 Int J Hum Genet, 16(3,4): 164-169 (2016)

Evaluation of M2/ANXA5 Haplotype and P53 Codon 72 Polymorphism in a Patient with Recurrent Pregnancy Loss, Ectopic Pregnancy and Recurrent Implantation Failure

N. Laleethambika[1*], Mahfrid N. Dharwadkar[2], K. S. Santhy[3], M. Sangeetha[4], D. Silambuchelvi[5] and V. Balachandar[1*]

[1]Human Molecular Genetics and Stem Cell Laboratory, Department of Human Genetics and Molecular Biology, Bharathiar University, Coimbatore 641 046, Tamil Nadu, India
[2]Department of Obstetrics and Gynaecology, Sri Devraj URS University, Tamaka Kolar 563 101, Karnataka, India
[3]Department of Zoology, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore 641 043, Tamil Nadu, India
[4]Vellalar College for Women, Thindal, Erode 638 012, Tamil Nadu, India
[5]Vamsam Fertility and Research Centre, Coimbatore 641 046, Tamil Nadu, India
E-mail: [1]<drlalithambika@yahoo.com>, [2]<geneticbala@yahoo.co.in>

KEYWORDS Annexin. Assisted Reproductive Technique. Donor Embryo Transfer. Intra Cytoplasmic Sperm Injection. Recurrent Implantation Failure. Recurrent Pregnancy Loss. Thrombhophilia

ABSTRACT Repeated implantation failure and recurrent pregnancy loss are foremost problems faced by couples undergoing assisted reproductive techniques (ART). Impairment of placental vascularisation often caused by thrombophilias has been the major cause for such consequences. Annexin A5 is a placental anticoagulant protein, which promotes good blood supply to the fetus throughout gestation. M2 haplotype of ANXA5 leads to reduced gene expression which may lead to hypercoaguability in inter villous space ultimately resulting in early pregnancy loss. In addition to this another gene p53 plays a vital role in inducing apoptosis as well as angiogenesis, it also regulates female reproduction besides blastocyst implantation through leukemia inhibiting factor. Here the researchers report a 25-year-old female with recurrent implantation failure and recurrent pregnancy loss subjected to genetic testing which revealed the prevalence of M2/ANXA5 haplotype and there is no p53 codon 72 polymorphism. The case here highlights the key role of Annexin and p53 gene in reproductive outcome.

INTRODUCTION

Recurrent implantation failure (RIF) is a scientific unit which refers to a condition when implantation has repeatedly failed to arrive at a stage decipherable by pelvic ultrasonography. Up till now there is no unanimously acknowledged definition for RIF, in spite of numerous publications scheduled under this topic (Urman et al. 2005; Das and Holzer 2012; Laufer and Simon 2012; Penzias 2012; Simon and Laufer 2012a, b). Recurrent pregnancy loss (RPL) is a discrete disorder defined by two or more failed clinical pregnancies (Van den et al. 2010). RIF and RPL are the significant reproductive problems affecting 5 percent of women under reproductive age who are otherwise healthy (Regan and Rai 2000; Rai and Regan 2006; Practice Committee of the American Society for Reproductive Medicine 2013). The frequency of RPL is supposed to be around 1 in 300 pregnancies depending on the incidence of sporadic pregnancy loss. On the other hand, epidemiologic studies have exposed that 1 percent to 2 percent of women experience RPL Stephenson (1996). Only 30 percent of all conceptions results in live birth (Macklon et al. 2002). At least three consecutive pregnancy losses before 20 weeks from the last menstrual period is said to be a recurrent miscarriage or habitual abortion. Implantation is said to be successful only when the gestational sac is visible in ultrasonography (Coughlan et al. 2014). It is estimated that up to 85 percent of embryo's transferred through assisted reproductive technique

