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Genetic association studies of angiogenesis- and vasoconstriction-related genes in women with recurrent pregnancy loss: a systematic review and meta-analysis

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TABLE OF CONTENTS

- Introduction
- Methods
  - Study selection
  - Statistical analysis
- Results
  - *VEGF* polymorphisms
  - *p53* (codon 72) polymorphisms
  - *eNOS* polymorphisms
- Discussion

BACKGROUND: Angiogenesis and an adequate blood supply are critical for several steps in human early pregnancy. Some studies have reported angiogenesis- and vasoconstriction-related genes are associated with recurrent pregnancy loss (RPL), but their sample size was limited. This study was conducted to investigate the genetic association between these angiogenesis- and vasoconstriction-related genes and idiopathic RPL, using meta-analyses.

METHODS: A systematic review of the published literature from MEDLINE and EMBASE databases was conducted and investigations of an angiogenesis- and vasoconstriction-related gene polymorphism in RPL reported more than three times were selected. Aggregating data from eligible studies were integrated into meta-analyses by means of random effects models.

RESULTS: Of 185 potentially relevant studies, 18 case–control studies comprising a total of 2397 RPL patients and 1760 controls were included into the meta-analyses. Among these genetic association studies were 4 reports of vascular endothelial growth factor (VEGF) (−1154G>A) polymorphisms, 4 reports of p53 (codon72) and 10 reports of endothelial nitric oxide synthase (eNOS) (B/A, Glu298Asp) with RPL. The integrated results showed that VEGF (−1154G>A), p53 (codon 72) and eNOS (Glu298Asp) polymorphisms were significantly associated with RPL, and their summary odd ratios [95% confidence interval (CI)] were 1.51 (1.13–2.03), 1.84(1.07–3.16) and 1.37 (1.11–1.69), respectively. The summary odd ratio of the eNOS (B/A) polymorphism in RPL was 1.15 (0.94–1.41), and failed to show significance at meta-analysis.

[†]These two authors contributed equally to the study.

CONCLUSIONS: Meta-analyses of available data showed significant associations between the *VEGF* (−1154G>A), p53 (codon72) and eNOS (Glu298Asp) polymorphisms and idiopathic RPL. These angiogenesis- and vasoconstriction-related genes jointly confer higher susceptibility to idiopathic RPL.

Key words: meta-analysis / recurrent pregnancy loss / VEGF / p53 / eNOS

Introduction

Recurrent pregnancy loss (RPL) is the occurrence of repeated pregnancies that end in miscarriage of the fetus, usually before 20 weeks of gestation. RPL affects about 1–5% of women who conceive (Baek *et al*., 2007). Various factors have been identified as being related to miscarriage, including uterine anomaly, chromosomal abnormalities, endocrine dysfunction, thrombophilia, immune disorders, lifestyle factors and maternal infections (Regan *et al*., 1989). However, in up to 50% of patients who experience RPL, the underlying causes remain undetermined (Li *et al*., 2002). In patients with idiopathic RPL, gene polymorphisms have been proposed as susceptibility factors that increase the risk of pregnancy loss compared with otherwise healthy women.

A normal pregnancy is dependent on adequate placental circulation and fetal vasculature. The development of a normal functioning vascular network requires complicated cooperation between different cell types and various growth factors in the processes of implantation, embryo development and placentation (Reynolds and Redmer, 2001; Schiessl *et al*., 2009; Reynolds *et al*., 2010). Abnormalities of placental vasculature may result in several gestational complications, including pregnancy loss, intrauterine fetal death, intrauterine growth restriction and pre-eclampsia (Khankin *et al*., 2010; Reynolds *et al*., 2010). Microarray data have shown a decreased expression pattern of angiogenesis-related genes in the chorionic villi of RPL patients (Choi *et al*., 2003).

Vascular endothelial growth factor (VEGF) is a potent angiogenic factor and a survival factor for endothelial cells during physiological and tumor angiogenesis, and functions in vasodilatation, vascular permeability and anti-apoptosis (Benjamin and Keshet, 1997; Ferrara *et al*., 2003). VEGF plays an essential role in fetal and placental angiogenic development; mice lacking the expression of VEGF die *in utero* due to inadequate vascular formation (Ferrara, 2004; Hiratsuka *et al*., 2005). VEGF also plays a critical role in oocyte maturation, decidualized endometrial vascularization, embryo implantation/development and placenta angiogenesis/vascularization in early gestation (Jackson *et al*., 1994; Kruessel *et al*., 2000, 2001; Zygmuni *et al*., 2003). In human early pregnancy, the state of chorionic villi vascularization is closely related to embryonic development, and diminished placental trophoblastic VEGF has been described in the decidual endothelium of spontaneous miscarriages (Vuorela *et al*., 2000). Several VEGF polymorphisms have been reported to affect VEGF activity and expression (Brogan *et al*., 1999; Renner *et al*., 2000; Awata *et al*., 2002; Mohammadi *et al*., 2003). One common polymorphism, (*−1154G/A*) in the promoter region, is reported to be associated with RPL (Papazoglou *et al*., 2005; Coulam and Jeyendran, 2008; Lee *et al*., 2010; Su *et al*., 2011); however, the genetic association studies are, so far, inconclusive.

