Embryo implantation is a complex process that requires a perfect balance between completely opposed mechanisms as apoptotic and cell proliferation processes, as well as inflammatory and anti-inflammatory processes that allow a correct maternal-fetal immunological tolerance and adequate tissue invasion and angiogenesis. In these processes, adequate expression levels of the proteins involved in the different mechanisms, as well as functional proteins, are required, so any genetic variant may alter the embryo implantation and fetal development.

Our data suggest an association between proline variant in position 72 of p53 protein and RPL and RIF. The proline codon at position 72 results in a protein that induces high levels of G1 arrest and therefore a decrease in cell proliferation (Hu, Feng, & Levine, 2009), yielding inadequate trophoblastic growth resulting in RPL or RIF. Likewise, p53 regulates the LIF expression which is important for proper endometrial receptivity. The proline variant in position 72 has a lower ability to induce the expression of LIF, causing lower implantation rates (Kang et al., 2009).

The correlation observed in our patients with the APOE E4 gene variant agrees with previous results reported in different meta-analysis (J. Li et al., 2014). The increased thrombotic risk produced by the E4 variant as well as a reduction of inflammatory cytokines could explain the aetiology of the relationship between the E4 variant of the APOE gene and RPL (Korkmazer, Ustunyurt, Tekin, & Cilingir, 2013). Cytokines, as well as angiogenic factors, play an

| Table 1. Genotype and allele frequencies for SNPs in p53, IL-11 and APOE in RPL patients. |
|-----------------|----------------|----------------|----------------|
|                 | CONTROL (n = 89) | RPL (n = 89) | p   | OR (95% CI) |
| p53 RR/RP (%)   | 93.3           | 86.5         | 0.136 | 2.156 (0.771–60.26) |
| PP (%)          | 6.7            | 13.5         |      |            |
| APOE E2E2/E2E3/E3E3 (%) | 86.5 | 70.8 | 0.010 | 2.648 (1.238–5.667) |
|                 | 13.5           | 29.2         |      |            |
| IL-11 AA/AG (%) | 93.3           | 86.5         | 0.136 | 2.156 (0.771–60.26) |
| GG (%)          | 6.7            | 13.5         |      |            |

| Table 2. Genotype and allele frequencies for SNPs in p53 and VEGF in RIF patients. |
|-------------------------------|-----------------|----------------|----------------|
|                              | CONTROL (n = 89) | RIF (n = 77) | p   | OR (95% CI) |
| p53 RR (%)                   | 65.2            | 40.3          | 0.001 | 2.778 (1.447–5.208) |
| RP/PP (%)                    | 34.8            | 59.7          |      |            |
| VEGF AA/AG (%)               | 61.8            | 46.8          | 0.048 | 1.842 (1.002–3.422) |
| GG (%)                       | 38.2            | 53.2          |      |            |
important role in modulating the maternal-fetal immune tolerance and the establishment of the vascular network. The genetic variants responsible for modifying the expression of the genes encoding these proteins will affect their own levels and therefore the mechanisms associated with them. In this sense, the IL-11 polymorphism (Saxena et al., 2015) as well as VEGF polymorphism (Magdoud et al., 2012), would produce insufficient levels of proteins that would cause difficulties for embryo implantation and/or the establishment of pregnancy.

Our results suggest patients carrying variants in p53 and VEGF would be at risk of RIF, and those carrying variants in APOE gene would suffer from RPL. To corroborate these results, prospective cohort studies with higher sample size are needed. In conclusion, this study has identified genetic variants associated with RIF and RPL. This information, together with additional markers, could allow the development of diagnostic tests to detect the risk of RIF and RPL and set the prognosis of the cycle before starting the treatment.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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