GENETICS

Association between estrogen receptor 1 (*ESR1*) and leukemia inhibitory factor (*LIF*) polymorphisms can help in the prediction of recurrent implantation failure

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Objective: To investigate an association between polymorphisms related to the implantation process that together could help in the prediction of recurrent implantation failure (RIF).
Design: Cohort study.
Setting: Private fertility center and reproductive genetics laboratory.
Patient(s): Forty-four women presenting RIF, who were included in study group (RIF group), and two control groups, one with 63 women who were attended at our service and became pregnant after the first IVF/intracytoplasmic sperm injection attempt (control group I) and other with 65 fertile women who had at least two children without any treatment and no history of miscarriage (control group II).
Intervention(s): None.
Main Outcome Measure(s): Genotyping was performed in the intron region of *TP63*, *VEGFA*, *MMP2*, *ESR1*, and *ESR2* genes and in the 3′ untranslated region of the *LIF* gene on genomic DNA using real-time polymerase chain reaction.
Result(s): The presence of *ESR1/AA* (rs12199722) and *LIF/GT* (rs929271) genotypes was more frequent in the RIF group, leading to a 7.9-fold increase in the chance of women presenting with RIF when compared with women who became pregnant on their first cycle of IVF/intracytoplasmic sperm injection and a 2.8-fold increase when compared with women who became pregnant without treatment.
Conclusion(s): The association between *ESR1* and *LIF* polymorphisms can help in the prediction of RIF. (Fertil Steril® 2018;■:■–■. ©2018 by American Society for Reproductive Medicine.)
Key Words: *ESR1*, *LIF*, recurrent implantation failure (RIF), IVF/ICSI

Implantation is a multifactorial event in which the embryo attaches itself to the luminal surface of the endometrium, followed by migration and invasion into its deep layer, becoming closely connected to the maternal endometrial surface to form the placenta (1). This poorly understood system represents a critical step in the reproductive process (2), and it depends on the interplay between the blastocyst and the endometrium (3). A receptive endometrium, a normal and functional embryo at the blastocyst developmental stage, and a synchronized dialogue between maternal and embryonic tissues are required for successful implantation (3). Implantation failure is considered to have occurred if there is a lack of ultrasonography evidence of an

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intrauterine gestational sac. It may occur very early (during attachment or migration states) or later, after successful migration of the embryo through the luminal surface of the endometrium, with the disruption of the process before the formation of an intrauterine gestational sac, owing to embryo or endometrial factors (4).

Recurrent implantation failure (RIF) is a clinical condition in which implantation has repeatedly failed to reach a stage recognizable by ultrasonography (4, 5). Although RIF is the most common cause of unsuccessful pregnancy after assisted reproductive technology, there is no consensus on its definition, despite many publications on this topic (4–9). There are many causes of RIF, among which we can mention gamete/embryo factors (oocyte quality, sperm quality, parental chromosomal anomalies), maternal age, uterine factors (congenital uterine anomalies, acquired intracavity conditions), hydrosalpinx, immunologic factors, thrombophilic conditions, hormonal or metabolic disorders, and genetic abnormalities (4–7). In women with unexplained implantation failure, despite good hormonal response, good-quality embryos, satisfactory endometrial development, and no identifiable pathology, suboptimal endometrial receptivity is considered a key factor in inhibiting embryo implantation (2). The implantation process requires the interaction of at least four types of cells: stromal, epithelial, endothelial, and immune cells (10).

Because genetic factors seem to be highly associated with RIF (11), a growing number of candidate genes have been investigated concerning their potential roles in that etiology. Single nucleotide polymorphisms (SNPs) have being used as markers owing to their large number in virtually all populations, improving the ability to diagnose RIF, thereby increasing a couple's chance of conception. In preliminary analysis, we selected some reproduction-related genes to identify SNPs that could be important to the implantation process. Using next-generation sequencing we identified six polymorphisms that were somehow related to RIF and were used in this study: tumor protein p63 (*TP63*) rs12486772, vascular endothelial growth factor A (*VEGFA*) rs3025010, matrix metallopeptidase 2 (*MMP2*) rs2287076, estrogen receptor 1 (*ESR1*) rs12199722, estrogen receptor 2 (*ESR2*) rs1952585, and leukemia inhibitory factor (*LIF*) rs929271.

