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Angiotensin-converting enzyme DD genotype, angiotensin type 1 receptor CC genotype, and hyperhomocysteinemia increase first-trimester fetal-loss susceptibility

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Complications of pregnancy have been found to be related with thrombophilic polymorphisms that explain about 30% of obstetric complications. We evaluated the angiotensin converting enzyme (ACE) and the angiotensin type 1 receptor (AT1R) gene polymorphisms in the renin–angiotensin system (RAS) as possible risk factors for fetal loss. Fifty-nine women with a history of three or more first-trimester fetal losses and 70 healthy women with a history of normal pregnancies were enrolled in this study. Thrombophilic factors, ACE insertion/deletion (I/D) and AT1R A1166C polymorphisms, prothrombin G20210A and factor V Leiden mutations were analyzed. At univariate and multivariate analysis, a significant association between ACE DD and AT1R CC genotype and fetal loss was observed. The effect of the ACE DD genotype on the risk of fetal loss was higher in AT1R C allele carriers. The prevalence of hyperhomocysteinemia (Hcy) (defined as baseline plasma levels higher than the 95% percentile; cut-off, 10.5 μmol/l per l) was significantly higher in women with fetal loss, and an association between Hcy and fetal loss was detected. All patients showed normal antithrombin, protein C, protein S, and plasminogen activator inhibitor-1 (PAI-1) values. The presence of one risk factor not associated with others was found in 33 out of 59 patients (56%); ACE DD genotype was the most prevalent risk factor. Our results identify new possible predictive markers for fetal loss in RAS polymorphisms and Hcy. Large-scale studies are warranted to attribute clinical relevance to these polymorphisms as risk factors for complicated pregnancies. *Blood Coagul Fibrinolysis* 11:657–662 © 2000 Lippincott Williams & Wilkins.

Keywords: fetal loss, thrombophilic factors, angiotensin converting enzyme polymorphism, angiotensin type 1 receptor polymorphism

Introduction

Obstetrical complications such as severe pre-eclampsia, fetal growth retardation, stillbirth and fetal loss contribute to maternal and fetal morbidity and mortality. The causes have not been completely elucidated, but they have recently been associated with abnormal placental vasculature [1] and disturbances of hemostasis [2]. The renin–angiotensin system (RAS) is one of the main factors regulating

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blood pressure, fluid and electrolyte balance. Placental and fetal membranes are important production sites of the angiotensin converting enzyme (ACE) and angiotensinogen (AGT), and contain high concentrations of angiotensin II receptors [3]. In addition, angiotensin II may increase the production and secretion of the plasminogen activator inhibitor-1 (PAI-1) by endothelial cells [4]. ACE may also increase platelet function because ACE inhibitors have been shown to reduce platelet aggregation in humans [5].

An insertion/deletion (I/D) polymorphism in the ACE gene has been associated with serum ACE activities: subjects with DD ACE genotype had higher levels than those with the ACE II genotype, and intermediate levels were observed in heterozygote ID [6]. In pregnancy-induced hypertension, elevated serum ACE activity was found [7–9], but no effect of ACE gene polymorphism on pregnancy outcome or the incidence of pregnancy-induced hypertension has been found in Caucasian women. In a series of Caucasian women with pregnancy-induced hypertension, a significant association of pre-eclampsia with the AGT M235T polymorphism has been observed. No data are available on the A1166C polymorphism in the gene encoding for the angiotensin II type 1 receptor (AT1R).

A higher prevalence of thrombophilic polymorphisms such as factor V (FV) Leiden mutation and factor II (FII) G20210A mutation has been reported in women with fetal loss, pre-eclampsia, abruptio placentae and fetal growth retardation [10,11]; however, genetic thrombophilic polymorphisms account for about 30% of obstetrical complications [12].

In this study, we have evaluated RAS ACE and AT1R polymorphisms as possible risk factors for pathological pregnancies and for their possible contribution to the occurrence of fetal loss.

Materials and methods

From April 1998 to March 1999, we enrolled 59 consecutive women with a history of fetal loss referred to the Prenatal Medicine Unit of the University of Florence. Women fulfilled the following inclusion and exclusion criteria. Inclusion criteria were three or more first-trimester (7–12 weeks of gestation) fetal losses. All were in good general health without previous history of venous or arterial thromboembolic disease, fetal growth retardation and abruptio placentae. The exclusion criteria were hypertension, previous pre-eclampsia, diabetes mellitus or thyroid dysfunction, the presence of congenital malformations or chromosomal abnormalities in the fetus, recent cytomegalovirus infection or drug abuse during pregnancy and essential thrombocytemia [13].

