MDM2 309 polymorphism is associated with missed abortion

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BACKGROUND: In this study, we assessed whether the single nucleotide polymorphism in the murine double minute 2 (MDM2) promoter (SNP309) was associated with the occurrence of missed abortion.

METHODS: Genotyping of MDM2 SNP309 polymorphism was conducted by polymerase chain reaction-restriction fragment length polymorphism with blood and villous samples from 95 women diagnosed as having 1st trimester missed abortion.

RESULTS: The MDM2 SNP309 G/G genotype was associated with a higher risk of missed abortion compared with the T/T + T/G genotype in blood ($P = 0.010$; odds ratio (OR): 2.164; 95% confidence interval (CI): 1.207–3.878) and villous samples ($P = 0.043$; OR: 2.767; 95% CI: 1.092–7.011).

CONCLUSIONS: The MDM2 SNP309 G/G genotype may be a genetic risk factor for missed abortion.

Key words: missed abortion / MDM2 / polymorphism / pregnancy

Introduction

Missed abortion refers to a pregnancy in which there is a fetal demise without outside intervention, but the uterine activity is absent to expel the products of conception before 20 weeks of gestation (Griebel et al., 2009). Multiple etiologic factors, including parental chromosomal abnormalities, immunological factors, endocrinological disorders, uterine abnormalities, hereditary thrombophilia, infections and environmental factors, have been identified for missed abortion, and these conditions may occur in ~50% of all women with miscarriages (Clifford et al., 1994; Hatasaka, 1994). Apart from the above etiologic factors, gene polymorphisms have been proposed as a susceptibility factor that increases the chance of miscarriage in otherwise healthy women (Pietrowski et al., 2005).

Apoptosis has been shown to be critically important for the successful development of normal pregnancy (Jerzak and Bischof, 2002; Savion et al., 2002; Choi et al., 2003). During pregnancy, various genes co-ordinately regulate apoptosis and proliferation (Levy and Nelson, 2000). In particular, p53, a key regulator of apoptosis, has been shown to be expressed in first-trimester placenta (Quenby et al., 1998). Enhanced p53 expression and activity subsequently lead to induction of murine double minute 2 (MDM2) gene that may act either as an oncogene (Fakharzadeh et al., 1991; Dubs-Poterszman et al., 1995; Jones et al., 1998) or as a growth inhibitor (Brown et al., 1998). Specifically, enhanced MDM2 levels have been shown to cause p53 degradation in surviving cells and, consequently, the attenuation of the p53-mediated DNA damage response (Zauberman et al., 1995; Ries et al., 2000). This interplay between p53 and MDM2, characterized as the p53-MDM2 auto-regulatory feedback loop (Wu et al., 1996; LaRusch et al., 2007), plays an important role to induce either cell-cycle arrest at G1 or apoptosis in response to DNA damage (Ravi et al., 2000; Yuan et al., 2005).

Natural sequence variations in the MDM2 promoter may alter expression of the MDM2 protein. For example, a single nucleotide polymorphism in the MDM2 promoter has been shown to increase MDM2 expression by 2–3-fold (Toledo and Wahl, 2007). Recently, an intrinsic polymorphism in the MDM2 gene was shown to entail a T to G change at the 309th nucleotide in the first intron. Importantly, this MDM2 SNP309 G/G polymorphism enhanced promoter recognition by the transcription factor Sp1, which in turn caused elevated MDM2 expression and attenuation of the p53-mediated apoptotic response to cellular stresses including DNA damage (Bond et al., 2004; Hong et al., 2005).

To date, there is no evidence linking the MDM2 SNP309 polymorphism with events in pregnancy. In this study, we tested the hypothesis that this polymorphism may be associated with the risk of missed abortion.
Materials and Methods

Diagnosis
By ultrasound examination, the first-trimester missed abortion was defined as an intact gestational sac lacking any fetal cardiac activity (6 weeks after last menstrual period (LMP)), intrauterine gestational sac with the largest diameter exceeding 10 mm but devoid of yolk sac or an empty gestational sac with a confirmed gestational age of no < 6 weeks (Griebel et al., 2005).

Patients
This prospective observational study involved 95 pregnant women diagnosed with first-trimester missed abortion. All the pregnancies terminated in the first trimester <10 weeks from the LMP. All subjects underwent a standard diagnostic work-up to rule out any verifiable cause of missed abortion prior to inclusion into the study. The women were examined by ultrasoundography and had blood drawn for testing uterine abnormalities, chromosomal abnormalities, immunologic factors, infections, but all ended up with unexplained etiology. Among the 95 women who suffered from missed abortion, 36 had at least two prior miscarriages, 24 had one prior miscarriage and 35 experienced miscarriage for the first time. None of these women had had successful pregnancy. The control group consisted of 164 early pregnancy women with a healthy, viable intrauterine fetus and no prior miscarriage. Fetal cardiac activity and gestational age were confirmed by ultrasound. Written informed consent was obtained from all participating subjects. The study design was approved by the Ethical Committee of Shandong University.

