The LEPR (853A>G and 511A>G) Transitions may Enhance Idiopathic Recurrent Miscarriage: Evidences Based on Case-control and *in silico* Studies

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Abstract

Previous studies in human leptin receptor protein (LEPR) signaling are important in the establishment of fetal growth. Idiopathic recurrent miscarriage (IRM) may be the result of abnormal placental and fetal development. Thus single nucleotide polymorphisms (SNPs) of LEPR might be associated with IRM. In our case-control study, which conducted from 2017 to 2018 at the Milad Sari Genetic Detection Center and Razi Hospital (Ghaemshahr, Iran), 140 samples, including 70 cases with history of three or more IRM as before the 22nd week of gestation, and 70 controls with at least two live births and no history of pathologic pregnancies during reproductive period were studied. Polymorphisms of maternal LEPR 853A>G and 511A>G were assessed by PCR-RFLP and SSCP, respectively. Results showed that 853A>G SNP, contained frequent genotype AG (p= 0.002; OR= 0.391; 95% CI= 0.154-0.664) and G allele (p= 0.003; OR= 0.125; 95% CI= 0.032–0.489), revealed a significant protective association with IRM. Primary screening of 511A>G showed that 63 case-samples were AG genotype. PCR directed sequence showed this SNP contained frequent genotype for AG (p= 0.001; OR= 0.57; 95% CI= 0.22-0.147) and G allele (p= 0.006; OR= 0.34; 95% CI= 0.008–0.149), revealed a significant protective association with IRM. Based on our findings, LEPR (853A>G and 511A>G) gene transitions not only might enhance IRM but also could be useful genetic markers in susceptibility and severity of recurrent miscarriage.

Introduction

Idiopathic recurrent miscarriage is a common problem that affects 10 to 15% of known clinical pregnancies (Yan et al., 2012). It has been reported that 0.5 to 1% of couples suffer from idiopathic recurrent miscarriage, and in 40 to 50% of them, the reason is unknown (Chin et al., 2013). Idiopathic recurrent miscarriage (IRM) or idiopathic recurrent spontaneous abortion (IRSA) is determined as the loss of gestation before 20 weeks (Matthiesen et al., 2012), that defined by two or three continuous miscarriage (Kolte et al., 2015; Silver et al., 2011). The factors involved can be referred to infections (Nigro et al., 2011), antiphospholipid syndromes (Da et al., 2017), maternal disease (Yang et al., 2017), thrombophilias (Kar et al., 2017), genetic disorder (AlShaikh et al., 2011). Some of the genes involved in miscarriage including: Zpi (Pang et al., 2013), Vegf (Messaoudi et al., 2013), Stat3 (Su et al., 2011), Progen (Shin et al., 2013), Nos3 (Fraga et al., 2014), P53 (Shahsavari et al., 2015), Kdr (Muller et al., 2013), and LEPR (Dias et al., 2012). The leptin receptor protein (LEPR), which known as OB-R, and CD295 (Baumann et al., 1996), is located on the

choromosom1p31.3 with 24 exons (Bartha et al., 2005). Leptins, which secreted by the adipose tissue, attached to the receptors which are located in the hypothalamus and follicular-ovarian cells. These peptide hormones regulate homeostasis of energy (Considine et al., 1996). The LEPR protein categorized as interleukin-6 from the family of type 1 cytokines with six isoforms: LEPR-a, b, c, d, e, and f (Zhou et al., 2015). Isoforms of LEPR-a, c, d, and f are short but LEPR-b isoform is long. The LEPR gene involves in some diseases including: type2 diabetes mellitus (Wang et al., 2017), breast cancer (Cleveland et al., 2010), prostate cancer (Alshaker et al., 2015), lung cancer (Unsal et al., 2014) and idiopathic recurrent miscarriage (Muller et al., 2016). Previous studies have reported that the role of LEPR in idiopathic recurrent miscarriage could vary among different populations. Expression of LEPR-b and LEPR-a are effective in stimulation of LH in the rat ovaries (Ramirez et al., 2017). Leptin receptor has been known in the vascular endothelial cells of a fetal vessel of chorionic villi during the early pregnancy (Muhlhauser et al., 1996). Researchers have also suggested that single nucleotide polymorphisms (SNPs) of the LEPR 853A>G (as

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Table 1. Oligomers used as primers and PCR conditions.

SNP	Primers Names	Oligomers (5'→ 3')	Cycles and thermal conditions	PCR product
853A>G (rs1137101)	*LEPR1-f* *LEPR1-r*	5'-TCCTGCTTTAAAAGCCTATCC 5'-GTAGATGTAGTACTGGGGGTA	35 cycles of 94°C for 5 min, 94°C for 30s, 58°C for 30s, and 72°C for 30s; final extension at 72°C for 5 min	417 bp
511A>G (rs1137100)	*LEPR2-f* *LEPR2-r*	5'- CTTTTGCCTGCTGGACTCTC 5'- AAACTAAAGAATTTACTGTTGAAACAAATGTC	35 cycles of 94°C for 5 min, 94°C for 30s, 58°C for 30s, and 72°C for 30s; final extension at 72°C for 5 min	217 bp

known: Gln223Arg; A177266G; 853Q>R; rs1137101) in the exon 7 (Figure 1A) and LEPR 511A>G (as known as: Lys109Arg; A155194G; 511A>G; rs1137100) in exon 5 (Figure 1A) can be related to idiopathic recurrent miscarriage (Muller et al., 2016; Riestra et al., 2011). SNPs in the translation process can cause different structural changes in the mRNA (Ng and Henikoff et al., 2006). The nonsynonymous single nucleotide polymorphism (nsSNP) is as a gene variation which can affect in the structure and stability, ligand binding specifications, catalysis and post-translational modification (PTM), protein-protein interaction (PPI) and hydrophobicity content of proteins (Aftabi et al., 2016; David et al., 2012). In biomedical sciences, the current significance and effectiveness of bioinformatics and silica tools have recently been studied (Mehmood et al., 2014; Chou et al., 2015). In the present study, the association of 853A>G and 511A>G with idiopathic recurrent miscarriage and *in silico* analysis were investigated.