[*]Address for correspondence:
N. Laleethambika[1] and
Dr. V. Balachandar[2]
Human Molecular Genetics and Stem Cell Laboratory
Department of Human Genetics and Molecular Biology
Bharathiar University, Coimbatore 641046,
Tamil Nadu, India
Phone: +91 9843728544; +91 422 2428514
Fax: +91 422 2422387
E-mail:[1]<drlalithambika@yahoo.com>,
[2]<geneticbala@yahoo.co.in>

could not be implanted in endometrial cavity successfully (Andersen et al. 2005). Even though there are various etiologies, genetic factors appear to be highly associated with reproductive loss (Stephenson 1996; Goodman et al. 2009). Tumor suppression protein TP53 gene also known as genome guardian plays a vital role in cell cycle regulation, apoptosis, maintaining genomic stability, protecting DNA from hypoxia induced damage (Pim and Banks 2004; Pflaum et al. 2014). The p53 tumor suppressor gene is located on chromosome 17p13 which contains 11 exons. In previous studies it has been noted that the low expression of this gene is associated with implantation failure (Smith 2000). This gene also plays a significant role in early embryonic development and angiogenesis as it regulates many proteins engaged in inflammation, transcription and growth control (Hong et al. 2014; Pflaum et al. 2014). TP53 gene regulates an increased expression of cytokine leukemia inhibiting factor (LIF) in the uterus, which occurs coincidentally with the onset of blastocyst implantation (Stewart et al. 1992; Chen et al. 2000). A single-nucleotide polymorphism of codon 72p53 gene resulting in an arginine to proline transition affects the biological activity and leads to difference in LIF expression (Pim and Banks 2004). Earlier reports suggest P72 polymorphism is a risk for RIF (Tang et al. 2011).

Annexin A5 originally named as placental anticoagulant protein is ubiquitously expressed phospholipid-binding annexin found abundantly on apical surfaces of the synctiotrophoblasts covering placental villi (Rogenhofer et al. 2012). M2/ANXA5 is a combination of polymorphisms in the proximal core promoter region of the annexin A5 gene located at 4q27. It has been found to be associated with increased risk of early pregnancy loss among European and Asian population, therefore, anticipated as an established genetic determinant of RPL at the earliest stages of pregnancy (Bogdanova et al. 2007; Tiscia et al. 2009; Miyamura 2011; Rogenhofer 2012; Tuttelmann 2013). Here the researchers report a clinical case with recurrent implantation failure and pregnancy loss in ART cycles, with a family history of PCOD and recurrent miscarriage in maternal aunt.

Objective

The aim of the study was to determine the probable genetic cause for adverse pregnancy outcome to be either M2/ANXA5 Haplotype or P53 Codon 72 Polymorphism in this particular case.

CASE REPORT

Non-consanguineous married couple was referred for chromosomal analysis with a history of recurrent spontaneous abortions in the first trimester. The wife was 25 years old, and husband 32 years. They were healthy and phenotypically normal (male height - 170cm and weight 70kg, female height - 156cm and weight 58kg). There was no history of exposure to known teratogens. The study was approved by the Institutional ethical committee. An informed consent was obtained from the couple before investigation.

Reproductive history of the couple revealed Azoospermia and right Orchidectomy due to Orchitis in male partner. Female history revealed two first trimester miscarriages during the past two years, all being consecutive repeated abortions. The cause and genetic status of recurrent abortions was not known. Uterine cavity was evaluated by three dimensional ultrasonography and revealed a normal size (7.2 X 3.8 X 3.2 cm) and echo pattern. Both ovaries were polycystic, multiple small follicles of same size arranged peripherally with prominent central stroma.

Hormonal profile for female partner evaluated on the 3rd day of the menstrual cycle, demonstrated normal values for Follicle stimulating hormone (FSH) 7.8mIU/ml (1.5-8 mIU/ml), Prolactin 10.8 ng/ml (3-21 ng/ml), TSH 3.5 UIU/ml (0.35-5.5 UIU/ml) and elevated values for Luteinizing hormone (LH) 11.2 mIU/ml (1.5-9.5 mIU/ml). The patient have underwent three cycles of donor intra uterine insemination with history of two miscarriages, the couple had attempted controlled ovarian hyperstimulation and intracytoplasmic sperm injection with donor sperm elsewhere, but the cycle was cancelled due to poor oocyte quality.