It is known that the *TP63* gene is a regulator of the quality and maturation of oocytes (12). In addition, Feng et al. (13) suggested that TP63 may have distinct functions in reproduction other than maintaining female germ cell integrity, because they identified an association of one SNP with both young and older patients. The *VEGFA* gene is important for almost every aspect of blood vessel formation and function, mediating angiogenesis and vasculogenesis (14). It has been shown that polymorphisms in the *VEGFA* gene correlate with variation in VEGFA protein production (15), which could result in decreased angiogenesis and blastocyst invasion, leading to implantation failure. The *MMP2* gene belongs to a family of zinc-dependent proteinases (MMPs) that can be found widely distributed in human tissues. They are able to degrade nearly all extracellular matrix proteins and are necessary for cell migration, the healing process, development, scar formation, and other tissue changes (16). They also play important roles in reproductive physiology, remodeling extracellular matrix during ovarian follicular growth and ovulation (17). *ESR1* and *ESR2* genes are responsible for codifying two estrogen receptors (ERα and ERβ, respectively), which mediate estrogen (E) effects that are well known on follicle growth, maturation, oocyte release (18), and endometrial preparation for implantation (19). The LIF protein is an important mediator of embryo implantation (20), and the influence of polymorphisms in the *LIF* gene has been analyzed by several groups, helping in the understanding of the implantation process. High concentration of LIF in follicular fluid has been correlated with good embryo quality, suggesting its role in ovulation and early embryonic development (21). It was also associated with embryo implantation and endometrial receptivity, being part of a highly coordinated orchestra of the reproductive process (22). Levels of LIF protein are increased at the time of expected implantation in fertile women (23). On the other hand, lower LIF levels have been associated with women presenting with unexplained infertility (24, 25).

Genetic factors combined or alone can affect the implantation process; therefore, our objective was to identify the best association between six polymorphisms (*TP63* rs12486772, *VEGFA* rs3025010, *MMP2* rs2287076, *ESR1* rs12199722, *ESR2* rs1952585, and *LIF* rs929271) that could help in the prediction of RIF.

MATERIALS AND METHODS

Ethical Approval

The study was authorized by the local ethics committee (the Ethics Committee in Research of the Reference Center for Women Health; project reference: 045/11) and was carried out according to the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from all recruited subjects.

Study Population

A cohort study was conducted with blood samples from 44 infertile women (aged 35.3 $\pm$ 2.6 years [mean $\pm$ SD]) presenting RIF and two different control groups: group I, 63 women (aged 33.3 $\pm$ 4.1 years); and group II, 65 women (aged 52.2 $\pm$ 10.7 years). The study population consisted of patients who were subjected to IVF/intracytoplasmic sperm injection (ICSI) protocols recruited at our Human Reproduction Center between January 2014 and December 2016 (RIF group). Recurrent implantation failure was defined as failure to achieve pregnancy after three or more IVF/ICSI attempts and five or more cleavage-stage good morphological quality embryos transferred (4, 5). All women were $\leq$39 year old (at the time of the ETs) and had normal couple karyotype. The exclusion criteria were defined as the presence of uterine defects by ultrasound, hysterosalpingography, and hysteroscopy, evidence of hydrosalpinx by ultrasonography, infections, endocrine problems, coagulation defects or thrombophilia, and autoimmune defects (including antiphospholipid anti-bodies).

The enrolment criteria for the control group I included patients who were subject to IVF/ICSI protocols at the same center and became pregnant on the first attempt and had at least one live birth. Control group II included fertile women (postmenopausal volunteers) with no history of miscarriage or infertility treatment who had at least two live births. The inclusion criteria of postmenopausal volunteers had the purpose to avoid possible miscarriages or future infertility problems after recruitment for the study. These inclusion criteria were based on published research (26, 27). None of the patients in the study group or control group I underwent PGS/PGTA (preimplantation genetic screening/preimplantation genetic testing for aneuploidy) in their treatments.