Materials and Methods

Blood sample collection

In a case-control trial for a total of 140 women, citrate blood samples were collected from 70 cases with 20-45 years old, with history of ≥ 3 successive miscarriages; and 70 controls, with 19-50 years old, with history of ≥ 3 successful gestations. In the case group, patients with a known cause of pregnancy loss (chromosomal impairment, chronic disease, infection, hormonal defect, antiphospholipid antibodies, thromboembolic disease, cervical inadequacy, or other obstetric complications that could cause abortion fetus (such as hypertension, preeclampsia, eclampsia, gestational diabetes mellitus) were excluded (Ramirez et al., 2017). In the control group, all women with a history of abortion fetus, thrombotic changes associated with other obstetric complications, preterm placental ablation, intrauterine fetal death, and chronic diseases were also excluded (Ramirez et al., 2017).

All participants were from Milad Sari Genetic Detection Center and Razi Hospital (Ghaemshahr, Iran), between 2016 up to Aug. 2018. These study approved by the ethics committees of the University of Mazandaran (#IR.UMZ.REC.1397.028) and informed consent was obtained from each subject before participation.

Amplification of SNPs flanking fragments

The genomic DNA was isolated from all blood samples by a commercial DNA extraction kit (Cinnagen Co, Iran). For amplification of LEPR-SNPs flanking fragments, 4 oligomers were designed by Oligo7 software and fragments amplified by PCR program at Mastercycler EP Gradient thermal cycler conditions (Table 1). All of the PCR reactions were performed at a final volume of 25 μl, which consisted of 10 pM of each primer, 0.75 mM of MgCl2, 0.2 mM of mix-dNTPs and 0.06 U of *Taq* DNA polymerase.

PCR-RFLP and SNP genotyping

LEPR 853A>G polymorphism was analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method using primers and restriction enzyme. 2.5 μl of PCR products digested with 0.3 U of *Msp*I restriction enzyme (Fermentas Co, Germany) and 1X buffer at 37 °C for 16 h. Digested fragments electrophoresed in 1.5% agarose gel and stained by 1μg/ml ethidium bromide stock solution. Agarose gel electrophoresis and gel staining were performed through Green and Sambrook (2012) methods. At least three PCR direct-sequences products were evaluated (Takapouzist Co., Iran) for verification of PCR-RFLP results.

Statistical analysis

For various alleles and genotypes in case and control groups, odds ratio (OR) with 95% confidence interval (95% CI) was calculated. The
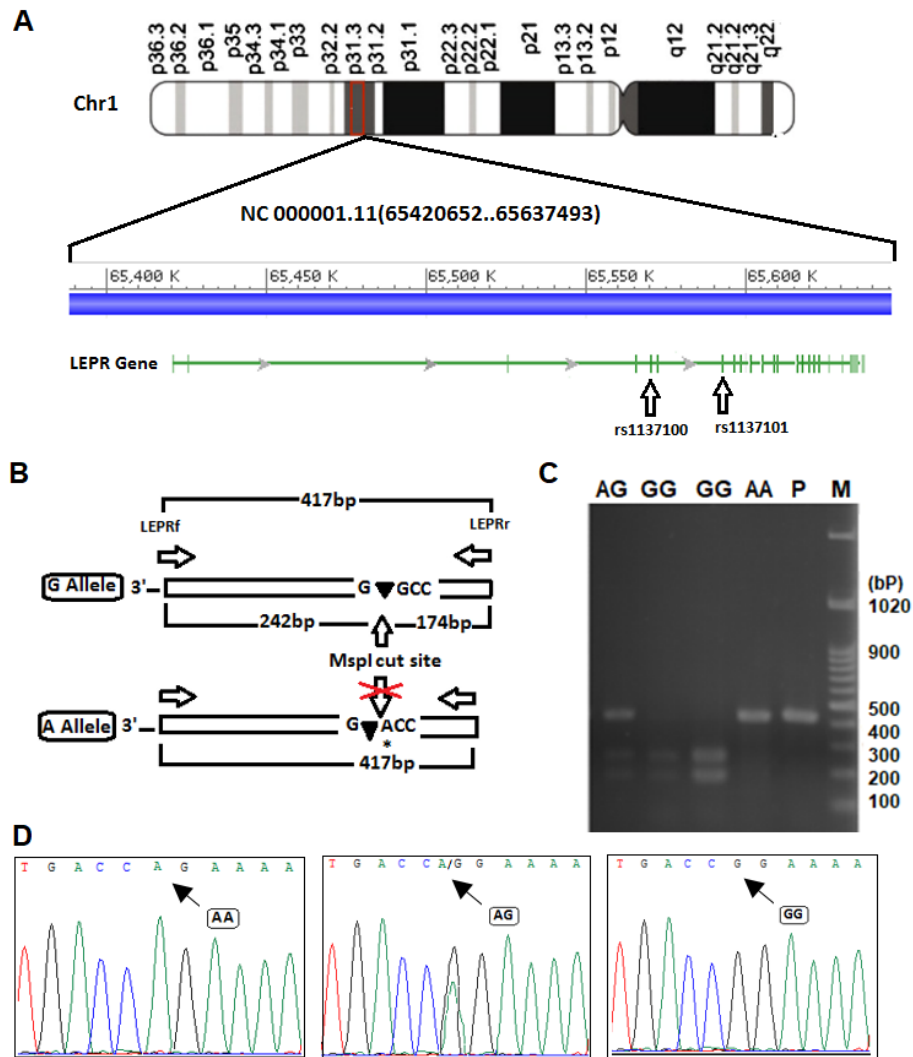
Figure 1. *LEPR* gene map, PCR-RFLP, and DNA sequencing results: (A) Human *LEPR* gene map, from NCBI database, the SNPs of rs1137101 and rs1137100 located in exon7 (position 177266) and exon5 (position 155194), respectively; (B) *Msp*I restriction diagrammatic map of PCR products; (C) *Msp*I restriction map of PCR products in the 1.5% agarose gel electrophorese, which stained by Ethidium bromide, AA, AG and GG lanes indicate genotypes, P= PCR products without *Msp*I restriction enzyme, M= Marker DNA ladder100bp; (D) Electropherogram results of PCR direct sequence which showed genotypes.

Chi-square test was applied to compare the allele and genotype frequencies in the case and control groups. Moreover, Hardy-Weinberg equilibrium (HWE) in both of the case and control groups was tested. The p-value of less than 0.05 was considered as statistically significant. These statistical calculations were performed by SPSS statistical package version 16.