They all underwent a thorough investigation that was negative for potential causes of fetal demise including fasting glucose, basal follicle stimulating hormone (FSH), luteinizing hormone (LH) and estradiol levels on day 3 of a natural cycle, thyroid-stimulating hormone (TSH) and prolactin levels, and antinuclear factor. Screening tests for antiphospholipid antibodies included lupus anticoagulant sensitive activated antithromboplastin time, thromboplastin titration index, diluted Russel Viper Venom test, and immunoglobulin (Ig)G and IgM anticardiolipin and antiβ2-glycoprotein I. If one of these assays was positive, the patient was not included in the study The control group consisted of 70 healthy women matched for age, gravidity and smoking habits with a history of normal pregnancies.

All the subjects of the patient and control groups were Caucasian women from Central Italy (Tuscany). Personal and familial history of thromboembolic disease was determined by interviewing patients and controls. Both cases and controls were not pregnant at the time of investigation, and were not taking oral estroprogestinic preparations during the study. Six out of 59 patients had taken the pill before the index pregnancy. Informed consent was obtained in all cases and controls.

Peripheral venous blood samples were collected from the antecubital vein, with minimal stasis, in vacutainer tubes containing 0.129 mol/l sodium citrate, the final blood/anticoagulant ratio being 9 : 1. The samples were centrifuged at $600 \times g$ for 10 min at 4°C, and the supernatant was drawn off and immediately stored at −80°C until analysis.

Mutations of the FV and FII gene were analysed as previously described [14,15]. The FV gene mutation was detected by polymerase chain reaction (PCR) amplification of genomic DNA followed by digestion with the restriction enzyme Mnl I. The G20210A mutation of FII gene was detected by PCR amplification with a mutagenic primer: the amplified fragment was digested by Hind III.

Antithrombin (AT) and protein C (PC) activities were determined with an Electra 1000C coagulation analyser using the chromogenic commercial kit (Antithrombin III, Protein C; DADE, Düdingen, Switzerland). The normal range was 80–120% for AT and 60–130% for PC. Protein S (PS) activity was measured by the clotting method with an ACL 300 Research coagulation analyser (Instrumentation Laboratory Company, Milan, Italy) using a commercial kit (Protein S; Instrumentation Laboratory).

The normal range was 55–130%. PAI-1 activity (control range, 3–15 IU/ml) was determined according to Chmielewska *et al.* [16], using a commercial kit (Spectrolyse (fibrin); Bio-Pool, Umea, Sweden).

The plasma levels of total homocysteine (free and protein bound) were determined by high-performance liquid chromatography (LKB 2248 pump; Pharmacia, Uppsala, Sweden) and fluorescence detection (Waters 474, Italy Waters, Vimodrone (MI) Italy). Median and range of control women were 7.5 and 3–15 μmol/l per l, respectively.

ACE and AT1R polymorphisms were analyzed after genomic DNA extraction from peripheral blood leukocytes using a QIAmp Blood Kit (QIAGEN, Hilden, Germany). The ACE I/D polymorphism was genotyped according to Rigat *et al.* [17]. DNA was amplified with 5% dimethylsulfoxide (DMSO) in the reaction mixture at an annealing temperature of 60°C in order to reduce the incidence of mistyping ID as DD. Moreover, each DD genotype was subjected to a second PCR amplification without the 5% DMSO at an annealing temperature of 67°C and using a primer pair that recognizes the insertion-specific sequence. These modifications were made to reduce underestimation of heterozygotes [18]. The A1166C polymorphism of the AT1R gene was identified by PCR amplification and restriction fragment length polymorphism analysis, using primers and PCR conditions as described by Katsuja *et al.* [19].

Statistical analysis

Statistical analysis was performed by using the program Statistica 6.0 (StatSoft Italia s.r.l., Vigonza (Padova) Italy) for IBM. The ACE and AT1R allele and genotype frequencies in patients and controls were obtained by direct count. The genotype distribution and allele frequencies between groups were compared by the χ^2 test. Association between variables was tested using Fisher's exact test or the χ^2 test. The estimate of odds ratios (OR) was obtained by univariate regression analysis. Multiple regression analysis was used to evaluate the influence of ACE, AT1R, FV genotype and homocysteine levels on the occurrence of fetal loss. The Hardy–Weinberg equilibrium for genotype distribution was estimated by the χ^2 test. $P < 0.05$ was considered statistically significant.