All women recruited for this study were Brazilian, from all over the country. Despite the high rate of interracial marriage in the Brazilian population, most patients and healthy study participants described their skin color as "white."

DNA Samples and Genotyping

Genomic DNA for the entire studied population was extracted from peripheral blood samples using the QIAamp DNA blood mini kit (Qiagen), following the manufacturer's instructions.

Nucleotide changes were evaluated in duplicate by real-time polymerase chain reaction (PCR) using individual TaqMan SNP genotyping assays (Thermo Fisher) for each SNP (*TP63* rs12486772, *VEGFA* rs3025010, *MMP2* rs2287076, *ESR1* rs12199722, *ESR2* rs1952585, and *LIF* rs929271) and TaqPath ProAmp Master Mix, following the manufacturer's instructions, on a StepOne Real-time PCR System. The PCR conditions were as follows: 60°C for 30 seconds (pre-read); 95°C for 5 minutes (initial denature, enzyme activation), 40 cycles of 95°C for 15 seconds (denature), and 60°C for 1 minute (anneal/extend). Genotyping results were validated and confirmed by the automatic sequencer XL 3500 Genetic Analyzer (Applied Biosystems) using 20 samples of each genotype from each polymorphism (normal homozygous, heterozygous, and mutated homozygous) selected randomly. For the minor allele frequency of each polymorphism, all genotypes were sequenced.

Statistical Analysis

All data were analyzed using StatsDirect statistical software version 2.7.9 software. The following parameters were evaluated for each group analyzed: the woman's age, body mass index, time of infertility, cause of infertility, semen parameters, endometrial thickness, total dose of recombinant FSH, number of oocytes retrieved, number of oocytes retrieved in metaphase II, and fertilization rate. The differences in the frequencies of the SNPs genotypes, alleles, or both, in the RIF group and control groups were also evaluated.

To compare the means of continuous variables, the nonparametric Mann-Whitney test was used if the continuous variables were not normally distributed, and Student's *t* test and one-way analysis of variance were used if the continuous variables were normally distributed. The results are expressed as the arithmetic mean ± SD. For categorical variables, Fisher's exact test was used, and the results are expressed as percentages. In addition, logistic regression analysis was used to determine a significant association between all polymorphisms as a tool in the prediction of RIF. All statistical tests were considered significant at a level of $P<.05$.

RESULTS

Table 1 represents the main characteristics of study participants. The genotype and allele frequencies of each SNP (*TP63* rs12486772, *VEGFA* rs3025010, *MMP2* rs2287076, *ESR2* rs1952585, *ESR1* rs12199722, and *LIF* rs929271) are presented in Table 2. The *TP63* polymorphism showed no significant difference between the studied groups. Although there was no difference between the *VEGFA* genotypes (rs3025010), the T allele was more frequent in control group II. *MMP2/TC* genotype frequency was significantly higher in the RIF group compared with control group II. The *ESR2/AG* genotype was more frequent in control group II. The *ESR1/AA* genotype was significantly higher in the RIF group when compared with both control groups. On the other hand, the *ESR1/AG* genotype was significantly higher in both control groups. In the RIF group, the frequency of the *LIF/GT* genotype was significantly higher when compared with control group I; however, the *LIF/GG* genotype was significantly higher in control group I. When these six polymorphisms were used in combination with each other, we found that the association of the *LIF/GT* and *ESR1/AA* genotypes had a 7.9-fold increase in the chance of women being included in the RIF group when compared with women in control group I and a 2.8-fold increase when compared with women in control group II (Table 3). The other SNPs combinations did not show a significant association.

DISCUSSION

Because RIF is a clinical condition caused by diverse genetic and environmental factors, SNPs in some of these genes may affect individual susceptibility to RIF at different levels (28). This study demonstrated that, of the six polymorphisms investigated here, three showed a significant association with women presenting RIF when analyzed one by one.