PAGE conditions

Exon 7 PCR fragments were dissected by 12% polyacrylamide gel electrophoresis (PAGE; containing 40 mL polyacrylamide (30%) (29 g, acrylamide:1 g, bis-acrylamide); 5mL of TBE (10X), 350 mL of ammonium persulfate (APS) (10%) and 10 mL of 10% tetramethyl ethylenediamine (TEMED) madeupto100mLwith

double-distilled water in 90V for 4h at 48°C with TBE buffer (0.5X). Then, the polyacrylamide gel (12%) was stained with AgNO₃. Gel electrophoresis and gel staining were performed according to the methods of Green and Sambrook (2012).

SSCP conditions

For single-strand conformation polymorphism (SSCP) analysis of the 511A>G, PCR products were loaded on 10% polyacrylamide gel and after electrophoresis at 120V for 6 h at 4ºC in 1X TBE buffer, visualized by silver nitrate staining based on Green and Sambrook (2012) manual. Preparation of samples for SSCP was performed by 2.5 µl of PCR product which mixed with 7 µl SSCP dye (containing: 9.5 mL Formamide; 400 mL of 20 mM EDTANa2 (pH 8.0) and 100 ml of bromophenol blue

(1%) dissolved in absolute ethanol). This mixture was incubated in the Mastercycler EP for 10 min at 94°C. The samples were then located on a freezer -21°C for 20 min before being loaded in 10% polyacrylamide gel. At least three PCR direct-sequences products were evaluated (Takapouzist Co., Iran) for verification of SSCP results.

In silico analysis

The *in silico* servers were used to describe the possible effects of LEPR (853A>G and LEPR 511A>G) polymorphisms, including the following items: Polyphen2 (http://genetics.bwh.harvard.edu/pph2/): which predicts the probable effects of nsSNPs amino acid on the stability and function of human proteins. HumDiv (human mutation/divergency) and HumVar (human polymorphic variants) are two models on this server (Aftabi et al., 2016); PROVEAN (http://provean.jcvi.org/seq_submit.php): which predicts the effect of replacing the desired amino acid on the biological function of a protein; SNAP (https://www.rostlab.org/services/SNAP/): which is useable for screening of non-acceptable polymorphisms, and predicting the function of mutated protein (Bromberg and Rost, 2007); RNAsnp (http://rth.dk/resources/rnasnp/): which predicts the effect of SNPs on the local RNA secondary structure; dpPTM (http://dbptm.mbc.nctu.edu.tw/index.php): to reports the solvent availability of substrate, protein secondary and tertiary structures, domains and variations (Minguez et al., 2012); Cfssp (http://www.biogem.org/tool/chou-fasman/): for determination of protein secondary structures according to Chou and Fasman algorithm (Chou and Fasman, 1974); ProtParam (https://web.expasy.org/protparam/): to predict the effect of the changes of polymorphism on the primary physicochemical property and the local secondary structure of the protein (Khosronezhad et al., 2015; Aftabi et al., 2016); MUpro (https://www.ics.uci.edu/~baldig/mutation.html): that predicts value and energy change using SVM and sequence information (Cheng et al., 2006); SNPeffect 4.0 (www.snpeffect.switchlab.org): which usually focuses on the molecular specifications and polymorphism types in human proteins (Aftabi et al., 2016); BioGRID (http://thebiogrid.org/): that is a public database which archives and disseminates genetic and protein interplay data from model organisms and human (Chatr-Aryamontri et al., 2014); UbPred (http://www.ubpred.org/help.html): which predicts ubiquitination sites of protein

(Radivojac et al., 2010); SUMOplot™ (http://www.abgent.com/sumoplot): that predicts the possibility of the sumo consensus sequence and its score system is according to two standard of the direct amino acid, in compliance with sumo consensus sequence, and substitution of the consensus residues with amino acids exhibiting similar hydrophobicity (Aftabi et al., 2016).

Results

Genotyping of LEPR-853A>G

The *LEPR*-853A>G flanking fragments of samples were amplified by PCR. These 417 base pairs (bp) fragments were run in 1.5% agarose gel. The *Msp*I restriction pattern of PCR products showed three fragments: 417, 243 and 174bp were AG; without cut fragment was AA, and two fragments (243 and 174 bp) were GG genotypes (Figure 1B & 1C). The PCR-RFLP was verified by PCR direct sequencing of DNA sequences (Figure 1D).

Statistical analysis for 853A>G showed that the frequencies of homozygous (GG) and heterozygous (AG) genotypes in the case group were 51.4% and 17.1%, respectively. These frequencies in the control group were 32.8% and 4.28%, respectively. Therefore, there was a statistically significant difference between the control and case groups. The frequencies of genotypes and alleles of the 853A>G polymorphism in the case and control groups are shown in Table 2.

Genotyping of LEPR- 511A>G

For genotyping of *LEPR*- 511A>G transition, at first all samples screened and analyzed by SSCP on the PAGE (Figure 2A). Our results showed that 63 samples were hybrid, which were probably AG carriers. Then PCR products with polymorphic phenotypes were sent for DNA sequencing, which known as PCR directed sequencing and were sequenced by Tcaposis Co., Iran (Figure 2C).

Statistical analysis for 511A>G showed that the frequencies of homozygous (GG) and heterozygous (AG) genotypes in the case group were 70% and 28.57%, respectively. These frequencies in the control group were 30% and 24.28%, respectively. Therefore, there was a statistically significant difference between the control and case groups. The frequencies of genotypes and alleles of the 511A>G polymorphism in the case and control groups are shown in table 2.