Results

The characteristics of patients and controls are presented in Table 1. The ACE and AT1R genotype distribution was compatible with the Hardy–Weinberg equilibrium. The genotype and allele frequencies of RAS genes are shown in Table 2.

Table 1. Characteristics of study groups

	Patients ($n = 59$)	Controls ($n = 70$)
Age (years)	31 (23–40)	32.5 (21–41)
Gravidity	3 (3–5)	2 (2–5)
Smoker (%)	14	15

Values represent the median, with the range in parentheses.

ACE D allele frequency was significantly ($P = 0.013$) higher among women with fetal loss than in the control group (65 versus 50%). A significant association between the ACE DD genotype and pregnancy loss was observed [OR(DD/ID + II) = 2.26, 95% confidence interval (CI) = 1.09–4.67; $P = 0.03$]. The difference of ACE I/D genotype distribution between cases and controls was not statistically significant ($P = 0.07$).

The AT1R C allele frequency was higher in women with fetal loss (40 versus 28%; $P = 0.04$), and the genotype distribution was significantly different between cases and controls ($P = 0.005$). An association between AT1R CC genotype and fetal loss [OR(CC/AC + AA) = 5.62, 95% CI = 1.75–18.08; $P = 0.002$] was observed.

The effect of the ACE DD genotype on the risk of fetal loss was higher in AT1R C allele carriers. The OR for fetal loss associated with the ACE DD genotype varied from 1.69 (95% CI = 0.57–5.05; $P = 0.34$) in subjects without the C allele (AA

Table 2. Distribution of renin–angiotensin system genotypes and allele frequencies

	Patients ($n = 59$)	Controls ($n = 70$)	P value
ACE genotype			
DD	28	20	0.07
ID	21	30	
II	10	20	
ACE allele frequency			
D	0.65	0.50	0.013
I	0.35	0.50	
AT1R genotype			
CC	15	4	0.005
AC	17	31	
AA	27	35	
AT1R allele frequency			
C	0.40	0.28	0.04
A	0.60	0.72	

ACE, Angiotension converting enzyme; AT1R, angiotensin type 1 receptor.

homozygotes) to 2.81 (95% CI = 1.03–7.61; $P = 0.04$) in AT1R C allele carriers (AC heterozygotes and CC homozygotes).

Six out of 59 (10%) women with fetal loss were positive for FV Leiden mutation (heterozygotes or homozygotes). The prevalence of FV Leiden tended to be higher in patients than in controls, although this did not make statistical significance (Fisher exact test; $P = 0.14$) (Table 3). No difference was found in the prevalence of either FII G20210A mutation or PC, PS and AT deficiencies between patients and controls (Table 3).

High PAI-1 levels were seen in 6% of controls and in 10% of patients. The difference in percentage of high PAI-1 levels between controls (6%) and patients (10%) was not statistically significant (Fisher's exact test; $P = 0.51$). No significant association was found between ACE DD and AT1R CC genotype and PAI-1 plasma levels (Fisher's exact test; $P = 1.00$ and $P = 1.00$, respectively).

Twelve out of 59 patients (20%) had homocysteine plasma levels higher than the 95% percentile. We observed a significant difference in the prevalence of hyperhomocysteinaemia between women with pregnancy loss and control subjects (Fisher's exact test; $P = 0.016$) (Table 3), and an association between hyperhomocysteinemia and fetal loss [OR = 4.21, 95% CI = 1.28–13.88; $P = 0.02$] was detected.

In 14 out of 59 patients and in 31 out of 70 controls, none of the tested risk factors was found. The presence of one risk factor not associated with others was found in 33 out of 59 patients (56%); ACE DD genotype was the most prevalent risk factor. Figure 1 reports the number of patients with each of several different risk factors, singly and in combination.

By multiple regression analysis, ACE DD and AT1R CC genotype and hyperhomocysteinemia were found to be independent risk factors for fetal loss (Table 4).

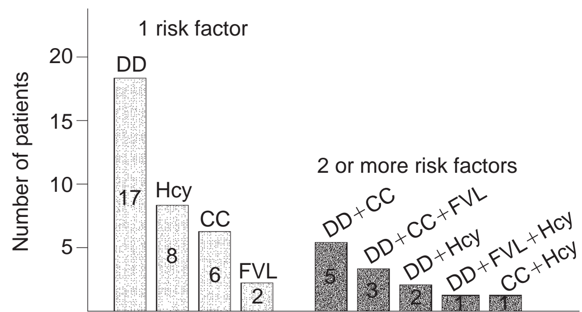

Figure 1. Number of patients with one or more thromboembolic risk factors. Hcy, Hyperhomocysteinemia; FVL, factor V Leiden.