p53 is known as a tumor suppressor gene, and it plays an important role in regulating the cell cycle, apoptosis and protecting the genome from hypoxia and DNA damage (Smeenk and Lohrum, 2010). In addition to the above functions, the p53 gene also plays a critical role in angiogenesis and embryonic development as it interacts with many proteins involved in growth control, inflammation and transcriptional regulation (Choi and Donehower, 1999; Ravi *et al*., 2000; Schetter *et al*., 2010). Mutant p53 was reported to induce angiogenesis by mediating hypoxia-inducible factor 1 alpha and the VEGF pathway (Ravi *et al*., 2000; Schetter *et al*., 2010), and inappropriate over- and under-expression of p53 shown to lead to embryonic lethality, and interrupt differentiation (Schmid *et al*., 1991; Choi and Donehower, 1999). A common p53 polymorphism at codon 72, encoding either proline or arginine, was reported to alter p53 functional activity (Dumont *et al*., 2003; Pim and Banks, 2004) and affect human fertility (Kang *et al*., 2009). The Arg72 variant has been shown to induce higher apoptotic activity than Pro72, and the Pro72 variant might thus cause inadequate trophoblast invasion (Dumont *et al*., 2003; Pim and Banks, 2004). These effects of the p53 variants would be expected to manifest early in pregnancy and could explain the role of p53 polymorphisms in pregnancy losses (Pietrowski *et al*., 2005; Coulam *et al*., 2006; Firouzabadi *et al*., 2009; Kaare *et al*., 2009).

Nitric oxide (NO) is known to mediate vascular smooth muscle relaxation, and the lack of endothelial-derived NO is associated with vasospasm and vascular infarction (Cauwels and Brouckaert, 2011; Gilchrist *et al*., 2011). Endothelial nitric oxide synthase (eNOS) is the main enzyme required for vascular NO production, by converting L-arginine to L-citrulline (Moncada and Higgs, 1993). eNOS is expressed in the terminal chorionic villous vessels and in the cyto- and syncytiotrophoblast layers during the first trimester, and the eNOS level increase as gestation progresses and in certain pathological conditions (intrauterine growth restriction and pre-eclampsia; Rossmanith *et al*., 1999; Ayuk *et al*., 2002). Reduced NO production can lead to impaired placental perfusion and a compromised oxygen and nutrient supply to the fetus. In mice, lipopolysaccharide (LPS)-induced abortion is mediated by placental NO production, and inhibition of NO release will successfully rescue LPS-induced abortion (Aisemberg *et al*., 2007). A 27 bp of the variable nucleotide tandem repeat (VNTR) polymorphism in intron 4 and the Glu298Asp polymorphism in exon 7 were shown to influence the plasma NO level and were associated with clinical phenotypes in pre-eclampsia and cardiopulmonary disease (Tsukada *et al*., 1998; Tesauro *et al*., 2000; Yoshimura *et al*., 2000; Bashford *et al*., 2001). These two common polymorphisms were also investigated for their possible associations with RPL (Tempfer *et al*., 2001; Hefler *et al*., 2002; Buchholz *et al*., 2004; Makino *et al*., 2004; Suryanarayana *et al*., 2006; Karvela *et al*., 2008; Zammiti *et al*., 2008; Al Sallout *et al*., 2010; Shin *et al*., 2010).

In view of the importance of angiogenesis and vascular tone reactivity in human pregnancy, we investigated the role of angiogenesis- and vasoconstriction-related genes in RPL. We chose three candidate genes (VEGF, p53 and eNOS) after a literature review; their associations with idiopathic RPL were not conclusive. Therefore, we

evaluated the susceptibilities of the VEGF, p53 and eNOS gene polymorphisms to RPL risk using meta-analysis.

Methods

Study selection

A systematic review of the literature, with no language restrictions, was conducted with an EMBASE and a MEDLINE search using the OVID SP and PubMed (last updated Nov 2010) databases. We used the terms 'abortion' (which also included keywords: pregnancy with abortive outcome; spontaneous abortions; miscarriages; vaginal expulsion of fetus; termination of pregnancy); 'pregnancy' (which also included keywords: mammalian gestation; gestation; pregnancy function) and 'polymorphism, genetic' in the OVID SP system and focused on studies of angiogenesis- and vasoconstriction-related genes in three or more independent populations. Three genes (VEGF, p53 and eNOS) were therefore selected in the first stage search, and then we restricted the searches to VEGF, p53 and eNOS as target genes into meta-analysis. Various combinations of the terms: ('abortion' OR 'miscarriage' OR 'pregnancy loss') AND ('polymorphism') AND ('VEGF' OR 'p53' OR 'eNOS' OR 'NOS3') were searched in EMBASE and MEDLINE databases. The searches were complemented with hand searches of the references of the retrieved articles. All studies were compared carefully to ensure that we avoided duplicating analyses of the same samples.