The couple was counselled and many treatment options were discussed, they opted for a cycle of intracytoplasmic sperm injection with donor sperm, controlled ovarian hyperstimulation was done, oocytes retrieved were not of good quality explained to the patient and they preferred donor embryo transfer which was done subsequent to obtaining written consent. Two weeks later embryo transfer, serum β HCG was measured and the value was < 2mIU/ml. Followed by that the couple underwent 6 cycles of donor

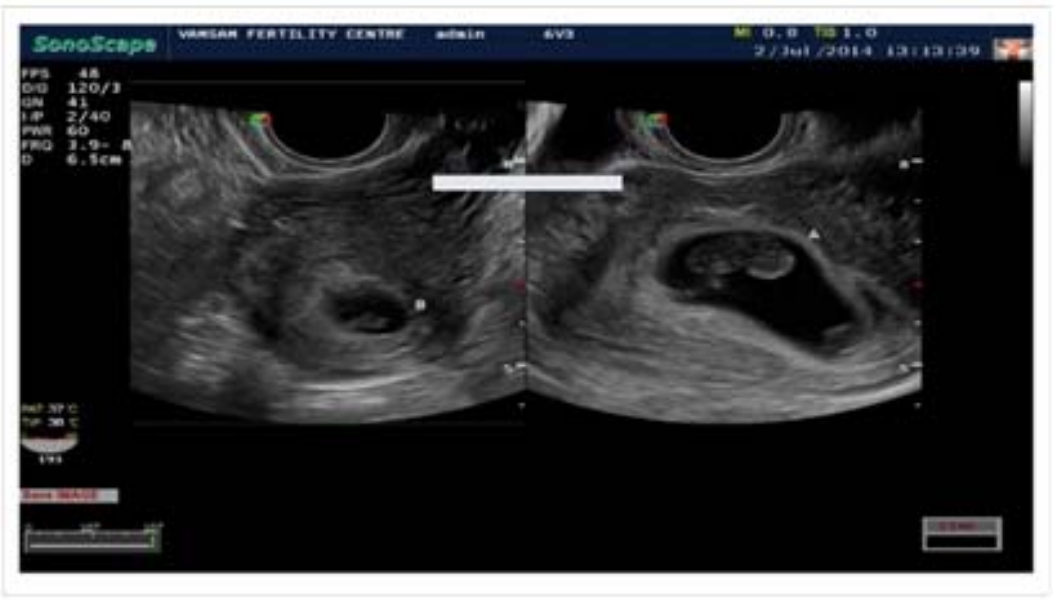

Fig. 1. Failed intrauterine dichorionicdiamniotic twin gestation at 7-8 weeks

embryo transfer with adequate interval between each cycles, resulting in failed implantation in 3 cycles and pregnancy loss in three cycles. Last cycle showed positive outcome with twin gestation (Fig. 1) but went in for missed abortion. In all the cycles 2-3 good quality embryos were transferred, each time patient developed good endometrium with triple line, Doppler showed good endometrial vascularity and embryo transfer was smooth in each cycle. The beta HCG values and the ultrasound reports are given (Table 1). Hence, by considering all the above situations, the patient was advised to undergo Cytogenetic as well as the Molecular genetic test to confirm whether the recurrent pregnancy loss is due to some inherited mutations which might be running in the family.

The researchers collected peripheral blood from the woman to check for any kind of chromosomal anomalies in 17p13 as well as for 4q27 region. Based on Phytohaemaglutinin-stimulated peripheral blood lymphocyte culture cytogenetic analysis was carried out. Lymphocyte culturing and GTG - banding were performed following standard protocols as described by the AGT Cyto genetics Laboratory Manual (Moorhead et al. 1960; Seabright 1971). Karyotyping was found to be normal for both the partners. DNA was extracted from peripheral blood, using the QIAmp DNA blood mini kit (Qiagen, Hilden, Germany) and stored in 100 µl aliquots at 4°C for further analyses. Genotyping of extracted DNA was done, to rule out M2/ANXA5 haplotype and p53 codon polymorphism. The result showed the prevalence of M2/ANXA5 haplotype and there is no p53 codon 72 polymorphisms in this patient. According to these findings, it was detected that even though both the genes are responsible for RIF and RPL, M2/ANXA5 haplotype is the sole cause for the reproductive failure in this patient.

