Recurrent pregnancy failure is associated with a polymorphism in the p53 tumour suppressor gene

Detlef Pietrowski¹,³, Hertha Bettendorf¹, Eva-Katrin Riener¹, Christoph Keck¹, Lukas A.Heffer², Johannes C.Huber² and Clemens Tempfer¹,²

¹Department of Obstetrics and Gynecology, University of Freiburg School of Medicine, Freiburg, Germany and ²Department of Obstetrics and Gynecology, University of Vienna School of Medicine, Vienna, Austria

³To whom correspondence should be addressed at: University of Freiburg School of Medicine, Freiburg, Hugstetter Strasse 55, D-79106 Freiburg, Germany. E-mail: pietrowski@frk.ukl.uni-freiburg.de

BACKGROUND: The p53 tumour suppressor gene is a well-known factor regulating apoptosis in a wide variety of cells and tissues. Alterations in the p53 gene are among the most common genetic changes in human cancers. In addition, recent data provide evidence that p53 plays a critical role in mediating pregnancy by regulating steroid hormone activation. In idiopathic recurrent miscarriages (IRM), causes and associations are much debated as the exact pathophysiological mechanisms are unknown. In this study, we assess whether an established polymorphism in the p53 gene is associated with the occurrence of IRM.

METHODS: Genotyping was performed by PCR-based amplification of the p53 Arg and Pro variants at codon 72 in 175 cases of IRM and 143 controls.

RESULTS: We observed a statistically significant association between carriage of the Pro allele and the occurrence of IRM (P < 0.03, odds ratio 1.49, confidence interval 1.04–2.14). Distribution of genotypes was in Hardy–Weinberg equilibrium.

CONCLUSIONS: Our results indicate an over-representation of the Pro allele of the p53 gene in women with IRM, giving support to the theory that p53 has a potential role during pregnancy.

Key words: apoptosis/genotyping/idiopathic recurrent miscarriage/p53/pregnancy

Introduction

Recurrent miscarriages, defined as at least three consecutive miscarriages, are an aetiological enigma. There is much debate about cause and association since the exact pathophysiological mechanisms of this disorder are unknown. Presumed aetiological factors include endocrine dysfunction such as hypothyroidism and luteal phase inadequacy, chromosomal aberrations, uterine abnormalities and infectious disorders, and these factors are present in ~50% of all women with recurrent miscarriage (Clifford et al., 1994; Hatasaka, 1994). In cases of idiopathic recurrent miscarriage (IRM), polymorphisms have been proposed as susceptibility factors, increasing the chances of miscarriage in otherwise healthy women (Tempfer et al., 2001; Pietrowski et al., 2003).

Pregnancy is dependent on adequate placental circulation. The development of a normal functioning placental vascular network requires a remarkable degree of coordination between different vascular endothelial cell-specific growth factors and cell types and is exquisitely dependent upon signals exchanged between these cells. Abnormalities of placental vasculature may result in a number of gestational pathologies including pregnancy loss, intrauterine fetal death (IUFD), intrauterine growth restriction (IUGR), placental abruption, and pre-eclampsia (Salafia et al., 1995).