Eligibility criteria included the following: (i) Case–control design with the genotyping of women with and without RPL; (ii) RPL defined as two or more losses in the first two trimesters of pregnancy; (iii) unexplained or idiopathic pregnancy losses, that is, pregnancy losses with 'known' causes being excluded; (iv) genotypes identified by DNA analysis (polymerase chain reaction); (v) genotyping of the common polymorphisms in the candidate genes. If a study as a whole did not meet the inclusion criteria, but a subset of the subjects qualified, only the subset was included. When the study populations overlapped, we generally retained the study with the most extensive data to avoid duplication. We excluded those studies that did not provide adequate information on selection criteria and the actual distribution of polymorphisms in each group. Case reports or case series, and review articles were excluded.

The following information was recorded from the retrieved studies: authors' names, publication year, country where the study was conducted, study design, definition of RPL, inclusion criteria for RPL patients and normal controls, methods used for genotyping and the distribution of polymorphism genotypes in each group. Authors of primary reports were contacted for additional information, if necessary. Data extraction was performed by the two authors independently. In case of disagreement, consensus was obtained by joint review of the study. The quality of the included studies was assessed using the following criteria modified from the previous report (Sotiriadis *et al.*, 2007):

(i) Description of the case and control groups (adequate, inadequate).
(ii) Assessment and validation of miscarriage in the patients (adequate, inadequate, not stated). Adequate validation would include confirmation by scan or pathological examination; inadequate validation would include recollection of the patient as the only evidence or a biochemical pregnancy without ultrasound evidence of pregnancy.
(iii) Description of the laboratory procedures for the genotyping (adequate, inadequate).
(iv) Elimination of confounding factors in patients (not described, inadequate, adequate). Adequate elimination refers to the exclusion of the proven causes of recurrent miscarriage (chromosomal abnormalities of the couples, uterine abnormalities, antiphospholipid antibodies, protein C/S and antithrombin-III deficiency).
(v) Equal assessment for confounding factors in the case and control groups (equal, unequal, not stated).

Statistical analysis

The selected data were analyzed statistically using the R programming language (R-2.12.0). For each genetic variant study, individual and summary odds ratios (ORs) and 95% confidence intervals (CIs) were calculated for both dominant and recessive genetic models, using random effects (DerSimonian–Laird method) (DerSimonian and Laird, 1986). A *P*-value of <0.05 was considered to be significant. Tests for among-study heterogeneity were performed using Cochran's Q statistics for each meta-analysis, and a *P*-value of <0.1 was considered in-homogeneous among included studies. Publication bias was assessed by using funnel plots and Egger's test (Egger *et al.*, 1997).

Results

The initial search identified 185 potentially relevant studies, of which 25 full articles were retrieved for thorough evaluation and 18 were considered eligible for meta-analysis. All included studies were English language literature, except one in the Chinese language (Fan *et al.*, 2007). More than three association studies were found for VEGF −1154G>A polymorphisms (four studies, Table I), p53 codon 72 (four studies, Table II), eNOS A/B polymorphisms (nine studies, Table III) and eNOS Glu298Asp polymorphisms (six studies, Table III). The majority of studies focused on variants that were of likely or proved functional significance, or had plausible disease associations that were described previously. Of these included studies, all were case–control designs and were conducted in 13 countries: three in the USA, two each in Austria, Greece and Korea, and one each in 9 other countries (Tables I–III). Cases were generally recruited in referral centers for women with a history of RPL, and the controls were selected from ethnically matched women with at least one or two live births without a history of pregnancy loss in most studies. Most studies recruited patients with pregnancy losses before 20 weeks of gestational age, but two studies recruited those before 25 weeks (Buchholz *et al.*, 2004; Al Sallout *et al.*, 2010) and one before 30 weeks (Zammiti *et al.*, 2008; Table III). The number of cases varied from 46 to 350, with a mean of 153, and the numbers of controls varied from 20 to 200, with a mean of 101. In 8 studies, two or more pregnancy losses were used to define RPL, and in another 10 studies, three or more pregnancy losses were used. The majority of these studies excluded 'known' causes for RPL, but the exclusion criteria varied.

VEGF polymorphisms

The most commonly studied allelic variant in VEGF was the −1154G>A polymorphism. Four eligible studies included a total of 534 patients and 430 controls for analysis (Table I; Papazoglou *et al.*, 2005; Coulam and Jeyendran, 2008; Lee *et al.*, 2010; Su *et al.*, 2011). Under the dominant genetic model, no significant among-study heterogeneity was observed (Cochran's Q statistics: $\chi^2 = 2.45$, df = 3, *P*-value = 0.48; Higgins statistics: $I^2 = 0\%$), and the summary ORs and 95% CI for random effects showed significant association between the VEGF (−1154G>A) polymorphism and RPL [the summary OR (95% CI) = 1.51 (1.13–2.03)] (Fig. 1). However, under the recessive genetic model, the among-study heterogeneity was obvious ($P = 0.003$), and the association was not significant

Table I Characteristics of studies on association between VEGF(−1154G > A) and RPL.