Table 1: Clinical outcome of ART cycles

Cycle No.	*No. of embryos transferred*	*Day of transfer*	*Beta HCG values*	*Clinical pregnancy*	*Ongoing pregnancy*
I	3 Grade I	Day 2	12.15 mIU/ml	No	No
II	2 Grade I	Day 3	7.85 mIU/ml	No	No
III	2 Grade I	Day 3	100.2 mIU/ml	Biochemical	No
IV	3 Grade I	Day 3	76.18 mIU/ml	Biochemical	No
V	2 Grade I	Day 3	120.3 mIU/ml	Clinical abortion	No
VI	3 Grade I	Day 5	724.3 mIU/ml	Failed twin gestation	No

DISCUSSION

The disproportion in cell differentiation caused by apoptosis or abnormal angiogenesis in either cytotrophoblasts or blood vessels may lead to miscarriage (Goodman et al. 2009). Later than conception, human beings endure extremely high early embryonic mortality rate (Van den et al. 2012). Larsen et al. (2013) and Moore (1998) suggested that one-third to one-half of zygotes fail to mature into blastocysts, and 40 percent formed blastocyst will not implant. Along with some known causes such as maternal and paternal age, parental chromosomal abnormalities, infectious diseases, uterine anomalies, endocrine dysfunction, autoimmune disorders, environmental toxins the etiology in about 50 percent of RPL cases remains unknown (idiopathic RPL) which may be related to immunological factors (Laird et al. 2003; Rai and Regan 2006). Miscarriages in the first trimester are found to be more frequent in PCOS women due to disequilibrium in the hemostatic system demonstrated by elevated concentrations of clotting factors FVIII, FX, and Vwf (Shan et al. 2013). A pilot study conducted by Rogenhofer et al. showed M2/ANXA5 acting as an independent RPL risk factor in PCOS patients and its correlation with first trimester pregnancy loss (Rogenhofer et al. 2013). Once implanted the embryo should achieve sufficient trophoblastic proliferation to induce its own blood supply by adequate angiogenesis for normal development (Dimitriadis et al. 2005; Folmes and Terzic 2014).

In spite of advancement in the field of assisted reproductive techniques (ART), the success rate of implantation after embryo transfer hasn't risen as high as acceptable. Only 20 percent of transferred embryos obtained from invitro fertilization implant in uterus inspite of the selection of apparently normal embryos for transfer (ICMART 2002; Nyboe et al. 2005). Combined statistical data from papers reporting implantation rates in diverse ART clinics sturdily suggest that the highest implantation rate is between 40 percent and 60 percent (Rinehart 2007). Both synchronous expansion and communication between hatching blastocyst and endometrium is essential for successful embryo implantation (Timeva et al. 2014). The p53 tumor suppressor protein safeguards the germinative cells and embryos against carcinogens by regulating leukemia inhibitory factor, important cytokine for blastocyst implantation (Hu et al. 2007; Hu 2009; Paskulin et al. 2012; Kusama et al. 2015).

In general, unsuccessful pregnancy in most women are due to RIF and RPL. Several studies have explored the association between p53 polymorphism and RIF (Firouzabadi et al. 2009; Lledo et al. 2014). Studies have also linked p53 polymorphisms among reproductive capability and cancer in humans (Hu 2009; Santoro et al. 2014). High levels of G1 cell cycle arrest induced by P53 codon P72 lead to decreased proliferation (Hu et al. 2009). This may direct to inadequate trophoblastic growth and then implantation failure. Moreover the lower level of apoptosis induced by this polymorphism cause erroneous cell and tissue growth leading to miscarriage (Paskulin et al. 2012).The endometrial receptivity is chiefly aided by the regulatory role of p53 on Leukemia inhibitory factor (LIF), a cytokine responsible for regulating implantation (Hu et al. 2007). Reduced uterine LIF levels and decreased implantation rates was observed in women with P72 polymorphism (Kang et al. 2009). The ANXA5 protein restricted at the apical surfaces of the syncytiotrophoblast shield the fetomaternal boundary from the coagulation of maternal blood (Wang et al. 1999). M2 haplotype of ANXA5 gene produces lower levels of transcripts than the standard haplotype leading to lower levels of ANXA5 protein on the surface of the trophoblast inducing increased susceptibility to coagulation and RPL (Bogdanova et al. 2007).