The p53 tumour suppressor gene encodes a multifunctional transcription factor that is activated by stress stimuli, including DNA damage and hypoxia. It is a well-known factor regulating apoptosis in a wide variety of cells and alterations in p53 are among the most common genetic changes in human cancers. However, beside its role as a tumour suppressor gene, p53 plays a critical role in regulating angiogenesis (Ravi et al., 2000; Yuan et al., 2002). Alterations in the p53 gene product have been shown to be a potent inducer of angiogenesis via the Hypoxia inducible factor 1α (Hif1α) and vascular endothelial growth factor (VEGF) pathway (Dameron et al., 1994). Recently, it was shown that p53 is a potential mediator of pregnancy by estrogen and progesterone activation (Sivaraman et al., 2001). Dysfunction of p53 may lead to accumulation of cytoplasmic p53 which in turn may lead to immunity to abnormally expressed p53 as revealed by autoantibodies in the blood. Alterations in the p53 gene product may be caused by polymorphic sites within the gene. A common sequence polymorphism is located within the proline-rich domain of p53 encoding either proline or arginine at position 72. These variants have been reported to differ in functional activity. The Arg72 variant is believed to be a better suppressor of cellular transformation and was found to be more susceptible to degradation by the human papilloma virus (HPV) 18 E6 protein (Storey et al., 1998; Thomas et al., 2000).
1999). These studies imply that there is a functional difference between the codon 72 polymorphic variants and suggests that a closer analysis of the Pro72 and Arg72 variants is warranted. We hypothesized that the polymorphism in the p53 gene is associated with IRM by influencing p53-mediated function during pregnancy. We thus investigated the frequency of the p53 codon 72 variants in a Middle-European white population of women with a history of IRM and in control population of women with no history of recurrent miscarriage.

Materials and methods

Patients
Between February 2000 and August 2003, 214 women with recurrent miscarriage (RM) referred to the Outpatients Clinic for Recurrent Miscarriages at the University of Vienna School of Medicine were screened for this study. Diagnosis of RM was based on a documented history of at least three spontaneous, consecutive miscarriages before 20 weeks of gestation with the same partner. All of these women underwent a standard diagnostic work-up to rule out a verifiable cause of the recurrent miscarriages prior to inclusion into the study. Diagnostic procedures included hysteroscopy, paternal and maternal karyotype, cervical cultures for chlamydia, ureaplasma and mycoplasma, a comprehensive hormonal status (estradiol, progesterone, testosterone, dehydroepiandrosterone, androstendione, FSH, LH), and evaluation of antiphospholipid syndrome with IgM and IgG anticardiolipin antibody assessment and lupus anticoagulant testing. Of 214 women, six did not meet the criteria for RM. Of the remaining 208 women, 33 were excluded from this study because a suspected cause for RM was identified during the diagnostic work-up, i.e., an abnormal karyotype in four cases, uterine anomalies in 22 cases, and antiphospholipid syndrome in seven cases. Women with local cervical infections, defined as positive cultures for chlamydia, ureaplasma and mycoplasma, were not excluded from the study.

To avoid confounding by ethnicity, only white Caucasian women were included in the study and control groups. To avoid confounding by genetic admixture, only women whose parents were of the same ethnicity were included in the study and control groups. A total of 175 women was included in the study group.

Primary RM was defined as no history of a pregnancy carried beyond 20 weeks of gestation and at least three spontaneous miscarriages. Secondary RM was defined as a history of at least one pregnancy carried beyond 20 weeks of gestation and at least three spontaneous, consecutive miscarriages before 20 weeks of gestation with the same partner.

The control group consisted of 143 women with at least one live birth and no history of RM. Controls were recruited from the Outpatients’ Clinic for Post-menopausal Disorders at the University of Vienna School of Medicine. The reason for referral was evaluation of, counselling on, and therapy of peri- and post-menopausal signs and symptoms. All women in the control group were post-menopausal to rule out possible future miscarriages after inclusion in the study. Written informed consent was obtained from participating women. Local ethics committees approved the study.

Genetic studies
Blood was drawn from the antecubital vein and DNA was extracted using the Puregene System (Gentra Systems, USA). DNA were stored at 4°C until analysed. Analysis of the p53 genotype at codon 72 was performed as described by Nagpal et al. (2002) with some modifications. We used the primers GCCAGAGGCTGCTCCCCC and CTGGTCACAGACTT for amplification of the Pro codon and primers TCCCCCTTCGCTCCCCA and CTGGTGCA-GGGGCCACGC for amplification of the Arg codon.