It has already been shown that *TP63* is associated with different reproductive functions (12, 13). Additionally, an association of one SNP (rs12486772) of this gene and women presenting RIF was shown in our preliminary analysis using next-generation sequencing, being the reason for using this SNP in this work. Here we did not find any association between the *TP63* polymorphism (A>G, rs12486772) and the three groups studied, perhaps owing to the limited number of women involved in this study.

In relation to the *VEGFA* gene, we have previously reported an association of the *VEGFA -1154 G/A* (rs1570360) polymorphism and RIF (29), but in the present study the SNP *VEGFA* (*T>C*, rs3025010) was not correlated to the group of women presenting RIF, even though an association between the T allele and women who had a child without any fertility treatment was identified.

MMP2 has been demonstrated to act in the implantation window (period of maximal endometrial receptivity, 6–9 days after ovulation), and women presenting idiopathic infertility

TABLE 1

Main characteristics of patients presenting RIF and control groups (I and II).

Characteristic	RIF group (n = 44)	Control group I (n = 63)	Control group II (n = 65)	*P* value
Age (y)	35.3 ± 2.6[a,b]	33.3 ± 4.1[a,c]	52.2 ± 10.7[b,c]	.004[a] <.0001[b,c]
Total of ET	11.6 ± 5.8	2.0 ± 0.4	NA	<.0001
Total of implantation failure	4.8 ± 1.8	0	NA	–
BMI (kg/m^2)	23.0 ± 4.9[a,b]	24.0 ± 4.0[a,c]	28.1 ± 4.6[b,c]	.46[a] .22[b] .26[c]
Time of infertility (y)	4.6 ± 2.9	4.1 ± 3.0	NA	.25
Cause of infertility				
Idiopathic	16 (36.4)	17 (27.0)	NA	–
Male	13 (29.5)	26 (41.2)	NA	–
Endometriosis	5 (11.4)	8 (12.7)	NA	–
Tuboperitoneal	5 (11.4)	8 (12.7)	NA	.81
Tuboperitoneal + endometriosis	2 (4.5)	1 (1.6)	NA	–
Male + endometriosis	2 (4.5)	2 (3.2)	NA	–
Male + tuboperitoneal	1 (2.3)	1 (1.6)	NA	–
Semen parameters				
Total sperm count (×10^6/mL)[d]	74.4 ± 55.3	62.8 ± 57.9	NA	.25
Motility (%)[d]	58.6 ± 20.4	56.8 ± 15.6	NA	.11
Normal spermatozoa (%)[e]	0.7 ± 1.0	0.5 ± 0.7	NA	.51
DNA fragmentation (%)[f]	13.0 ± 6.6	13.7 ± 8.3	NA	.86
Underprotamination (%)[g]	57.7 ± 13.4	57.7 ± 13.6	NA	.96
Apoptosis (%)[h]	18.4 ± 5.5	20.6 ± 6.3	NA	.13
Endometrial thickness (mm)	10.7 ± 3.0	11.5 ± 3.2	NA	.62
Total dose r-FSH (IU)	2,181 ± 719	2,032 ± 651	NA	.25
Retrieved oocytes (n)	9.1 ± 5.4	10.2 ± 5.7	NA	.44
Oocytes in metaphase II (n)	7.0 ± 4.1	7.7 ± 4.3	NA	.89
Fertilization rate (%)	73.4 ± 19.4	74.4 ± 20	NA	.91

Note: Values are mean ± standard deviation or number (percentage). AFC = antral follicle count; BMI = body mass index; AMH = antimüllerian hormone; NA = not applicable.

[a] Comparison between RIF group and Control group I.
[b] Comparison between RIF group and Control group II.
[c] Comparison between Control group I and Control group II.
[d] According to the World Health Organization (39).
[e] According to motile sperm organelle morphology examination (40).
[f] The percentages of DNA fragmentation by TUNEL assay.
[g] Abnormal chromatin packaging by chromomycin A3.
[h] Apoptosis by Annexin-V.