Author (year)	Country (ethnicity)	Cases	Controls	Genotype frequency GG:GA:AA (number of samples)	Quality assessment
Papazoglou et al. (2005)	Greece (Greek)	52 patients with 3 or more unexplained consecutive pregnancy losses	82 healthy, post-menopausal controls with at least two live births and no history of pregnancy loss	(case) 18:19:15 (control) 42:28:12	1: adequate; 2: not stated; 3: adequate; 4: adequate; 5: unequal
Coulam and Jeyendran (2008)	USA (a variety of ethnics)	152 women with 2 or more consecutive spontaneous abortions	65 fertile women with at least two live births and no history of pregnancy loss	(case) 26:101:25 (control) 10:51:4	1: adequate; 2: not stated; 3: adequate; 4: adequate; 5: unequal
Lee et al. (2010)	Korea (Korean)	215 patients with 2 or more unexplained consecutive pregnancy losses	113 healthy controls with at least one live birth and no history of pregnancy loss	(case) 130:80:5 (control) 81:23:9	1: adequate; 2: not stated; 3: adequate; 4: adequate; 5: unequal
Su et al. (2011)	Taiwan (Chinese Han)	115 women with 2 or more unexplained consecutive pregnancy losses	170 healthy controls with at least one live birth and no history of pregnancy loss	(case) 74:37:4 (control) 124:39:7	1: adequate; 2: adequate; 3: adequate; 4: adequate; 5: unequal

Quality assessment codes: 1 = description of cases and controls; 2 = assessment and validation of the RPL in the patients; 3 = description of laboratory methods; 4 = Elimination of confounding factors in patients; 5 = equal assessment of confounding factors in both groups.

(Table IV). Tests for publication bias did not detect a significant bias (Egger's *P*-value = 0.33).

P53 (codon 72) polymorphisms

Four studies with a total of 523 RPL women and 387 normal controls investigated the p53 polymorphism, and were included in the analysis (Table II; Pietrowski *et al*., 2005; Coulam *et al*., 2006; Firouzabadi *et al*., 2009; Kaare *et al*., 2009); the estimated ORs are given in Fig. 2. The heterogeneity among each study was not significant in both the dominant and recessive models (Cochran's Q statistics: $\chi^2 = 0.1$, df = 3, *P*-value = 1.00; Higgins statistics: $I^2 = 0\%$, random effects in the recessive genetic model). Meta-analysis showed that women who carried the p53 (codon 72) polymorphism had a higher risk of RPL in the recessive model: the summary OR (95% CI) is 1.84 (1.07–3.16) for the random effects model (Fig. 2). Publication bias was not detected in the included studies (Egger's *P*-value = 0.32).

eNOS polymorphisms

The two most commonly studied eNOS polymorphisms in RPL are the VNTR polymorphism in intron 4 (shown as B/A or 4/5 repeats) and the Glu298Asp polymorphism in exon 7. The largest study (Zammiti *et al*., 2008) was composed of 350 RPL women and 200 healthy controls. The B/A and Glu298Asp polymorphisms investigated in this study were assessed separately owing to their inclusion of RPL that occurred within 30 weeks, whereas most studies included only those within 20 or 25 weeks of gestation Table III.

A total of nine studies with 1210 RPL women and 876 controls investigating the association between the eNOS (B/A) polymorphism and RPL were included in the analysis (Tempfer *et al*., 2001; Buchholz *et al*., 2004; Makino *et al*., 2004; Suryanarayana *et al*., 2006; Fan *et al*., 2007; Karvela *et al*., 2008; Zammiti *et al*., 2008; Al Sallout *et al*., 2010; Shin *et al*., 2010). The heterogeneity among these included studies did not show significance (Cochran's Q statistics: $\chi^2 = 10.4$, df = 8, *P*-value = 0.25; Higgins statistics: $I^2 = 0\%$), and the associations between the eNOS (B/A) polymorphism and RPL were not significant, with or without inclusion of the Zammiti *et al*. (2008) study, under dominant and recessive genetic models (Table IV). Publication bias was not detected in the included studies (Egger's *P*-value = 0.94).

The six studies investigating the association between the eNOS (Glu298Asp) polymorphism and RPL included 1231 RPL patients and 751 normal controls (Hefler *et al*., 2002; Suryanarayana *et al*., 2006; Fan *et al*., 2007; Karvela *et al*., 2008; Zammiti *et al*., 2008; Shin *et al*., 2010). The heterogeneity among these included studies did not show significance (Cochran's Q statistics: $\chi^2 = 3.64$, df = 5, *P*-value = 0.60; Higgins statistics: $I^2 = 0\%$; Fig. 4). Under the dominant genetic model, the eNOS (Glu298Asp) polymorphism was significantly associated with RPL using random effects analysis; whereas the association between the eNOS (Glu298Asp) polymorphism and RPL was not significant under the recessive genetic model (Table IV). Regardless of whether the Zammiti study was included, the eNOS (Glu298Asp) polymorphism was associated with RPL, and the summary OR was about 1.37 (Table IV). Therefore, evaluating the association between the eNOS (Glu298Asp) polymorphism and RPL under the dominant genetic model was more appropriate and consistent. Publication bias was not detected in the included studies (Egger's *P*-value = 0.35).

