# Elevated expressions of p53, CDKNA1, and Bax in placental villi from patients with recurrent spontaneous abortion

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**Abstract.** – OBJECTIVE: This study assessed the relationship between the p53-dependent apoptosis and recurrent spontaneous abortion (RSA).

PATIENTS AND METHODS: Thirty women with recurring miscarriages were enrolled as experimental group, and 30 women with normal reproduction served as control group. Immunohistochemistry was used to evaluate expression of p53 in villous tissue specimens. Further, expressions of CDKN1A and Bax mRNAs were evaluated by qPCR. TUNEL assay was utilized to document cell apoptosis.

**RESULTS:** Expression of p53 was significantly increased in chorionic villi of patients in experimental group (p < 0.05 vs. control group). Further, CDKN1A and Bax mRNA levels were elevated in experimental group (p < 0.05 vs. control group), and the cell apoptosis index was increased as well.

**CONCLUSIONS:** The p53-CDKN1A and p53-Bax signaling pathways appear to be activated in RSA. Thus, the apoptosis pathways controlled by p53 may be involved in the pathogenesis of RSA.

Key Words:

Recurrent spontaneous abortion, Apoptosis, p53, CDKN1A, Bax, mRNA, TUNEL, qPCR, Immunochemistry.

# Introduction

Recurrent spontaneous abortion (RSA) is defined as two or more consecutive pregnancy losses before the 20<sup>th</sup> week of gestation from the last menstrual period. It occurs in approximately 1% to 5% of women of reproductive ag<sup>1</sup>. The ethiopathogenesis of RSA is complicated and includes genetic defects, anatomical anomalies, endocrine disorders, immune factors, infection, thrombosis, and environmental factors<sup>1</sup>. At present, the pathogenesis is not clear in more than 50% of the patients. The p53 gene can induce cell growth retardation, apoptosis, defects in cell differentiation and DNA repair. Apoptosis is the basis of spontaneous abortion<sup>2</sup>. The p53 gene regulates downstream genes such as Bax and CDKN1A (cyclin-dependent kinase inhibitor 1A), which are both important in the apoptosis signaling pathways. Further, p53 may mediate expression of CDKN1A, leading to cell arrest in the G1 phase and subsequent cell apoptosis. Bax also promotes apoptosis via mitochondrial apoptosis pathway.

In the present study, we tested chorionic villus tissue of normal early pregnancy and unexplained RSA for cell apoptosis and expression of p53, as well as its downstream targets CDKN1A and Bax.

# **Patients and Methods**

#### Patients

Thirty patients (mean 31.4 (range 22-40) years old and pregnancy of 58.1 (45-77) days with unexplained RSA were included in the experimental group. All these women had a history of spontaneous abortion. The urinary hCG enzyme test was positive, and ultrasound inspection showed intrauterine pregnancy with no embryonic heartbeat. All patients had regular menstrual cycle, normal karyotype, no genital infection, and were negative for anti-cardiolipin, antinuclear, antisperm, and anti-endometrial antibody. No endocrine disease, no tuberculosis, and cardiovascular diseases history were present, and the patients had no poisonous or harmful exposure.

As control group, we included 30 patients who were 28.1 (22-40) years old with pregnancy of 46.2 (35-70) days and normal reproductive func-

tion. These patients were also positive for urinary hCG; ultrasound inspection showed intrauterine pregnancy, normal embryonic heart beat, and normal fetal development.

The study was approved by the Human Ethics Committee, and all study individuals signed written informed consents.

#### Specimen Collection and Preparation

All chorionic villus specimens were obtained by vacuum aspiration. Each specimen was divided into two parts. One part was flash-frozen in liquid nitrogen and used for qPCR as described below. Another part was fixed with 10% formalin overnight at room temperature and paraffin embedded. Slices of 5  $\mu$ m were prepared, mounted on glass slides with 3-aminopropyltriethoxysilane, and used for immunohistochemical staining.