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have higher expression and activity of it (30). In our study, we showed that women with the *MMP2/TC* genotype (*T*>*C*, rs2287076) had a higher chance of presenting RIF when compared with women who had children without any fertility treatment.

Additionally, we found that patients with the *ESR1/AA* genotype (*A*>*G*, rs12199722) tend to present RIF, and women with the *ESR1/AG* genotype are more likely to have children after the first IVF/ICSI cycle or with no treatment. The *ESR2*/AG genotype (rs1952585) was prevalent in women who had a child without treatment. The uterus is a primary target of estrogen for various functions during the reproductive cycle and pregnancy; estrogen activates a wide array of tissue- and organ-specific physiologic responses by binding to its receptor, and modulating uterine events, preparing the endometrium for embryo attachment and implantation (31). Single nucleotide polymorphisms in these genes could modify estrogen action, modifying the way the endocrine system acts in the implantation process. Gene-array analysis revealed more than 300 affected genes in patients with IVF failure, 8% of them being estrogen-dependent (32). We did not find any other study associating these polymorphisms and infertility, although its association has been already analyzed with some others disorders, such as restrictive anorexia nervosa (33) and colorectal cancer (34).

In our previous study we found an association between the *LIF* (rs929271) polymorphism and implantation efficiency and pregnancy outcomes (35). In this study we strengthen the information that women with the *GG* genotype have more chance of becoming pregnant after the first attempt of IVF/ICSI, and women with the *GT* genotype tend to present RIF.

Five of the six polymorphisms studied here were located in intronic regions (*TP63* rs12486772, *VEGFA* rs3025010, *MMP2* rs2287076, *ESR1* rs12199722, and *ESR2* rs1952585). Evidence of different studies suggests that some intronic regions contain transcription factor binding sites that may be important for the regulation of expression and/or alternative splicing of the genes (36). This untranslated regions affects *cis*-acting elements that control messenger RNA stability, translation efficiency (including micro-RNA biding sites), and messenger RNA localization (37). The last polymorphism (*LIF* rs929271) was located in the 3′ untranslated region, which may affect the gene expression, transcription factor

TABLE 2

Genotype and allele frequencies.

SNP	RIF group (n = 44)	Control group I (n = 63)	*P* value	RIF group (n = 44)	Control group II (n = 65)	*P* value
TP63 (rs12486772)						
Genotype						
AA	30 (68.2)	40 (63.5)	.26	30 (68.2)	35 (53.8)	.29
AG	11 (25.0)	22 (34.9)		11 (25.0)	26 (40.0)	
GG	03 (6.8)	01 (1.6)		03 (6.8)	04 (6.2)	
Allele			>.99			.31
A	71 (80.7)	102 (81)		71 (80.7)	96 (73.8)	
G	17 (19.3)	24 (19)		17 (19.3)	34 (26.2)	
VEGFA (rs3025010)						
Genotype			.17			.06
TT	14 (31.8)	22 (34.9)		14 (31.8)	35 (53.9)	
CT	19 (43.2)	34 (54.0)		19 (43.2)	22 (33.8)	
CC	11 (25.0)	07 (11.1)		11 (25.0)	08 (12.3)	
Allele			.27			.01
T	47 (53.4)	78 (61.9)		47 (53.4)	92 (70.8)	
C	41 (46.6)	48 (38.1)		41 (46.6)	38 (29.2)	
MMP2 (rs2287076)						
Genotype			.36			.01
TT	18 (40.9)	19 (30.2)		18 (40.9)	39 (60.0)	
TC	24 (54.6)	37 (58.7)		24 (54.6)	17 (26.2)	
CC	02 (4.5)	07 (11.1)		02 (4.5)	09 (13.8)	
Allele			.25			.53
T	60 (68.2)	75 (59.5)		60 (68.2)	95 (73.1)	
C	28 (31.8)	51 (40.5)		28 (31.8)	35 (26.9)	
ESR2 (rs1952585)						
Genotype			.51			.02
AA	34 (77.3)	46 (73.0)		34 (77.3)	40 (61.5)	
AG	08 (18.2)	16 (25.4)		08 (18.2)	25 (38.5)	
GG	02 (4.5)	01 (1.6)		02 (4.5)	00 (0.0)	
Allele			>.99			.37
A	76 (86.4)	108 (85.7)		76 (86.4)	105 (80.8)	
G	12 (13.6)	18 (14.3)		12 (13.6)	25 (19.2)	
ESR1 (rs12199722)						
Genotype			.02			.01
AA	28 (63.6)	25 (39.7)		28 (63.6)	22 (33.8)	
AG	13 (29.6)	35 (55.5)		13 (29.6)	33 (50.8)	
GG	03 (6.8)	03 (4.8)		03 (6.8)	10 (15.4)	
Allele			.11			.005
A	69 (78.4)	85 (67.5)		69 (78.4)	77 (59.2)	
G	19 (21.6)	41 (32.5)		19 (21.6)	53 (40.8)	
LIF (rs929271)						
Genotype			.003			.05
TT	10 (22.7)	24 (38.1)		10 (22.7)	29 (44.6)	
GT	32 (72.7)	26 (41.3)		32 (72.7)	33 (50.8)	
GG	02 (4.6)	13 (20.6)		02 (4.6)	03 (4.6)	
Allele			>.99			.13
T	52 (59.1)	74 (58.7)		52 (59.1)	91 (70.0)	
G	36 (40.9)	52 (41.3)		36 (40.9)	39 (30.0)	