Table II Characteristics of studies on association between p53(codon72) and RPL.

Author (year)	Country (ethnicity)	Cases	Controls	Genotype frequency Arg/Arg:Arg/Pro: Pro/Pro (number of samples)	Quality assessment
Pietrowski *et al.* (2005)	Austria (White Caucasians)	175women with at least three spontaneous consecutive miscarriages before 20 weeks of gestation	143 peri- or post-menopausal women with at least one live birth and no history of miscarriage	(case) 83:70:22 (control) 83:50:10	1: adequate; 2: not stated; 3: adequate; 4: adequate; 5: unequal
Coulam *et al.* (2006)	USA (a variety of ethnics)	205 women with two or more consecutive spontaneous abortions	21 fertile women with at least two live birth and no more than one elective abortion	(case) 141:55:9 (control) 13:8:0	1: adequate; 2: inadequate; 3: adequate; 4: adequate; 5: unequal
Firouzabadi *et al.* (2009)	Iran (Iranians)	97 women experiencing two or more consecutive spontaneous abortions	32 fertile women with at least two healthy children	(case) 23:41:33 (control) 4:21:7	1: adequate; 2: not stated; 3: adequate; 4: adequate; 5: unequal
Kaare *et al.* (2009)	Finland (Caucasians)	46 women with three or more consecutive miscarriages	191 women with at least one successful pregnancy and no known history of miscarriage	(case) 21:22:3 (control) 106:77:8	1: adequate; 2: adequate; 3: adequate; 4: adequate; 5: unequal

Quality assessment codes: 1 = description of cases and controls; 2 = assessment and validation of the RPL in the patients; 3 = description of laboratory methods; 4 = Elimination of confounding factors in patients; 5 = equal assessment of confounding factors in both groups.

Discussion

In this systematic review of studies on the involvement of angiogenesis- and vasoconstriction-related genetic polymorphisms in RPL etiology, meta-analyses of three genes with four polymorphisms: VEGF (−1154G>A), p53 (codon 72), eNOS (B/A) and eNOS (Glu298Asp), were performed, as these had been investigated in at least four studies. Our integrated results showed that VEGF (−1154G>A), p53 (codon 72) and eNOS (Glu298Asp) were significantly and consistently associated with RPL, and their summary ORs (95% CI) were 1.51 (1.13–2.03), 1.83 (1.06–3.18) and 1.38 (1.09–1.74), respectively. The summary OR of the eNOS (B/A) polymorphism in RPL was 1.09 (0.90–1.32), and failed to show significance after meta-analysis.

The criteria for study inclusion and exclusion are critical parts of a meta-analysis and can substantially affect results. The most obvious difference would be the definition of RPL. Although RPL is strictly defined as three or more miscarriages before 20 weeks of gestation, many groups chose to include women with two consecutive miscarriages as well. As for the four polymorphisms that we investigated in this study, we included reports with both definitions of RPL, but this did not alter the results after subgroup analysis (data not shown). This issue has been also discussed in both an individual study from our group (Su *et al.*, 2011) and a meta-analysis study of RPL (Kovalevsky *et al.*, 2004), and both showed similar results after comparing subgroups with more than two consecutive pregnancy losses and three consecutive ones. However, the OR of the three or more miscarriage group with regard to the VEGF (−1154G>A) polymorphism was actually higher than the summary OR of the two or more miscarriage group (1.98:1.51; Fig. 1).

Another potential difference would be the inclusion criteria of gestational age at pregnancy loss. All but one of the analyzed studies included miscarriage before the second trimester. The largest case–control study on the association of the eNOS (B/A and Glu298Asp) polymorphism with RPL included miscarriage of the early third trimester (Zammiti *et al.*, 2008). We could not dissect each genotype in this study by selecting the miscarriages of the first two trimesters only; therefore, our investigation was carried out separately through analysis with or without this study. However, the final results were not altered, which may indicate that there were not that many cases of RPL in the third trimester in this study (Zammiti *et al.*, 2008).

Some other potentially less obvious biases may arise from the definition of 'unexplained miscarriage' and 'normal controls'. Although most studies claimed that they included only patients with unexplained miscarriage, the examinations offered for each group varied. The proven causes depended on which factors were under examination in the given study. Moreover, the criteria for the so-called normal controls could include those with no history of miscarriage, with previous live births, or a combination of the two, but the controls did not undergo the same investigation as the RPL patients. Maternal age is one of the factors that could exacerbate the effect of genetic polymorphisms, therefore some studies adjusted this factor and calculated higher ORs. Some of the included studies did not provide this information, and we could therefore not integrate this variable into the meta-analysis. However, the among-study heterogeneity indicates the above factors are unlikely to have apparent effects on the results of the meta-analyses.