# Expression of p53

Dewaxed tissue slices were processed by conventional immunohistochemistry methods. The p53 antibody (Santa Cruz Biotechnology, Dallas, TX, USA) was diluted 1:300 and incubated overnight at 4°C with tissue slices. The horseradish peroxidase-conjugated antibody (GBI, Los Angeles, CA, USA) was then incubated with slices for 2 hours at room temperature, followed by the DAB (3,3'-diaminobenzidine) color development reagent (10 min). Nuclei were counterstained with hematoxylin. The expression of p53 was evaluated in a double-blind manner. Expression of p53 in the nuclei, seen as claybank particles, was ranked positive. The number of positive was calculated, and percentage of positive cells determined in relationship to total cells. The ranking was as follows: a slide with < 5% positive cells was ranked as negative (-), 5% - 25% as weak positive (+), 25% - 50% as moderately positive (++), and > 50% as strong positive (+++). In addition, selected three fields (at magnification of  $\times 200$ ) of each slide were used to take images. The gray level of villus tissue area was evaluated using Image J software (National Institutes of Health, Bethesda, MD, USA).

#### Expression of CDKN1A and Bax mRNA

The CDKN1A and Bax mRNA expression was quantified using qPCR (quantitative Polymerase Chain Reaction). Total RNA was extracted from flash-frozen villus tissue using Trizol (Invitrogen, Carlsbad, CA, USA; 100 mg tissue/1 mL Trizol according to the manufacturer's protocol. RNA was reverse transcribed and used in qPCR along with the SYBR Premix Ex Taq kit (TaKaRa, Otsu, Japan). The primer sequences are shown in Table I. The samples were denaturated at 95°C for 30 sec, and then underwent 40 cycles of 95°C, 30 sec, 60°C, 30 sec, and 84°C, 1 sec. Fluorescence was read at the last step of each cycle. The  $2^{-\Delta Ct}$  method was used to calculate expressions of the CDKN1A and Bax mRNA in relation to expression of glyceraldheyde 3-phosphate dehydrogenase (GAPDH) mRNA as endogenous control.

## Apoptosis Assay

Apoptosis was detected using TUNEL assay (DeadEnd Fluorometric TUNEL System kit, Promega, Madison, WI, USA), according to the manufacturer's instructions. The slides were mounted using SlowFade Gold Antifade Reagent with DAPI (4',6-diamidino-2-phenylindole, Invitrogen). The blue (DAPI, nuclear counterstain) and green fluorescent images were captured and analyzed using Image J software. Under fluorescence microscopy, DAPI detects all nuclei, while cells exhibiting green fluorescent particles within the nucleus are apoptotic cells. Fluorescent images were taken 520 nm (green/TUNEL) and 430 nm (blue/DAPI), and merged using Photoshop CS5 (Adobe Systems, San Jose, CA, USA). Analysis of fluorescence ratio was done using Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MD, USA).

#### Statistical Analysis

To analyze study findings, SPSS13.0 statistical software (SPSS Inc., Chicago, IL, USA) was used. Study groups were compared using the t test.

## Results

#### Expression of p53 Gene in Villus Tissue

Immunohistochemistry results showed that expression of p53 was mainly present in the cell nuclei (Figure 1). p53 was negatively expressed in villus tissue of one patient from control group and weakly positive in 29 patients. By contrast, all samples from the experimental group patients were positive, out of which 7 were weakly positive, 3 intermediately and 20 strongly positive (Table II).

A semi-quantitative analysis of p53 expression in both study groups is shown in Figure 2 (p = 0.0013, experimental vs. control group).



Figure 1. Immunochemistry analysis of p53 expression in embryonic villus tissue of healthy pregnant women with induced abortion (*a*, *b*), and villus tissue from patients with RSA (*c*, *d*). p53 is seen as brown particles and is located both in cytoplasm and nucleus. The arrow shows cells positive for p53. Magnification  $\times 200$ .

| Table I. Primer sequen |
|------------------------|
|------------------------|

| Gene   | Sense or antisense primer | Primer sequences                             | Amplicon length |
|--------|---------------------------|--|-----------------|
| CDKN1A | Sense<br>Antisense        | GCAGCGGAACAAGGAGT<br>GGAGAAACGGGAACCAG       | 251 bp          |
| Bax    | Sense<br>Antisense        | CCCCCGAGAGGTCTTTTTCC<br>TGTCCAGCCCATGATGGTTC | 160 bp          |
| GAPDH  | Sense<br>Antisense        | GGCTACAGCAACAGGGTG<br>TTTGGTTGAGCACAGGGT     | 257 bp          |

# Expression of CDKN1A and Bax mRNA in Villus Tissue

Expression of CDKN1A mRNA was significantly higher in experimental group compared with control group (p = 0.0153; Figure 3). Further, Bax mRNA levels were also significantly higher in experimental group (p = 0.0189 vs. control group; Figure 4).