Note: Values are number (percentage).

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biding, sequencing of RNA that is noncoding, and gene splicing (38). Although we do not know exactly the way each polymorphism acts, we could see that they affect in some way the function of the gene. Gene expression studies may help elucidate how each of these polymorphisms may influence the action of the genes.

Even though the *VEGFA* (rs3025010), *MMP2* (rs2287076), and *ESR2* (rs1952585) polymorphisms showed significant difference when analyzed separately, the association between them and other polymorphisms studied here did not present a significant difference, and further studies with related genes are necessary for a better understanding of this genetic pathway that may influence endometrial receptivity. However, the association between the *LIF* (rs929271) and *ESR1* (rs12199722) polymorphisms seemed to be significantly related to RIF, with a 7.9-fold increase in the chance of women present RIF when compared with women with background of successful IVF/ICSI treatment and 2.8-fold increase when compared with women with spontaneous fertility. This association, after further studies to confirm the results, could be used as an extra tool by physicians to indicate alternative treatments, such as oocyte or embryo donation. To the best of our knowledge, this is the first study that shows that this association can influence the implantation process.

TABLE 3

Association between *ESR1* (12199722) and *LIF* (929271) polymorphisms in the risk of RIF.

Variable	RIF group (n = 44)	Control group I (n = 63)	*P* Value	OR	95% CI
ESR1/AA + LIF/GT	20 (45.5)	06 (9.5)	< .0001	7.9	2.6–26.6
Other genotypes combinations	24 (54.5)	57 (90.5)			
	RIF group (n = 44)	Control group II (n = 65)			
ESR1/AA + LIF/GT	20 (45.5)	15 (23.0)	.02	2.8	1.1–6.9
Other genotypes combinations	24 (54.5)	50 (77.0)			

Note: Values are number (percentage). CI = confidence interval; OR = odds ratio.

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In conclusion, in this work we demonstrated that there is an association between the *ESR1* (rs12199722) and *LIF* (rs929271) polymorphisms and women presenting RIF. Although isolated SNPs were more significant among women with a background of RIF compared with women with spontaneous fertility, the *ESR1*/*LIF* association was stronger between women with a background of RIF compared with women with a background of successful IVF/ICSI treatment. Because the basic idea is to increase the chance of attaining a correct diagnosis of RIF, further validation of this association is required (i.e., increasing the number of cases between other fertility centers; expanding to different ethnic groups) to provide more information regarding the potential use of it. Additionally, the understanding of the way that each polymorphism acts in the implantation process would be enlightened with studies of gene expression.

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