By combining data from published studies, we can increase the sample size and statistical power of effect and exclude the modest associations of several of the candidate polymorphisms. Ethnic variation and genetic admixture need to be considered in evaluating the genetic background of RPL, and could significantly influence the results of genetic association studies (Dumont *et al.*, 2003). Most studies made an effort to reduce this error by recruiting the same ethnicity in the study and control groups. Although allele/genotype

Table III Characteristics of studies on association between two common eNOS polymorphisms and RPL.

Author (Year)	Country	Cases	Controls	Genotype	Genotype frequency (number of samples)	Quality assessment
Tempfer et al. (2001)	Austria (White Caucasians)	105 women with at least three spontaneous, consecutive miscarriages before 20 weeks of gestation	91 post-menopausal women with at least one live birth and no history of miscarriage	eNOS (B/A)	**BB(wild):BA:AA** (case) 65:39:1 (control) 69:22:0	1: adequate; 2: not stated; 3: adequate; 4: adequate; 5: unequal
Hefler et al. (2002)	USA (White Caucasians)	130 women with at least three spontaneous, consecutive miscarriages before 20 weeks of gestation	67 healthy, post-menopausal women with at least two live births and no history of miscarriage	eNOS (Glu298Asp)	**GG:GT:TT** (case) 60:57:13 (control) 32:27:8	1: adequate; 2: not stated; 3: adequate; 4: adequate; 5: unequal
Buchholz et al. (2004)	Germany	179 women with at least two unexplained consecutive spontaneous abortions before 25 weeks of gestation	126 healthy women with at least one normal term deliveries after uneventful pregnancies and no history of miscarriages	eNOS (B/A)	**BB:BA:AA** (case) 123: 52:4 (control) 82:39:5	1: adequate; 2: not stated; 3: adequate; 4: adequate; 5: unequal
Makino et al. (2004)	Japan (Japanese)	85 cases with a history of two or more unexplained embryonal losses before 10 weeks' gestation	76 women without obstetrical complications or any history of miscarriage (including 26 and 50 women with and without live births)	eNOS (B/A)	**BB:BA:AA** (case) 70:15:0 (control) 62:14:0	1: adequate; 2: not stated; 3: adequate; 4: adequate; 5: unequal
Suryanarayana et al. (2006)	India (Indians)	145 South Indian women with three or more first trimester miscarriages. (all are primary aborters with no live child)	99 healthy control women with at least one successful pregnancy outcome and no history of miscarriages or pregnancy-associated complications	eNOS (B/A) eNOS (Glu298Asp)	**BB:BA:AA** (case) 101:43:1 (control) 71:28:0 **GG:GT:TT** (case) 91:47:7 (control) 69:27:3	1: adequate; 2: not stated; 3: adequate; 4: adequate; 5: unequal
Fan et al. (2007)	China (Chinese Hans)	140 women who had at least two unexplained spontaneous abortions	140 women with at least one live birth and no history of miscarriages, pregnancy-associated complications or other systemic diseases	eNOS (B/A) eNOS (Glu298Asp)	**BB:BA:AA** (case) 100:38:2 (control) 115:24:1 **GG:GT:TT** (case) 98:37:5 (control) 106:30:4	1: adequate; 2: not stated; 3: adequate; 4: adequate; 5: unequal
Karvela et al. (2008)	Greece (Greeks)	126 women who had at least three unexplained spontaneous abortions before 20 weeks of gestation	130 women with at least two live births and without history of abortions	eNOS (B/A) eNOS (Glu298Asp)	**BB:BA:AA** (case) 95:30:1 (control) 95:31:4 **GG:GT:TT** (case) 53:57:16 (control) 62:58:10	1: adequate; 2: not stated; 3: adequate; 4: inadequate; 5: unequal
Zammiti et al. (2008)	Tunisia (Tunisians)	350 women with three or more unexplained consecutive pregnancy losses	200 healthy women with uncomplicated pregnancies	eNOS (B/A) eNOS (Glu298Asp)	**BB:BA:AA** (case) 231:97:22 (control) 146:46:8 **GG:GT:TT** (case) 256:83:11 (control) 157:39:4	1: adequate; 2: not stated; 3: adequate; 4: adequate; 5: unequal
Al Sallout et al. (2010)	Palestine (the same ethnicity in Gaza Strip)	100 women with at least 3 unexplained consecutive spontaneous miscarriages before 25 weeks of gestation	100 healthy women had delivered at least one healthy, term infant and had no history of pregnancy loss	eNOS (B/A)	**BB;BA:AA** (case) 65:30:4 (control) 67:32:0	1: adequate; 2: not stated; 3: adequate; 4: not clearly described; 5: unequal

Continued

Table III *Continued*

Author (Year)	Country	Cases	Controls	Genotype	Genotype frequency (number of samples)	Quality assessment
Shin *et al.* (2010)	Korea (Korean)	340 women with at least three consecutive spontaneous abortions	115 women had at least one live birth and no history of pregnancy loss	eNOS (B/A) eNOS (Glu298Asp)	**BB:BA:AA** (case) 275:63:2 (control) 90:24:1 **GG:GT:TT** (case) 266:60:14 (control) 103:12:0	1: adequate; 2: not stated; 3: adequate; 4: not described; 5: unequal

Quality assessment codes: 1 = description of cases and controls; 2 = assessment and validation of the RPL in the patients; 3 = description of laboratory methods; 4 = Elimination of confounding factors in patients; 5 = equal assessment of confounding factors in both groups. Common genotypes of eNOS provided in each study were shown in boldface.