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Table 2. Genotype and allele frequencies of LEPR 853A>G and 511 A>G in case and control samples

| Genotype	No. and percentage		*p-value* [OR (95% CI)]
	Case (n = 70)	Control (n = 70)	
A: *LEPR* 853A>G			
AA	22 (30)	44 (62.8)	-
AG	36(51.4)	23 (32.8)	0.002 [0.319 (0.154–0.664)]
GG	12 (17.1)	3 (4.28)	0.003 [0.125 (0.032–0.489)]
AG+GG	64 (91.42)	183 (261.42)	0.003 [0.391 (0.209–0.732)]
A	84 (60)	40 (57.14)	0.000 [.0338 (0.197–0.578)]
G	54 (38.57)	38 (27.14)	-
B: *LEPR* 511A>G			
AA	8(11.4)	32(45.71)	-
AG	42(70)	21(30)	0.001 [0.57(0.22-0.147)]
GG	20(28.57)	17(24.28)	0.006 [0.34(0.008-0.149)]
AG+GG	60(85.71)	68(61.81)	0.001 [0.32(0.012-0.019)]
A	72(79.28)	81(88.57)	0.009 [0.44(0.054-0.079)]
G	52(37.14)	29(20.71)	
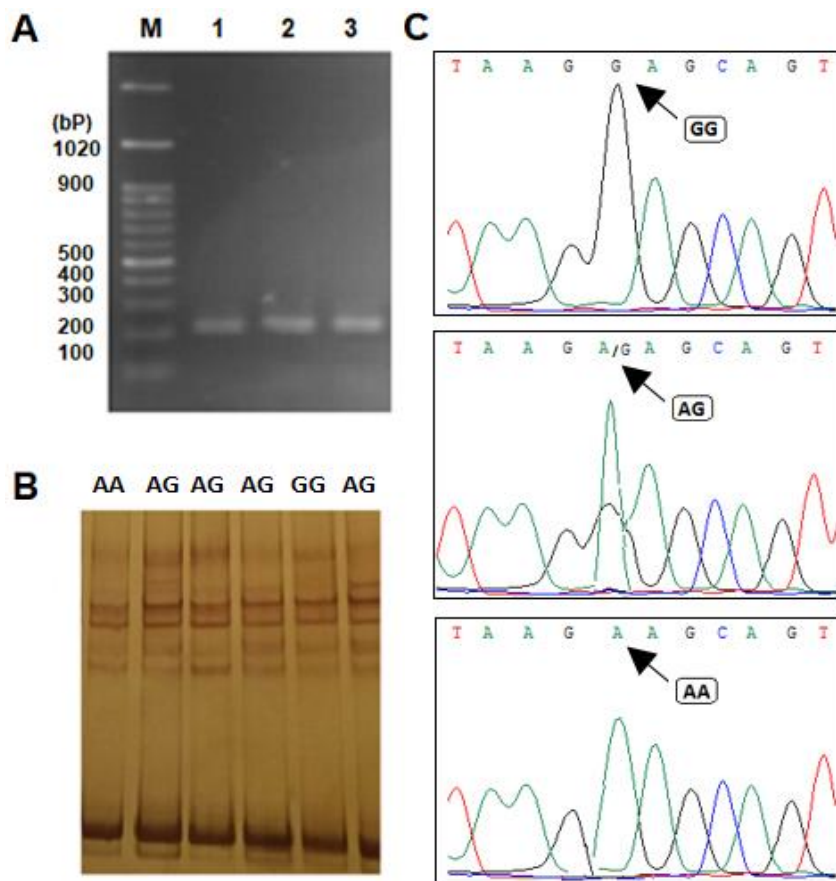
Figure 2. PCR, SSCP, and DNA sequencing results: (A) 217 bp PCR product is shown in 1% agarose gel, M= 100 bp DNA marker; (B) SSCP result shows polymorphic form in polyacrylamide gel; (D) Electropherogram results of PCR direct sequence.

In silico analysis

Polyphen-2 predicted two SNPs, LEPR 853A>G and LEPR 511A>G transition to be benign in both HumDiv and HumVar models analyzing (Figure 3) HumDiv and HumVar scores for 853A>G which are respectively 0.404 and 0.109, and for the 511A>G are 0.077 and 0.021 (Table 3).
The PROVEAN indicated two SNPs, 853A>G and 511A>G substitution as neutral. Variants with a score below -2.5 are considered deleterious and above -2.5 are considered neutral (Choi and Chan et al., 2015). The SNAP server predicted 853A>G and 511A>G mutations are effects (Table 3). The summary of RNAsnp results for LEPR 853A>G and LEPR 511A>G are presented in Table 3. In addition, base pair possibilities of the local region were

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Table 3. The result of *in silico* analysis for 853A>G and 511A>G SNPs

Server	Scores			Prediction
a. Polyphen-2:				
853A>G	HumVar model: 0.001 (sensitivity: 0.89; specificity: 0.90)			Prediction: Benign
	HumDiv model: 0.000 (sensitivity: 0.91, specificity: 0.69)			Prediction: Benign
511A>G	HumVar model: 0.001 (sensitivity: 0.93; specificity: 0.85)			Prediction: Benign
	HumDiv model: 0.000 (sensitivity: 0.95, specificity: 0.56)			Prediction: Benign
b. PROVEAN				
853A>G	Score: -1.404, Prediction Cutoff: 2.5			Neutral
511A>G	Score: -0.478, Prediction Cutoff: 2.5			Neutral
c. SNAP				
853A>G	Score: 65, Expected Accuracy:80%			Prediction: effect
511A>G	Score: 38, Expected Accuracy:66%			Prediction: effect
d. RNAsnp	Folding Window	Local region	Distance	*p*-value
853A>G	653-1053	806-855	0.0093	0.8132
511A>G	311-711	492-709	0.1532	0.1174

[a]Polyphen-2: predicts the possible impact of nsSNPs derived amino acid substitutions on the stability and function of human proteins employing machine learning classification; [b]PROVEAN: predicts whether an amino acid substitution or indel has an impact on the biological function of a protein; [c]SNAP: is a neural-network based method that uses *in silico* derived protein information in order to make predictions regarding the functionality of mutated proteins; [d]RNAsnp: is a server to predict the effect of SNPs on local RNA secondary structure based on the RNA folding algorithms.

determined with extreme differences located between base pairs 1655 and 1705 (Figure 2A). The p-value more than 0.2, represents a black color region that does not show significant structural changes. Given that amount, the p-value of 0.8132 is for the 853A> G, which does not show a structural change, but p-value equal to 0.1174 for 511A>G, as it is less than 0.2, shows a significant structural change (Figure 2B-C), wild sequences and global mutations (Figure 4D-E).