# Apoptosis in Chorionic Villus

The TUNEL assay showed that presence of apoptosis in trophoblast cells of chorionic villus tissue in both of control and experimental groups (Figure 5), however, the number of apoptotic cells was significantly higher in experimental group (p = 0.024; Figure 5).



**Figure 2.** Staining intensity of positive cells in control and experimental groups. \*\*p = 0.0013.



**Figure 3.** Expression CDKN1A mRNA in villus tissue of control (a) and experiment groups (b). \*p = 0.0153.

# Discussion

Spontaneous abortion is a common complication of pregnancy, accounting for 10%-15% of all pregnancies. However, once pregnant women had spontaneous abortion for the first time, the risk of repeated miscarriage is as high as 33.1%<sup>3</sup>. Apoptosis is a programmed cell death process and also part of all steps of embryonic development. In recent years, the relationship between apoptosis and pregnancy is being studied more frequently. During normal pregnancy, cell proliferation and apoptosis are well-balanced in placental villi and decid-



**Figure 4.** Expression Bax mRNA in villus tissue of control (A) and experimental (B) groups. \*p = 0.0189.

ual cells. Abnormal apoptosis may lead to pathological pregnancy and spontaneous abortion<sup>4</sup>. It has been reported that apoptosis is more extensive in patients with RSA than in women with normal pregnancy<sup>5</sup>. Therefore, excessive apoptosis appear to be the cause of this pregnancy complication.

The p53 gene induces cell growth retardation, apoptosis, and affects cell differentiation and DNA repair<sup>6</sup>. Recently, it was found that p53 is also involved in spontaneous abortion<sup>7</sup>. Thus, polymorphism of p53 codon 72 increases the incidence of recurrent miscarriage<sup>8</sup>. Specifically, women carrying the C<sup>®</sup>A or A<sup>®</sup>A genotypes have



Figure 5. TUNEL assay of villus tissue from control (A) and experimental (B) groups. Magnification ×100.

**Table II.** Expression of p53 in study groups.

|                    | Ranking |    |    |     |
|--------------------|---------|----|----|-----|
| Group              | -       | +  | ++ | +++ |
| Control group      | 1       | 29 | 0  | 0   |
| Experimental group | 0       | 7  | 20 | 3   |

*Footnote:* Rankings are statistically different between two study groups (p < 0.0001).

a twice as high risk of RSA compared with women with the C<sup>®</sup>C genotype<sup>9</sup>. In our present study, we found that expression of p53 in villus tissue of women with RSA was significantly higher than in control individuals. It can be speculated that overexpression p53 promotes apoptosis and, thereby, plays an important role in RSA.

p53 acts mainly through specific downstream targets<sup>10</sup>, including CDKN1A and Bax.When p53 affects expression of CDKN1A, cell arrest in the G1 phase ensues, eventually leading to cell apoptosis. The Bax gene family, which includes Bcl-2 and Bax, regulates apoptosis via mitochondrial pathway<sup>11,12</sup>. Bcl-2 is an antiapoptotic protein, while Bax promotes apoptosis. There are reports of higher Bax expression in the trophoblast, endometrial, and matrix and decidual cells of early spontaneous abortion<sup>13</sup>, and Bax is also overexpressed in placental tissue in pregnancies complicated with preeclampsia or diabetes. In our study, both CDKN1A and Bax were expressed at significantly higher levels suggesting that p53 induces apoptosis by activating both these signaling proteins.

#### Conclusions

The p53-CDKN1A and the p53-Bax pathways were found to be activated in tissues of RSA and appear to be involved in the increased apoptosis in these tissues. Since specific treatments for RSA are lacking, our findings may provide a theoretical basis for novel therapies.

#### **Declaration of Funding Interests**

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# **Conflict of Interest**

The Authors declare that there are no conflicts of interest.

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