Specimens
Blood samples were drawn from the antecubital vein and stored at 4°C before genomic DNA isolation.
Sixty villous samples from the missed-abortion group were collected by curettage or manual vacuum aspiration. Sixty-four villous samples from the control group were obtained by vacuum aspiration from women undergoing elective abortion at 7–10 weeks of gestation for social reasons. All the villous samples were stored at −80°C before genomic DNA isolation.

DNA preparation
DNA was extracted by using a DNA isolation kit (Tiangen, Beijing, People’s Republic of China). The DNA content and purity of each sample were analyzed by ultraviolet spectrophotometry (the E260/280 ratio ranging between 1.6 and 1.8), and a 10 ng DNA aliquot of each sample was used for polymerase chain reaction (PCR) amplification. DNA samples were routinely stored at −20°C.

PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of MDM2 SNP309 polymorphism
Primers used in this study have been described: 5′-CGCGGGAGT TCAGGTAAAG-3′ and 5′-AGCTGGAGACAAGTCAGGACTTAA-3′ (Ohmiya et al., 2006). In each 25 µl reaction, 10 ng genomic DNA was mixed with 1.25 U Taq Platinum Polymerase (Tiangen), 250 µmol/l each dNTP, 25 mmol/l Tris–HCl (pH 8.7), 10 mmol/l KCl, 2 mmol/l MgCl2 (Tiangen) and 10 µmol/l of each primer (previously described). The PCR conditions of MDM2 SNP309 were as follows: 94°C for 7 min, 35 cycles of 94°C for 40 s, 60°C for 40 s, 72°C for 40 s and a final extension of 72°C for 7 min. The assay for MDM2 polymorphism utilized an RFLP site for the enzyme MSPA1I (5′...CNG CKG...3′) (NEB, Beijing, People’s Republic of China). After restriction, 10 µl of digested samples were subjected to electrophoresis on 3% agarose gels. The gels were stained with ethidium bromide and photographed using an ultraviolet light transilluminator.

The possible outcomes were as follows: (i) if only one DNA fragment of 237 bp was observed, the patient was considered as T homozygous (T/T); (ii) if two DNA fragments of 189 and 48 bp were observed, the patient was considered as G homozygous (G/G); (iii) if three DNA fragments of 237, 189 and 48 bp were observed, the patient was considered as heterozygous (T/G). Several PCR products were sequenced to further validate the PCR results.

Statistical analysis
χ² test was used to analyze the genotype distribution of MDM2 SNP309 polymorphism between the missed-abortion and control groups. T-test was used to analyze the association of MDM2 SNP309 genotypes with the onset age of missed abortion women and controls. The odds ratio (OR) was used to measure the association between the genotype frequencies and risk of missed abortion. All P-values are two-tailed and 95% confidence intervals (CIs) were calculated. Significant difference was defined as P < 0.05.

Results
The average age of women in the missed-abortion group was 29.9 (18–44) years, whereas the average age of controls was 27.4 (18–41) years. There was no significant association of the MDM2 SNP309 G/G or T/T genotype with the age of women experiencing missed abortion (P = 0.469 and P = 0.581, respectively) (Table I). Therefore, the MDM2 SNP309 genotypes were not associated with a nearly age for missed abortion.

MDM2 SNP309 genotypes of blood samples from both groups are shown in Table II. The G/G genotype was found more frequently among women who had missed abortion (32.63%) than among controls (21.05 versus 28.33%); conversely, both the homozygous G/G and heterozygous T/G genotypes were less common among women with missed abortion than among controls (53.66% versus 46.32%) (Table I).

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Missed abortion (years)</th>
<th>Controls (years)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>G/G</td>
<td>28.24</td>
<td>26.88</td>
<td>0.469</td>
</tr>
<tr>
<td>T/T</td>
<td>28.67</td>
<td>31.07</td>
<td>0.581</td>
</tr>
</tbody>
</table>

*Data were calculated by t-test.

MDM2 SNP309 genotypes of villous samples from both groups were shown in Table III. Similar to the blood samples, the G/G genotype was found more frequently in the villous samples of women with missed abortion (28.33%) than of controls (12.50%); conversely, both the T/T and T/G genotypes were less common among women with missed abortion than among controls (31.67 versus 40.625% and 46.32 versus 53.66%).

Table I MDM2 SNP309 genotypes associate with the age of women experiencing missed abortion

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40.00 versus 46.875%, respectively). A significant difference was observed in the comparison of the G/G genotype with the T/T + T/G genotypes (P = 0.043; OR: 2.767; 95% CI: 1.092–7.011), but not of the T/G genotype compared with the G/G + T/G genotypes (P = 0.352; OR: 0.677; 95% CI: 0.324–1.417) among women with missed abortion and controls.

**Discussion**

Apoptosis and cell proliferation are coordinately regulated during pregnancy (Halperine et al., 2000). One key regulator may be p53, a potent transcription factor that controls expression of multiple target genes involved in cell-cycle progression and apoptosis (Sivaraman et al., 2001; Carvajal et al., 2005). MDM2 is a p53 target gene, yet also feeds back to promote p53 degradation. Thus, loss of MDM2 results in constitutively active p53, which may have dire consequences to the cell and embryo (Ueno et al., 2002). On the other hand, expression of MDM2 has been shown to be induced by DNA damage, causing a reduction in activities and levels of p53 proteins (Arva et al., 2005). This observation led to a model that induction of MDM2 is delayed to allow p53 to arrest the cell cycle, so that DNA repair can occur prior to the resumption of normal DNA synthesis (Yuan et al., 2005).