Dbptm for LEPR-k109 variant predicts the secondary structure of the sequence from the amino acid to the position 109 as a coil and the subsequent sequence from residue 111 to position 119 as a helix that is a normal secondary structure with addition SNP carrying sequence in this position. Also, in LEPR-Q223 predicts secondary structure at position 223 as a sheet and positions 224 to 241 like a coil that is normal. Then, to investigate the effects of SNPs on the secondary structure of the protein, Cfssp was used. Cfssp did not show any difference in the secondary structure in the LEPR K109R as well as in the LEPR Q223R.

ProtParam predictions for the physicochemical properties of the 511A>G and 853A>G are summarized in Table 4. The obtained results are similar for LEPR-K109 and LEPR-Q223, but different for LEPR-R109 in the instability index and gravy and LEPR-R223 in Gravy.

MUpro reported that the K109R and Q223R reduced the stability of the LEPR protein structure with a confidence score which is equal to -0.2 and 0.1, respectively. Also, MUpro prediction of the sign (direction) of energy changes using SVM and NN, decreases the stability of the LEPR protein structure with a confidence score which was equal to -0.1 and -0.5. SNPeffect three predict the effects of nsSNPs on a specification that has important roles in protein folding. TANGO algorithm shows the regional protein sequence. The WALTZ is an algorithm that accurately and specifically predicts amyloid-forming regions in protein sequences. LIMBO algorithm shows chaperone binding site for the Hsp70 chaperones, trained from peptide binding data and structural modeling. The results of the SNP effect for the two polymorphisms 853A>G and 511A>G indicated that dTANGO were equal to -4 and 0, respectively, which meant no change, and dWALTZ was equal to -253, which indicated a decrease and 0 which meant no change. dLIMBO was also for both polymorphisms equal to zero. Moreover, the BioGRID was used to check the network of human LEPR interactions, that defined by physical or genetic experiments. The results of BioGRID is shown in figure 5.

Ubpred predicted the probability of generating proper sumoylation sites after the presentation of K at position 511 and 410. UbPred predicted 10 low configurations, 5 medium confidence and 2 high confidence ubiquitination sites for two SNPs LEPR-K109 and LEPR-Q223. Also, for LEPR-R109 and LEPR-R223, UbPred showed new patterns of ubiquitination, 10 low confidence, 4 medium confidences, and 2 high confidence sites. The SUMOplot ™ analysis predicted 7 low and 7 high probability identical motifs for LEPR-K109 and LEPR-Q223 variants. Furthermore, a different pattern predicted 3 low and 6 highs for LEPR-R109 and LEPR-R223.
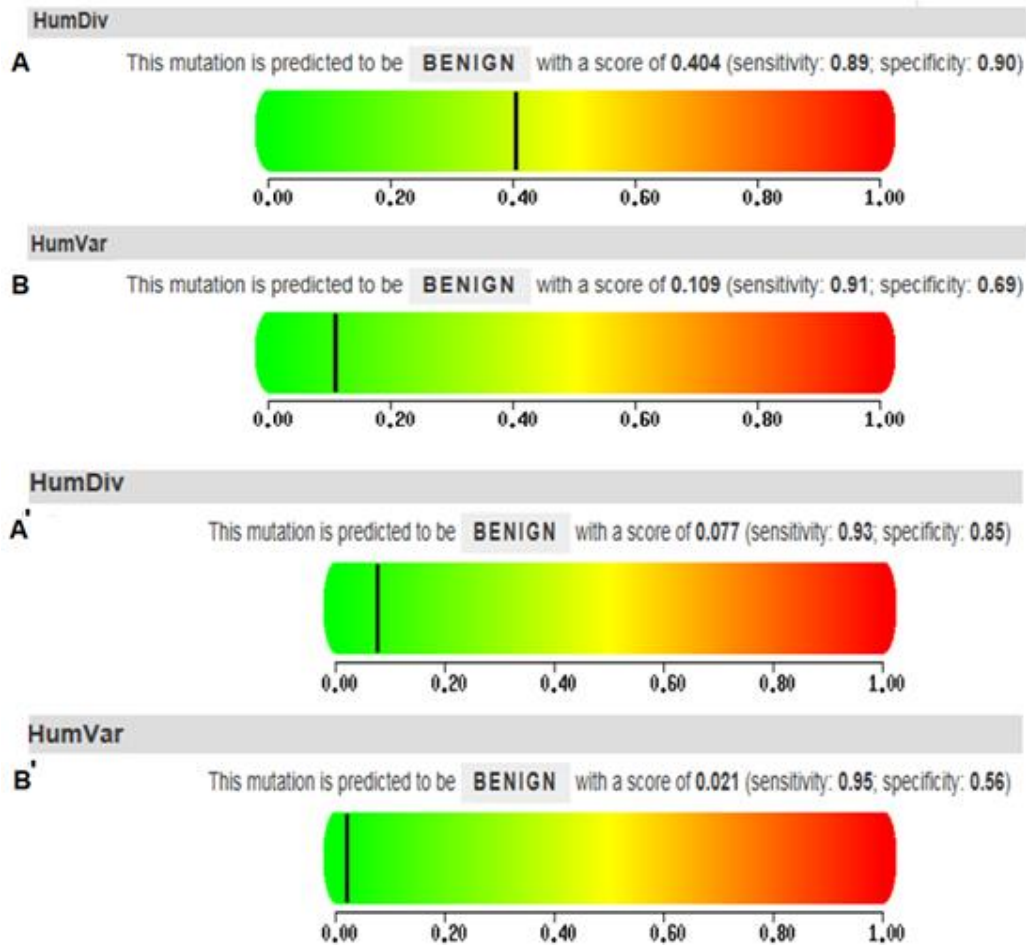
Figure 3. POLYPHEN.2 shows in LEPR-Q223R: HumDiv is sensitivity of 0.89 and in the 0.90 is specificity (A); HumVar is sensitivity of 0.91 and in the 0.69 is specificity and LEPR-K109R (B); HumDiv is sensitivity of 0.93 and in the 0.85 is specificity (A'); HumVar is sensitivity of 0.95 and in the 0.56 is specificity (B').