Here the researchers examined the presence M2/ANXA5 haplotype causing recurrent implantation failure and recurrent pregnancy loss. Based on proband genotyping parental screening was done, maternal aunt was also evaluated who had a history of polycystic ovaries with repeated abortions, her genotyping too revealed M2/ANXA5 haplotype.

CONCLUSION

The researchers concluded that in a varying proportion of cases chromosomal disorders contribute to the underlying basis of reproductive failure. Carriers of M2 haplotype of ANXA5, although appears phenotypically normal, they face repeated adverse pregnancy outcomes as a result of the formation of unbalanced gametes. To overcome the problems of recurrent implantation failure and recurrent pregnancy loss during

pregnancy the embryo should be able to achieve appropriate trophoblastic proliferation, enzyme digestion, and endometrial invasion thus enabling adequate blood supply by angiogenesis after implantation. Thus the results of this study support that, genetic factors could contribute to explain the causes of recurrent implantation failure and recurrent pregnancy loss after undergoing in-vitro fertilization (IVF) treatment. Therefore, it can be postulated that M2/ANXA5 haplotype and P53 codon polymorphism might be associated with higher incidence and existence of recurrent pregnancy wastages and further studies are needed to prove their significance.

ACKNOWLEDGMENTS

The authors are thankful to clinicians of Vamsam Fertility Center, Volunteers and the Authorities of Bharathiar University, Coimbatore, for support and encouragement.

REFERENCES

Andersen AN, Gianaroli L, Felberbaum R, De Mouzon J, Nygren KG 2005. Assisted reproductive technology in Europe, 2001. Results generated from European registers by ESHRE. *Hum Reprod,* 20: 1158-1176.

Bogdanova sN, Horst J, Chlystun M, Croucher PJ, Nebel A 2007. A common haplotype of the annexin A5 (ANXA5) gene promoter is associated with recurrent pregnancy loss. *Hum Mol Genet,* 16: 573-578.

Chen JR, Cheng JG, Shatzer T, Sewell L, Hernandez L, Stewart CL 2000. Leukemia inhibitory factor can substitute for nidatoryestrogen and is essential to inducing a receptive uterus for implantation but is not essential for subsequent embryogenesis. *Endocrinology,* 141: 4365-4372.

Coughlan C, Ledger W, Wang Q, Liu F, Demirol A 2014. Recurrent implantation failure: Definition and management. *Reprod Biomed Online,* 28: 14-38.

Das M, Holzer HE 2012. Recurrent implantation failure: Gamete and embryo factors. *Fertil Steril,* 97: 1021-1027.

Dimitriadis E, White CA, Jones RL, Salamonsen LA 2005. Cytokines, chemokines and growth factors in endometrium related to implantation. *Hum Reprod Update,* 11: 613-630.

Firouzabadi RD, Ghasemi N, Rozbahani MA, Tabibnejad N 2009. Association of p53 polymorphism with ICSI/IVF failure and recurrent pregnancy loss. *Aust NZ J Obstet Gynaecol,* 49: 216-219.

Folmes CD, Terzic A 2014. Metabolic determinants of embryonic development and stem cell fate. *Reprod Fertil Dev,* 27: 82-88.

Goodman C, Jeyendran RS, Coulam CB 2009. P53 tumor suppressor factor, plasminogen activator inhibitor, and vascular endothelial growth factor gene polymorphisms and recurrent implantation failure. *Fertil Steril,* 92: 494-498.

Hong B, van den Heuvel AP, Prabhu VV, Zhang S, El-Deiry WS 2014. Targeting tumor suppressor p53 for cancer therapy: Strategies, challenges and opportunities. *Curr Drug Targets,* 15: 80-89.