Recently, a T to G substitution at the 309th nucleotide in the first intron of the MDM2 gene (SNP309) has been found to be associated with lower age for tumor onset and accelerated tumor formation in both hereditary and sporadic cancers. Cell lines homozygous for the G allele of SNP309 were shown to have attenuated p53 transcriptional and apoptotic responses (Bond et al., 2004). In early pregnancy, the behavior of trophoblasts has been characterized as ‘pseudo-malignancy’ (Quenby et al., 1998). The present study examined whether the genetic polymorphism of MDM2 SNP309 was associated with the risk of missed abortion, and our results clearly support the notion that variations in MDM2 SNP309 correlate with missed abortion.

In our study, we observed differences in genotype frequencies between the blood and villous samples of the control group (T/T 28.05 versus 40.625%; T/G 53.66 versus 46.875%; G/G 18.29 versus 12.50%). As opposed to the genotypes of maternal blood samples, the genotypes of villous samples likely represent fetal genotypes as determined by both parents. Importantly, the G/G genotype was found more frequently among women with missed abortion (32.63 and 28.33%) than among controls (18.29 and 12.50%) in both blood and villous samples. This profile is reminiscent of its distribution among Chinese ESCC patients and controls (26.7 and 20.5%, respectively), previously reported by Hong et al. (2005). Likewise, the MDM2 G/G genotype has been shown to be associated with enhanced risk of developing poorly differentiated and advanced ESCC compared with the T/G or T/T genotype.

In early pregnancy, villous cells may suffer from severe cellular stresses including DNA damage. Under stress conditions, MDM2 proteins would accumulate to a greater extent in SNP309 G/G cells, leading to destabilization of p53. Deficiency in p53 subsequently results in impaired apoptosis. The development of placental trophoblasts is a dynamic process of cell proliferation and apoptosis (Pietrowksi et al., 2005), and the balance between proliferation and apoptosis may be disrupted if cells with the MDM2 SNP309 G/G genotype become more resistant to apoptosis. Unregulated accumulation of cells may then contribute to abnormal fetal development and missed abortion.

Advancing maternal age is associated with a higher frequency of abnormal embryos (Warburton and Fraser, 1964). In our study, the average age of women experiencing missed abortion was 29.9 years, compared with 27.4 years for the controls. Therefore, the frequency

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Missed abortion (n = 95) (%)</th>
<th>Controls (n = 164) (%)</th>
<th>P</th>
<th>χ²</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T/T</td>
<td>20 (21.05)</td>
<td>46 (28.05)</td>
<td>0.239</td>
<td>1.204</td>
<td>0.684 (0.376–1.246)*</td>
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<td>T/G</td>
<td>44 (46.32)</td>
<td>88 (53.66)</td>
<td>0.302</td>
<td>1.021</td>
<td>0.745 (0.449–1.237)*</td>
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<td>G/G</td>
<td>31 (32.63)</td>
<td>30 (18.29)</td>
<td>0.010</td>
<td>6.096</td>
<td>2.164 (1.207–3.878)*</td>
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*Calculation was performed following a dominant genotype model for T/T versus G/G and T/G.

**Table III** MDM2 SNP309 genotypes of villous samples among women with missed abortion and controls

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Missed abortion (n = 60) (%)</th>
<th>Controls (n = 64) (%)</th>
<th>P</th>
<th>χ²</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T/T</td>
<td>19 (31.67)</td>
<td>26 (40.625)</td>
<td>0.352</td>
<td>0.722</td>
<td>0.677 (0.324–1.417)*</td>
</tr>
<tr>
<td>T/G</td>
<td>24 (40.00)</td>
<td>30 (46.875)</td>
<td>0.473</td>
<td>0.349</td>
<td>0.756 (0.370–1.541)*</td>
</tr>
<tr>
<td>G/G</td>
<td>17 (28.33)</td>
<td>8 (12.50)</td>
<td>0.043</td>
<td>3.890</td>
<td>2.767 (1.092–7.011)*</td>
</tr>
</tbody>
</table>

*Calculation was performed following a dominant genotype model for T/T versus G/G and T/G.

**Table II** MDM2 SNP309 genotypes of blood samples among women with missed abortion and controls

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Missed abortion (n = 95) (%)</th>
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</tr>
</tbody>
</table>

*Calculation was performed following a recessive genotype model for G/G versus T/T and T/G.
of missed abortion may also be associated with the increased maternal age. The association of the G/G or T/T genotype with missed abortion at younger age, however, was not statistically significant.

In conclusion, our findings support the hypothesis that the MDM2 SNP309 G/G genotype is associated with an increased risk of missed abortion. Further research is necessary to elucidate the molecular mechanisms of MDM2 in missed abortion.

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