Discussion

One of the risk factors for pregnancy that causes idiopathic recurrent miscarriage is obesity (Lashen et al., 2004; Jannat Alipoor et al., 2017). Studies by Boots and Stephenson including 24,738 women from four studies, demonstrated that obesity may increase the risk of idiopathic recurrent miscarriage. The percentage of women with one or more miscarriages rose from 10.7% in women with a normal body mass index (BMI) to 11.8% in overweight women and 13.6% in obese women (Bhandari et al., 2016). Leptin is an adipocyte-derived hormone that influences the intake of food and energy consumptions by connecting to specific receptors in the hypothalamus LEPR that is expressed in the brain and hypothalamus, but it is expressed in peripheral tissues such as adipose tissue, liver, kidneys, pancreas, and gonads (Chung et al., 1997; Cohen et al., 2004). LEPR expression in several maternal tissues such as placenta, and fetal tissues indicates the physiologic and pathophysiologic importance of leptin and leptin receptors in normal pregnancy (Sagawa et al., 2002). *LEPR* gene is one of the major genes on the biological pathway of obesity. In 2002, it was shown that expression of LEPR-a and LEPR-b were effective in stimulating LH in rat ovaries (Sagawa et al., 2002). On the other hand, there are some transcription factors that regulate LEPR expression in ovulating granulosa, including nuclear receptor 5a$_2$ (Duggavathi et al., 2008), progesterone receptor (Lydon et al., 1995) and CCAAT/enhancer-binding protein (Sterneck et al., 1997). The expression of leptin and leptin receptor in trophoblastic cells indicate that it may increase the activity or synthesis of molecules that regulate trophoblast invasion (Gaus et al., 1997). Placental villi fragments have functional leptin receptors, which stimulate the system A of placental amino acid transport through Janus kinase, signal transporter and activator of transcription proteins signaling pathway.
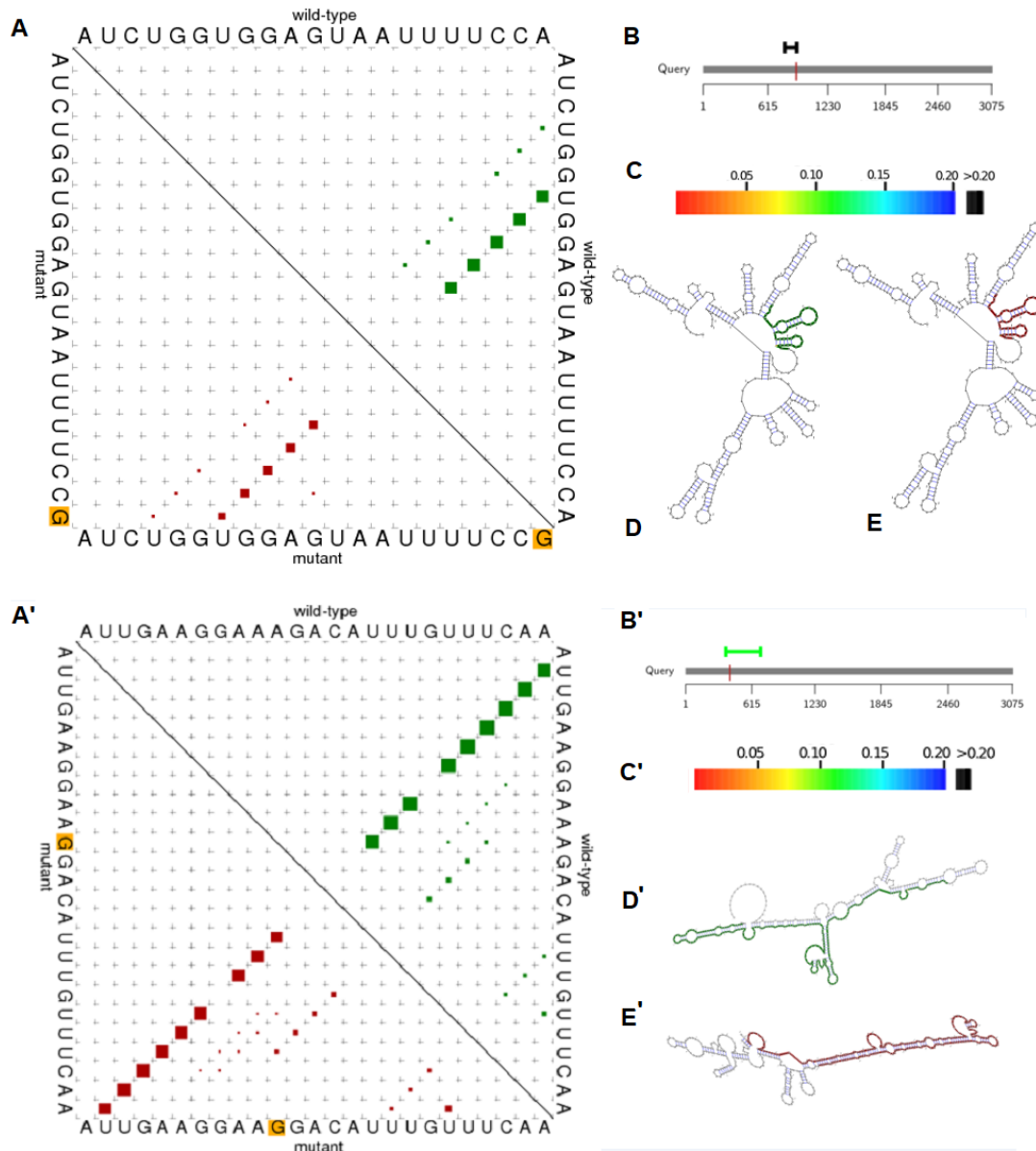
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Figure 4. The G1661A and G155194G transitions effects on LEPR-mRNA, analyzed by RNAsnp: RNAsnp analysis of LEPR-853A>G showed from A-D and RNAsnp analysis of *LEPR*- G155194G showed from A'-D', in local region with maximum differences in wild-type and mutant LEPR-mRNA. Base pair probabilities of the local region (653-1053bp for 853A>G, and 492-709 bp for 511A>G) were detected with maximum differences depicted. The upper and lower triangle of the matrix represents the base pair probabilities of wild-type and mutant sequences, respectively. The mutated nucleotide is shown in yellow (A, A'); Graphic summary of the analysis. The SNP-affected region is colored in black since the *p*-value greater than 0.2, which means not very significant change occurred in mRNA structure for 853A>G , and fewer than 0.2, which means very significant change occurred in mRNA structure for 511A>G (B, B'); *p*-value color direction (C, C'); The optimal secondary structure of global wild-type sequence (653-1053) depicted in green with the minimum free energy of -94.40 kcal/mol for 853A>G , and of -76.40 kcal/mol for 511A>G (D, D'); The optimal secondary structure of global mutant sequence, 653-1053, and 492-709, are shown in red with the minimum free energy equals to -93.30 kcal/mol for 853A>G , and -78.50 kcal/mol for 511A>G (E, E').