Hu W 2009. The role of p53 gene family in reproduction. *Cold Spring Harb Perspect Biol,* 1: a001073.

Hu W, Feng Z, Levine AJ 2009. The regulation of human reproduction by p53 and its pathway. *Cell Cycle,* 15: 3621-3622.

Hu W, Feng Z, Teresky AK, Levine AJ 2007. P53 regulates maternal reproduction through LIF. *Nature,* 450: 721-724.

International Committee for Monitoring Assisted Reproductive Technology (ICMART) 2002. World collaborative report on assisted reproductive technology. *Hum Reprod,* 24: 2310-2320.

Kang HJ, Feng Z, Sun Y, Atwal G, Murphy ME, Rebbeck TR 2009. Single-nucleotide polymorphisms in the p53 pathway regulate fertility in humans. *Proc Natl Acad Sci U S A,* 106: 9761-9766.

Kusama K, Yoshie M, Tamura K, Imakawa K, Tachikawa E 2015. EPAC2-mediated calreticulin regulates LIF and COX2 expression in human endometrial glandular cells. *J Mol Endocrinol,* 54: 17-24.

Laird SM, Tuckerman EM, Cork BA, Linjawi S, Blakemore AI, Li TC 2003. A review of immune cells and molecules in women with recurrent miscarriage. *Hum Reprod Update,* 9: 163-174.

Larsen EC, Christiansen OB, Kolte AM 2013. New insights into mechanisms behind miscarriage. *BMC Med,* 11: 154.

Laufer N, Simon A 2012. Recurrent implantation failure: Current update and clinical approach to an ongoing challenge. *Fertil Steril,* 97: 1019-1020.

Lledo B, Turienzo A, Ortiz JA, Morales R, Ten J, Llacer J, Bernabeu R 2014. Negative effect of P72 polymorphism on p53 gene in IVF outcome in patients with repeated implantation failure and pregnancy loss. *J Assist Reprod Genet,* 31: 169-172.

Macklon NS, Geraedts JPM, Fauser BCJM 2002. Conception to ongoing pregnancy: The “black box” of early pregnancy loss. *Hum Reprod Update,* 8: 333-343.

Miyamura H, Nishizawa H, Ota S, Suzuki M, Inagaki A, Egusa H 2011. Polymorphisms in the annexin A5 gene promoter in Japanese women with recurrent pregnancy loss. *Mol Hum Reprod,* 17: 447-452.

Moore KL (Ed.) 1988. *The Developing Human: Clinically Oriented Embryology*. 4th Edition. Philadelphia: WB Saunders Company.

Moorhead PS, Nowell PC, Mellman WJ, Battips DM, Hungerford DA 1960. Chromosome preparations of leukocytes cultured from human peripheral blood. *Exp Cell Res,* 20: 613–616.

Nyboe Andersen A, Goossens V, Bhattacharya S, Ferraretti AP, Kupka MS; European IVF-monitoring (EIM) Consortium, for the European Society of Human Reproduction and Embryology (ESHRE) 2009. Assisted reproductive technology and intrauterine inseminations in Europe, 2005: Results generated from European registers by ESHRE: ESHRE. The European IVF Monitoring Programme (EIM), for the European Society of Human Re-