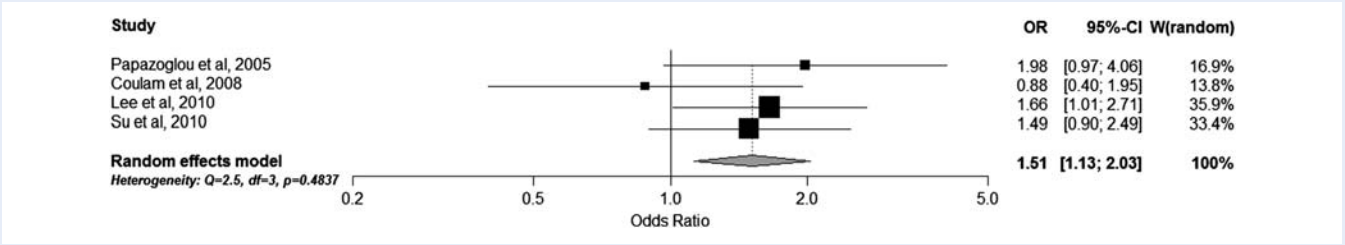

Figure 1 Association of VEGF polymorphism (−1154G/A) and RPL under a dominant genetic model. Results of individual and summary OR estimates, 95% CI and weights (W) of each study are shown. Horizontal lines represent 95% CI and dotted vertical lines represent the value of the summary OR.

Table IV Results of meta-analyses.

Genotype	Genetic model	Summary OR (95%CI)	Data from Zammitti *et al.* excluded
VEGF (−1154G>A)	Dominant	**1.51 (1.13–2.03)**	NA
	Recessive	1.24 (0.4–3.91)	
P53 (codon 72)	Dominant	1.13 (0.7–1.82)	NA
	Recessive	**1.84 (1.07–3.16)**	
eNOS (B/A)	Dominant	1.15 (0.94–1.41)	1.12 (0.88–1.42)
	Recessive	1.03 (0.54–1.95)	0.59 (0.23–1.52)
eNOS (Glu298Asp)	Dominant	**1.37 (1.11–1.69)**	**1.38 (1.08–1.77)**
	Recessive	1.34 (0.84–2.16)	1.30 (0.77–2.19)

Significant values were shown in boldface.

frequencies are similar in different studies of the same ethnicity, very different allele/genotype frequencies of the four polymorphisms exist in different ethnic groups (Tables I–III). For example, the p53 Arg/Pro allele frequencies are 0.75/0.25 in Caucasians (Pietrowski *et al.*, 2005; Kaare *et al.*, 2009) and 0.45/0.55 in Iranians (Firouzabadi *et al.*, 2009); the genotype frequencies of ArgArg/ArgPro/ProPro are 0.56–0.58/0.35–0.40/0.04–0.07 in Caucasians and 0.12/0.66/0.22 in Iranians. Even though genotype frequencies are diverse in different ethnicities, the integrated results still showed significant associations of VEGF (−1154G>A), p53 (codon 72) and eNOS (Glu298Asp) with RPL, suggesting these polymorphisms may have functional consequences in the etiology of RPL; however, the effects may vary in different populations.

Although the case–control study design is generally conducted in the majority of genetic association studies, the cohort design has more to recommend it. Cohort studies have several advantages over case–control studies in terms of exposure measurement. If exposure measurement occurs before disease occurrence, cohort studies are much less prone to differential measurement error. Exposure measurement error can lead to substantial bias in the estimated relative risk for the exposure-disease relation. Prospective data collection should also reduce measurement error due to poor recall of past exposures (White *et al.*, 1998; Clayton and McKeigue, 2001). However, this problem does not have much impact on measurement of genotype if the objective is simply to study the associations between genotype and disease risks, whereas it may lead to misleading results if the objective is to explore gene-environmental interactions (Clayton and McKeigue, 2001). Another factor that could affect the validity of case–control studies is choosing a control group, and this could also greatly affect a study's vulnerability to bias. Controls should come from the same population as the cases, and the same eligibility criteria need to be applied to potential cases and controls to avoid selection bias (Lijmer *et al.*, 1999; Schulz and Grimes, 2002). In the present meta-analyses, normal controls in some studies included peri-menopausal or post-menopausal women who were clearly not from the same population as their RPL patients (Tempfer *et al.*, 2001; Hefler *et al.*, 2002; Papazoglou *et al.*, 2005;

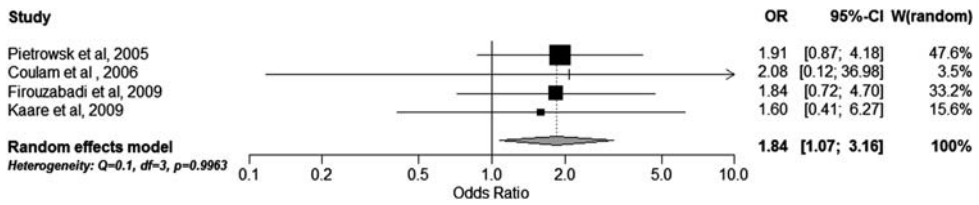

Figure 2 Association of p53 polymorphism (codon72) and RPL under a recessive genetic model. Results of individual and summary OR estimates, 95% CI and weights (W) of each study are shown. Horizontal lines represent 95% CI and dotted vertical lines represent the value of the summary OR.