Placental amino acids transport is decreased in gestation with fetal growth retardation (Jansson et al., 2002). LEPR might have a function in embryo-maternal cross-talk in the implantation window; as in patients with implantation damage role endometrial LEPR expression was lower. Before studies showed, the leptin level in miscarriage women, lower than in those with a successful pregnancy, significantly (Lea et al., 2000; Unkila et al., 2001). Cebpα/β deletions in the granulosa cells restricted human chorionic gonadotropin (hCG), and stimulate to the expression of *LEPR* (Fan et al., 2011). Leptin stimulates the

Table 4. ProtParam-computed physicochemical properties of LEPR-variants.

Variants	Theoretical pI	Extinction coefficients[a]	Instability index[b]	Aliphatic index	Gravy
K109	7.45	[d]198975 Abs 0.1% (=1 g/l) 1.941 and [e]197100 Abs 0.1% (=1 g/l) 1.923	43.53	89.64	-0.107
R109	7.45	[d]198975 Abs 0.1% (=1 g/l) 1.941 and [e]197100 Abs 0.1% (=1 g/l) 1.923	43.62	89.64	-0.108
Q223	7.45	[d]198975 Abs 0.1% (=1 g/l) 1.941 and [e]197100 Abs 0.1% (=1 g/l) 1.923	43.53	89.64	- 0.107
R223	7.45	[d]198975 Abs 0.1% (=1 g/l) 1.941 and [e]197100 Abs 0.1% (=1 g/l) 1.923	43.53	89.64	-0.108

[a] Extinction co-efficients are in units of M-1 cm-1, at 280 nm measured in water; [b]The calculated scores classifies both variants as unstable; [c] Grand average of hydropathicity; [d] Assuming all pairs of Cys residues form cystines; [e] Assuming all Cys residues are reduced.
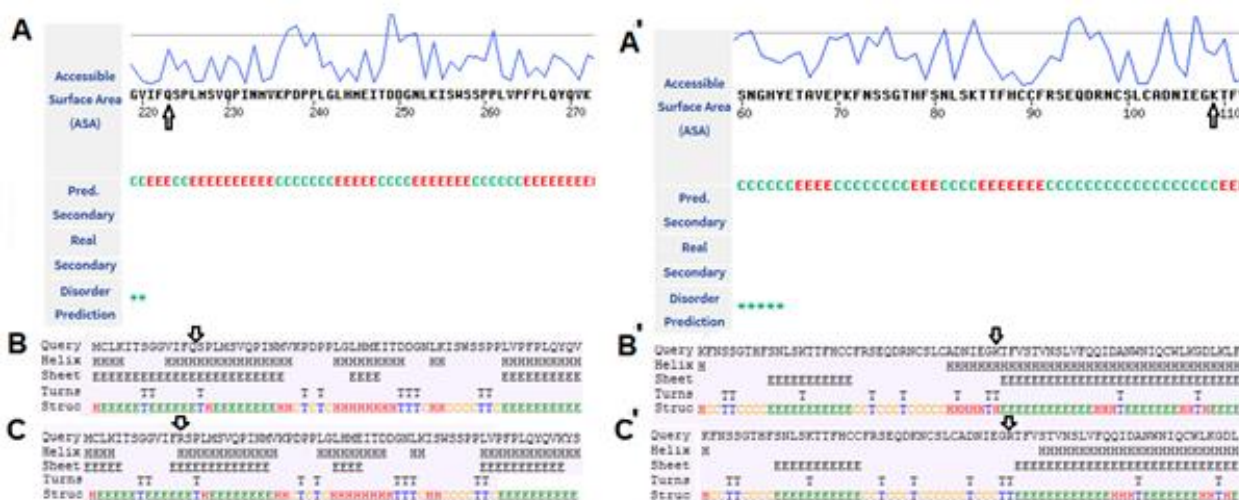

Figure 5. The dbPTM and Cfssp predictions of LEPR-variants structure: The dbPTM prediction of the acidic subdomain structure of LEPR (A, A'); Cfssp predicts a secondary structure for the LEPR-Q223 and LEPR-R109 positions that do not carry the SNP (B, B'); Cfssp predicts a secondary structure for the LEPR-R223 and LEPR-R109 positions that carry the sequence SNP (C, C').
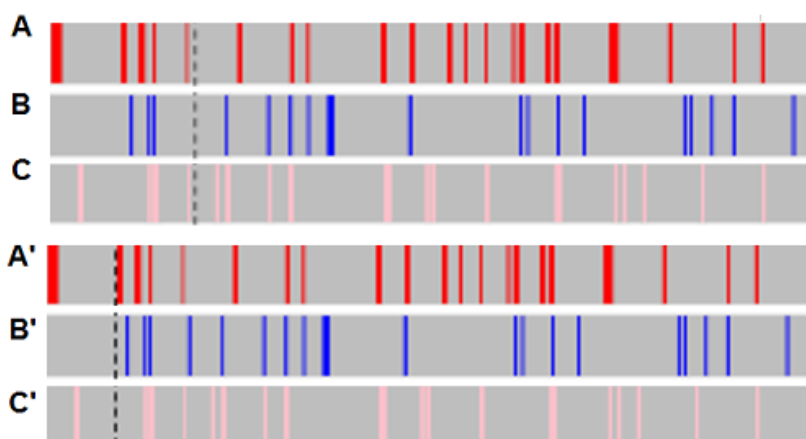

Figure 6. The Q223R and K109R substitutions effects on LEPR-protein folding, analyzed by SNPeffect: In bar representations of the predictions, wild type located on the top and mutant protein in the bottom. The dashed vertical line in the mutant, indicates the positions of the variants residue R223 and R109. The positions of the aggregating stretches visualized in red, blue and pink and it is clear that the variants residue could not disarrange the aggregating stretches; TANGO prediction of aggregation-prone regions (A, A'); WALTZ prediction of amyloid-forming regions (B, B'); LIMBO prediction of chaperone binding sites (C, C').