production and Embryology (ESHRE). *Hum Reprod,* 24: 1267-1287.
Paskulin DD, Cunha-Filho JS, Souza CA, Bortolini MC, Hainaut P 2012. TP53 PIN3 and PEX4 polymorphisms and infertility associated with endometriosis or with post-in vitro fertilization implantation failure. *Cell Death Dis,* 3: e392.
Penzias AS 2012. Recurrent IVF failure: Other factors. *Fertil Steril,* 97: 1033-1038.
Pflaum J, Schlosser S, Muller M 2014. p53 family and cellular stress responses in cancer. *Front Oncol,* 4: 285.
Pim D, Banks L 2004. P53 polymorphic variants at codon 72 exert different effects on cell cycle progression. *Int J Cancer,* 108: 196-199.
Practice Committee of the American Society for Reproductive Medicine 2013. Definitions of infertility and recurrent pregnancy loss: A committee opinion. *Fertil Steril,* 99: 63.
Rai R, Regan L 2006. Recurrent miscarriage. *Lancet,* 368: 601-611.
Regan L, Rai R 2000. Epidemiology and the medical causes of miscarriage. *Baillieres Best Pract Res Clin Obstet Gynaecol,* 14: 839-854.
Rinehart J 2007. Recurrent implantation failure: definition. *J Assist Reprod Genet,* 7: 284-287.
Rogenhofer N, Engels L, Bogdanova N, Tuttelmann F, Markoff A, Thaler C 2012. Paternal and maternal carriage of the annexin A5 M2 haplotype are equal risk factors for recurrent pregnancy loss: a pilot study. *Fertility and Sterility,* 98: 383-388.
Rogenhofer N, Engels L, Bogdanova N, Tüttelmann F, Thaler CJ 2013. Independent association of the M2/ANXA5 haplotype with recurrent pregnancy loss (RPL) in PCOS patients. *Metabolism,* 62: 1057-1060.
Santoro R, Strano S, Blandino G 2014. Transcriptional regulation by mutant p53 and oncogenesis. *Subcell Biochem,* 85: 91-103.
Seabright M 1971. A rapid banding technique for human chromosomes. *Lancet,* 2: 971-972.
Shan Y, Wang A, Sun Y, Jiang W, Pang B, An Z, Du X, Wang W, Huang Z 2013. Coagulation and fibrinolytic indices during the first trimester of pregnancy in women with polycystic ovary syndrome: a preliminary study. *Reproductive Sciences (Thousand Oaks Calif),* 20: 1390-1397.
Simon A, Laufer N 2012. Assessment and treatment of repeated implantation failure (RIF). *J Assist Reprod Genet,* 29: 1227-1239.
Simon A, Laufer N 2012. Repeated implantation failure: Clinical approach. *Fertil Steril,* 97: 1039-1043.
Smith SK 2000. Angiogenesis and implantation. *Hum Reprod,* 15: 59-66.
Stephenson MD 1996. Frequency of factors associated with habitual abortion in 197 couples. *Fertil Steril,* 66: 24-29.
Stewart CL, Kaspar P, Brunet LJ, Bhatt H, Gadi I 1992. Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature,* 359: 76-79.
Tang W, Zhou X, Chan Y, Wu X, Lu Y 2011. P53 codon 72 polymorphism and recurrent pregnancy loss: A meta-analysis. *J Assist Reprod Genet,* 28: 965-969.
Timeva T, Shterev A, Kyurkchiev S 2014. Recurrent implantation failure: The role of the endometrium. *J Reprod Infertil,* 15: 173-183.
Tiscia G, Colaizzo D, Chinni E, Pisanelli D, Scianname N, Favuzzi G 2009. Haplotype M2 in the annexin A5 (ANXA5) gene and the occurrence of obstetric complications. *Thrombosis and Haemostasis,* 102: 309-313.
Tuttelmann F, Ivanov P, Dietzel C, Sofroniou A, Tsvyatkovska TM 2013. Further insights into the role of the annexin A5 M2 haplotype as recurrent pregnancy loss factor, assessing timing of miscarriage and partner risk. *Fertility and Sterility,* 100: 1321-1325.
Urman B, Yakin K, Balaban B 2005. Recurrent implantation failure in assisted reproduction: how to counsel and manage. B. Treatment options that have not been proven to benefit the couple. *Reprod Biomed Online,* 11: 382-391.
Van den Berg MM, van Maarle MC, van Wely M, Goddijn M 2012. Genetics of early miscarriage. *Biochim Biophys Acta,* 1822: 1951-1959.
Van den Boogaard E, Kaandorp SP, Franssen MT, Mol BW, Leschot NJ 2010. Consecutive or non-consecutive recurrent miscarriage: Is there any difference in carrier status? *Hum Reprod,* 25: 1411-1414.
Wang X, Campos B, Kaetzel MA, Dedman JR 1999. Annexin V is critical in the maintenance of murine placental integrity. *Am J Obstet Gynecol,* 180: 1008-1016.