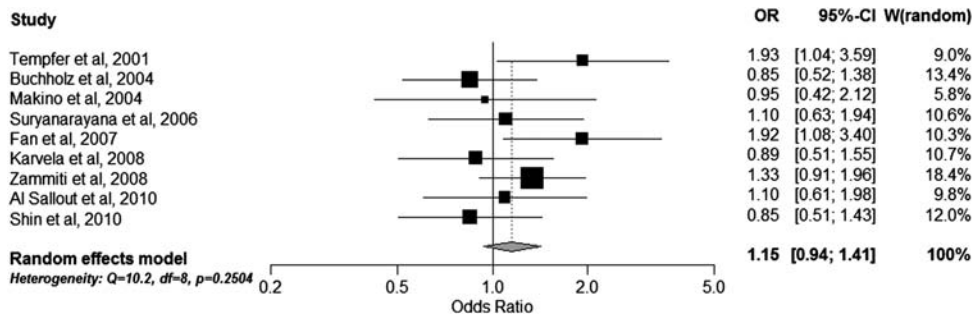

Figure 3 Association of eNOS polymorphism (B/A) and RPL under a dominant genetic model. Results of individual and summary OR estimates, 95% CI and weights (W) of each study are shown. Horizontal lines represent 95% CI and dotted vertical lines represent the value of the summary OR.

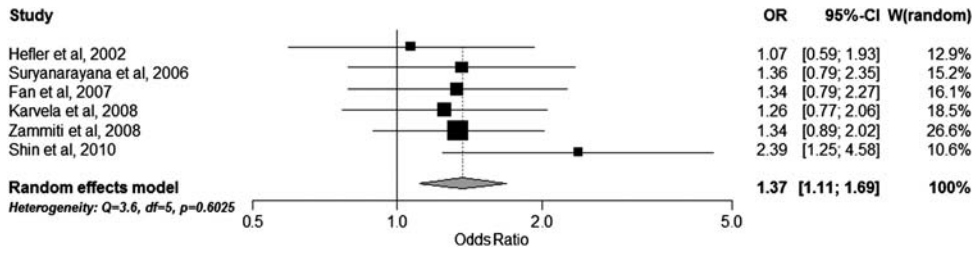

Figure 4 Association of eNOS polymorphism (Glu298Asp) and RPL under a dominant genetic model. Results of individual and summary OR estimates, 95% CI and weights (W) of each study are shown. Horizontal lines represent 95% CI and dotted vertical lines represent the value of the summary OR.

Pietrowski *et al*., 2005; Fan *et al*., 2007). Nevertheless, the genetic association effect on most common and complex disease is usually modest. For adequate statistical power and relatively low cost to detect such modest risk ratios, the case–control design is still more feasible than the cohort design (Clayton and McKeigue, 2001).

In the present meta-analyses, attempts were made to avoid general limitations such as selection bias and publication bias that might exist in the original papers. Selection criteria were rigorous and only those with a clear definition of RPL and with a reliable genetic method reported were considered. The search was comprehensive and systematic, and was performed by two authors independently. In all meta-analyses, a low probability of publication bias was observed after examination by funnel plots and Egger tests. Although no significant heterogeneities were observed among the included studies, we calculated the combined effects under random effect models. Regardless of the statistical methods applied previously, we tested all association studies with individual and combination ORs by using both dominant and recessive genetic models. In all meta-analyses, the combination of sample sizes in each study increased the statistical power and thus allowed a more precise estimate of risk evaluation.

In conclusion, the biological functions of these three genes are important in the regulation of angiogenesis and vasoconstriction in human pregnancy, and the present meta-analyses also show significant and consistently different associations between the VEGF (−1154G>A), p53 (codon 72), and eNOS (Glu298Asp)

polymorphisms and the occurrence of RPL. Although each polymorphism confers a small but significant increased risk, the data presented in this study add further evidence to the concept of idiopathic RPL as a polygenic disease. These angiogenesis- and vasoconstriction-related genes jointly contribute to idiopathic RPL, and the VEGF (−1154G>A), p53 (codon 72) and eNOS (Glu298Asp) polymorphisms may be useful clinical markers for evaluating the risk of RPL in routine testing.

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Authors' roles

M.-T.S.: Study conception, literature review, manuscript drafting and correspondence. S.-H.L. and Y.-C.C.: Literature review and statistical analysis.

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Conflict of interests

None of the authors have any conflict of interests to declare.

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