PCR conditions comprised an initial denaturing step at 94°C for 5 min, followed by 45 cycles of 94°C for 30s, 55°C for 30s and 72°C for 45 s and a final extension at 72°C for 5 min. Reaction products were fractionated on a 2% agarose gel and visualized after ethidium bromide staining with a raytest digital camera system (Raytest, Germany).

ELISA analysis
Enzyme-linked immunosorbent assay (ELISA) for p53 autoantibody detection was performed according to the manufacturer’s instructions (Biodiagnostics, Germany).

Statistical analysis
Differences in the frequencies of the p53 alleles in the study and control groups were analysed by use of χ²-test. The odds ratio (OR) was used as a measure of the strength of the association between allele frequencies and IRM. We calculated the power to detect a difference between IRM and control women for the p53 Arg→Pro polymorphism. For the calculation, we used a total of 318 women and we achieved a power of 30% to detect a 30% difference in genotype frequencies at an α of 0.05 using the Yates correction factor. All P-values are two-tailed and 95% confidence intervals (CI) were calculated. P < 0.05 was considered statistically significant.

Results
The characteristics of women with IRM and controls are given in Table I. Median age at the time of blood sampling was 57 years (range 42–68). The p53 Arg→Pro allele frequencies and genotypes of women with IRM and controls are given in Table II. p53 Arg→Pro allele frequencies among women with IRM and controls were 67.4 and 75.5% respectively for the Arg allele, and 32.6 and 24.5% respectively for the Pro allele. There was a statistically significant association between carriage of the Pro allele and the occurrence of IRM (P = 0.03, OR 1.49, CI 1.04–2.14). These allele frequencies are in accordance with previously published allele frequencies in Caucasian populations (Wennberg et al., 2002). The homozygous Pro/Pro genotypes as well as the heterozygous Arg/Pro genotypes were found more often among women with IRM (12.6 and 40% respectively) compared to controls (7 and 35% respectively). This difference, however, was not significant (P = 0.07, OR 1.53; CI 0.95–2.45) using a dominant genotype model (Arg/Arg versus Pro/Pro + Arg/Pro).

<table>
<thead>
<tr>
<th>Table I. Characteristics of women with idiopathic recurrent miscarriage (IRM) and controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Women with IRM</td>
</tr>
<tr>
<td>Age at miscarriage evaluation (years)</td>
</tr>
<tr>
<td>No. of miscarriages</td>
</tr>
<tr>
<td>No. of live births</td>
</tr>
<tr>
<td>No. of primary aborters (%)</td>
</tr>
<tr>
<td>No. of secondary aborters (%)</td>
</tr>
</tbody>
</table>

*Median (range).
The distribution of genotypes in our population was in Hardy–Weinberg equilibrium.

In a subset analysis comparing the genotype frequencies between women with primary and secondary IRM, no statistically significant difference was found. The genotype frequencies of the homozygous Pro/Pro genotypes and the heterozygous Arg/Pro genotypes were not significantly different between women with primary IRM (8.1 and 32.0% respectively) and secondary IRM (11.5 and 41% respectively) ($P = 0.1$).

Serum levels of p53 autoantibodies were determined in a subset of 30 women with IRM and 30 controls. We did not detect p53 autoantibodies in any of these serum samples.

### Discussion

We have investigated the association between a polymorphic site in the proline-rich region of the human p53 gene and the occurrence of IRM. We used a PCR-based assay for the separate amplification of the Arg and Pro codons of the p53 variants. Our results indicate that women carrying the Pro allele have a significantly higher risk of IRM than women with the Arg allele. The p53 Pro allele was reported to be associated with a lower potential to induce apoptosis compared to the p53 Arg allele (Dumont et al., 2003). However, it was also reported to induce a higher level of G1 arrest than the Arg 72 codon (Pim and Banks, 2004) and is associated with a higher risk for albuminuria (McDonald et al., 2002) among Aboriginal Australians. In addition, Savion et al. (2002) suggest a role for p53- and bcl-2-mediated apoptosis in pregnancy loss in mice.