transport of nutrients to the placenta, which is for fetal growth regulation (Unkila et al., 2001). Some researchers reported that 853A>G *LEPR* gene transition associated with idiopathic recurrent miscarriage (Muller et al., 2016; Riestra et al., 2011), while that this
association in Utah (of USA) population not observed (Chin et al., 2013). Since no study on 511A>G *LEPR* gene variants, this SNP as an adjacent SNP with 853A>G *LEPR* gene transition was selected. In this case-control study, we indicated that the AG genotype and G allele were significantly associated with increased risk of idiopathic recurrent miscarriage. We investigated the correlation of 853A>G and 511A>G SNPs in *LEPR* gene with idiopathic recurrent miscarriage in a case-control *in silico* analysis studies to obtain more precise results. Moreover, we performed an *in silico* analysis to demonstrate our findings more accurate. It seems that some single-gene diseases are dependent on missense mutations, especially on non-synonymous single nucleotide polymorphisms (nsSNPs). The

effects of nsSNPs on diseases and experimental tests to determine their effects on the structure of related mRNA and protein are usually challenging and difficult. For this purpose, we used *in silico* tools to determine the effects of nsSNPs on protein and mRNA features which may be beneficial in solving these problems (Nouri et al., 2014). To predict the effect of nsSNPs on the protein structure and function, three software (Polyphen-2, PROVEN, SNAP) were used. The results revealed that the 853A>G and 511A>G transitions were benign substitutions without significant effects on the protein structure (Adzhubei et al., 2013). We predicted the secondary structure of mRNA by the RNAsnp server for 853A>G and 511A>G transitions. The data showed that the substitution 511A>G reduced the minimum free energy of the mRNA. Therefore, conferring more stability to LEPR mRNA, may alter the *LEPR* gene expression. However, substitution 853A>G does not reduce the minimum free energy of mRNA (Sabarinathan et al., 2013). Also, the ProtParam server was used to
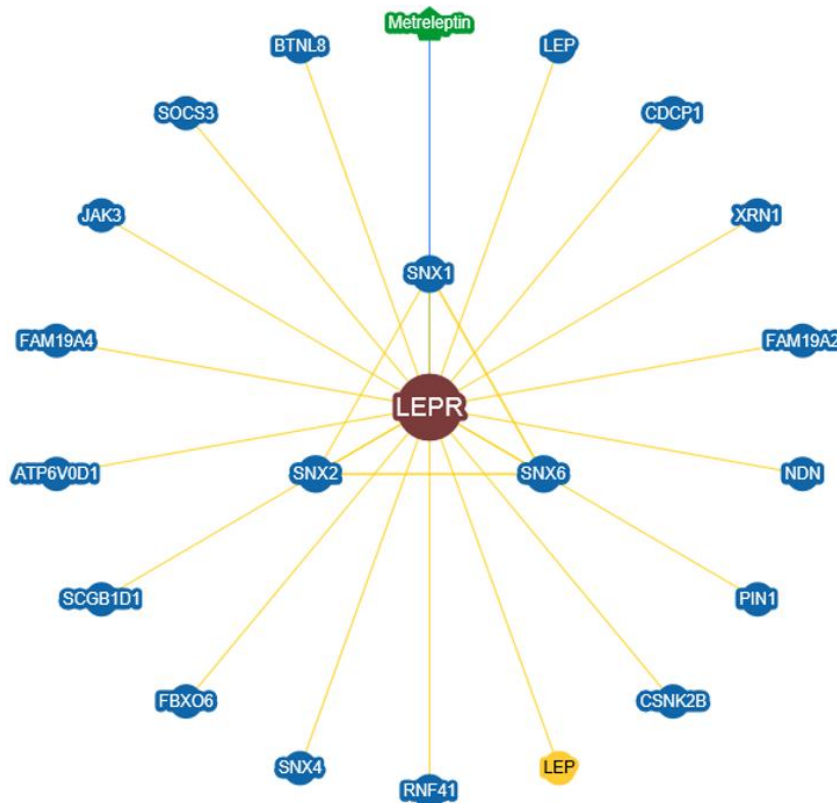

Figure 7. Human LEPR-interactions network obtained from BioGRID: Yellow and purple links demonstrate interactions discovered by physical and genetic experiments respectively; blue links also indicate LEPR-interacted chemicals as well; Proteins: LEP=Leptin; CDCP1= CUB domain-containing protein 1; XRN1= 5'-3' exoribonuclease 1; FAM19A2= Family with sequence similarity 19 member A2; NDN= Named data networking; PIN1= Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1; CSNK2K= Casein kinase; RNF41= E3 ubiquitin-protein ligase NRDP1; SNX4= Sorting nexin-4; FBXO6= F-box only protein 6; SCGB1D1= Segretoglobin family 1D member 1; FAM19A4= Family with sequence similarity 19 Member A4; JAK= Janus kinase 3; SOCS3= Suppressor of cytokine signaling 4; BTNL8= Butyrophilin like8; SNX1= Sorting nexin-1; SNX2= Sorting nexin-2; SNX6= Sorting nexin-6.

determine the physicochemical properties of the LEPR protein. The data indicated that the 853A>G and 511A>G transitions have no effect on the physicochemical features (Gasteiger et al., 2005). We used the dpPTM server to represent the secondary structure of the protein (Lu et al., 2013), and then the CFSSP server to show the effect of SNPs on the secondary structure. The Mupro, reports stability of the LEPR protein, which for both SNPs showed reduced stability (Cheng et al., 2006). Also, BioGRID showed 21 genes involved in LEPR protein (Chatr-Aryamontri et al., 2014). Ubpred predicted changes of ubiquitination sites for two SNPs LEPR-K109 and LEPR-Q223 (Radivojac et al., 2010) and also, for LEPR-R109 and LEPR-R223 as well. The SUMOplot ™ server was used to do sumo sequence analysis for LEPR-K109 and LEPR-Q223.

Conclusions

Based on our findings, LEPR (853A>G and 511A>G) gene transitions may enhance IRM. This notion came about because this loci, as a susceptibility locus, could be used for further case-control studies of IRM. So, these gene transitions might be a useful genetic marker for susceptibility and severity of recurrent miscarriage.

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