Discussion

This study is the first report demonstrating that RAS gene polymorphisms are associated with first-trimester fetal loss. ACE DD and AT1R CC genotypes account for more than 50% of fetal loss, whereas a thrombophilic condition accounts for about 10% and hyperhomocysteinemia for 20%. However, we observed that the AT1R C allele increases the risk associated with the ACE DD genotype, showing a synergistic effect on the occurrence of fetal loss, as previously described for coronary disease [20,21].

Tamura *et al.* [7] evaluated the role of ACE I/D polymorphism in pre-eclampsia, but the ACE DD genotype was not found to be a risk factor for pregnancy-induced hypertension. In this study, we found not only that the ACE gene polymorphism plays a significant role in the risk of first-trimester fetal loss, but also that a polymorphism in the gene encoding for angiotensin II type 1 receptor is associated with the risk of fetal loss. The multiple regression analysis provided evidence of the ACE DD and AT1R CC genotypes as independent predictors of fetal loss.

The RAS plays an important role in the regulation

Table 3. Risk factors for fetal loss considered in the present study

	Patients (n = 59)	Controls (n = 70)	P value
Factor V Leiden mutation	6 (10%)	2 (3%)	0.14
Hyperhomocysteinemia	12 (20%)	4 (6%)	0.016
Factor II G20210A mutation	1 (1.7%)	1 (1.4%)	1.00
Protein C deficiency	–	–	
Protein S deficiency	–	–	
Antithrombin deficiency	–	–	

Table 4. Odds ratio for fetal loss: results of the univariate and of the multivariate logistic regression analysis

Variables	Univariate logistic regression analysis			Multivariate logistic regression analysis		
	Odds ratio	95% CI	*P*	Odds ratio	95% CI	*P*
ACE DD genotype	2.26	1.09–4.6	0.03	2.37	1.58–3.15	0.03
AT1R CC genotype	5.62	1.75–18.08	0.002	5.63	4.41–6.84	0.006
Factor V Leiden mutation	3.85	0.75–19.85	0.14	2.34	0.44–4.24	0.38
Hyperhomocysteinemia	4.21	1.28–13.88	0.02	6.09	4.86–7.32	0.005

In the multivariate model, odds ratios for each variable are adjusted for the three other variables. CI, Confidence interval; ACE, angiotension converting enzyme; AT1R, angiotensin type 1 receptor.

of vascular fibrinolytic balance [22]. The ACE is strategically positioned to regulate the balance of the fibrinolytic elements by producing angiotensin II [22]. Angiotensin II, in addition to being a potent vasoconstrictor and to promoting smooth muscle cell proliferation and intimal thickening, may increase plasma PAI-1 levels in humans [23]. This latter effect might constitute a link between the RAS and vascular placental occlusion, although in the present study we observed high plasma PAI-1 levels in few patients. A further mechanism by which ACE can influence an occlusive tendency in placental vasculature is the release of tissue factor; Soejima *et al.* [24] demonstrated that the administration of ACE inhibitors significantly affects plasma tissue factor levels and, in an *in vitro* study, Nishimura *et al.* showed that angiotensin II increases tissue factor mRNA expression by cultured endothelial cells [25].

Recently, several reports demonstrate that thrombophilic polymorphisms are associated with an increased risk of spontaneous fetal loss, and that the combination of thrombophilic polymorphisms further increases the risk of unexplained fetal loss [10]. In this study, the prevalence of FV Leiden mutation in patients with a history of complicated pregnancy is lower than that previously reported by other studies and by ourselves [10,11,26]. This may be due to the investigation of second- and third-trimester fetal loss, whereas in this study we included only first-trimester fetal loss. The lack of association of FII G20210A mutation and fetal loss is in keeping with previous studies [10,11]. However, given the small size of the samples examined, the results cannot be considered conclusive.

Finally, this study adds further weight to the role of hyperhomocysteinemia in fetal loss, as suggested by the Hordaland Homocysteine Study [27] and by the association between MTHFR 677TT and pregnancy loss [10].

In conclusion, our results identify new possible predictive markers for fetal loss in RAS gene polymorphisms and in hyperhomocysteinemia. Large-scale studies are warranted to attribute clinical relevance to these polymorphisms as risk factors for complicated pregnancies.

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