Our findings of an increased risk of IRM for women carrying the Pro allele is in line with the hypothesis that changes of the proline-rich region of p53 may alter the potential of the protein to regulate processes in the cell involved in apoptosis or cell cycle arrest. Fetal growth and development depend on intact placental function. Maintenance of placental structure and differentiation is essential for the provision of adequate gas, nutrient and waste exchange between the fetus and its mother. Placental trophoblast and placental reorganization is a ongoing process during pregnancy. Therefore apoptosis and cell proliferation is frequently observed during pregnancy in blood vessel cells, cytotrophoblasts and trophoblasts of the placenta. Imbalances in these highly regulated processes of tissue or cell differentiation caused by an increased number of cells arrested at the G1 checkpoint, as may occur in the p53 Pro variants compared to the p53 Arg variants, might to some extent cause inadequate supply of nutrients, gases or waste exchange between mother and fetus, leading to preterm abortion.

A second possible explanation for the observed increase of IRM for p53 Pro carriers might be their higher potential to resist apoptosis. Because placental developmental is a dynamic process of cell proliferation and cell degradation, it might be a disadvantage if cells respond with a later onset or to a lower extent in terms of necessary physiological apoptosis in the p53 Pro allele carriers compared to the Arg carriers. The lower level of apoptosis in these carriers might lead to misguided growth of cells or tissues determined to be degraded by intrinsic apoptotic stimuli. However, these possible explanations are largely speculative and a more extensive study on how the p53 Pro variant contributes to preterm abortion is required.

It has to be acknowledged that the design of our study has limitations. The selection of the control group, namely post-menopausal women with proven fertility, may cause selection bias in that these women are not representative of the general population. In addition, a number of association studies investigating polymorphisms as possible risk factors for IRM have been published by our group and others (Tempfer et al., 2001; Pietrowski et al., 2003). Looking for associations between numerous different polymorphisms implies also detecting a significant association accidentally. However, the important impact the Arg/Pro polymorphism seems to exert on the placental and embryonic development warrants closer attention to this polymorphism in further studies and meta-analysis of comparable studies. This has to be taken into account when assessing the results of this study.

In summary, this is the first report which demonstrates an association of IRM with the p53 codon Pro72 variant in 318 Caucasian women. This study adds to the growing list of genetic factors that may increase the susceptibility to miscarriage in otherwise healthy women.

### Table II. Genotypes and allele frequencies of the p53 Arg → Pro polymorphism among women with idiopathic recurrent miscarriage and controls

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Women with IRM* ($n = 175, 350$ alleles)</th>
<th>Controls* ($n = 143, 286$ alleles)</th>
<th>$P^b$</th>
<th>$\chi^2$</th>
<th>OR (95% CI)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotypes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg/Arg</td>
<td>83 (47.4)</td>
<td>83 (58)</td>
<td>0.07</td>
<td>3.140</td>
<td>1.53 (0.95–2.45)</td>
</tr>
<tr>
<td>Arg/Pro</td>
<td>70 (40)</td>
<td>50 (35)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro/Pro</td>
<td>22 (12.6)</td>
<td>10 (7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alleles</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg</td>
<td>236 (67.4)</td>
<td>216 (75.5)</td>
<td>0.03</td>
<td>4.631</td>
<td>1.49 (1.04–2.14)</td>
</tr>
<tr>
<td>Pro</td>
<td>114 (32.6)</td>
<td>70 (24.5)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Values in parentheses are percentages.

*b$\chi^2$-Test.

*cCalculation was performed following a dominant genotype model for Arg/Arg versus Pro/Pro and Arg/Pro.

IRM = idiopathic recurrent miscarriage; $n$ = number of women in each group; OR = odds ratio; CI = confidence interval.
References


Submitted on June 8, 2004; resubmitted on November 3, 2004; accepted on November 30, 2004

851