

Increased apoptosis rate of human decidual cells and cytotrophoblasts in patients with recurrent spontaneous abortion as a result of abnormal expression of CDKN1A and Bax

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Abstract. In the present study, we analyzed the proliferation and apoptosis of trophoblasts and human decidual cells in patients with recurrent spontaneous abortion and the related cellular pathway mechanism. Thirty-four patients with recurrent abortion and 30 healthy pregnant women undergoing planned artificial abortion were selected. The trophoblast and decidual cells were collected by negative pressure aspiration technique. TUNEL method was used to detect the apoptosis rate. Immunohistochemical method was used for detection of TP53 protein. Quantitative real-time PCR was used for detection of the relative expression level of CDKN1A and Bax mRNA. It was found that the cell apoptosis rate in the recurrent miscarriage group was significantly increased and the expression levels of TP53 protein, CDKN1A and Bax mRNA were also significantly increased ($p < 0.05$). In conclusion, the trophoblast and decidual cells of patients with recurrent abortion were obviously apoptotic, which was probably related to abnormal expression of the CDKN1A and Bax genes mediated by TP53 protein through cellular pathways.

Introduction

The incidence of recurrent abortion is approximately 10-15% among all pregnancies. The rate of recurrent pregnancy loss can be as high as 50%, and recurrent spontaneous abortion brings serious injury and distress to the pregnant woman and her family (1). There are a number reasons for the medical condition including genetic defects, reproductive system abnormalities, endocrine disorders, immune disorders, infection,

thrombosis and environmental factors (2). In the clinic, the cause of pregnancy loss is unable to be determined in more than 55% of the patients, which makes treatment difficulty (3). The mechanism of recurrent abortion is considered to be related to the proliferation and apoptosis of human decidual cells and cytotrophoblasts (4). TP53 protein can induce cell growth arrest, apoptosis, differentiation and DNA repair, and activate or inhibit the expression of downstream genes such as Bax and CDKN1A (5). In the present study, we comparatively analyzed the cell apoptosis and cell signaling pathways of healthy patients and those with recurrent spontaneous abortion, providing a theoretical basis for clinical treatment.

Patients and methods

Patients. A total of 34 cases diagnosed with recurrent spontaneous abortion in our hospital were continuously selected from January 2015 to January 2016. Exclusion criteria included: a) no previous surgery and drug therapy; b) possibility of genetic defects; c) reproductive system abnormalities or endocrine disorders; d) immune system disorders or infections; e) thrombosis and environmental factors with normal function of male reproductive system; and f) semen and no fetal haemolytic disease. The age of patients ranged from 23 to 37 years with a mean age of 26.6 ± 5.2 years. Gestational weeks were 5-16 weeks, with an average of 9.2 ± 3.7 weeks. Thirty healthy pregnant women planning an artificial abortion were chosen as control. The controls had an age range of 20 to 35 years and an average age of 24.3 ± 5.6 years. Gestational age ranged from 4.5 to 12 weeks, and an average age of 8.3 ± 3.9 weeks. The age and gestational weeks of the women were compared between the two groups and the differences were not statistically significant ($p > 0.05$).

Specimen collection. The tissues were obtained through negative pressure aspiration biopsy. Each sample was divided into two parts. One part was frozen and stored in liquid nitrogen at -196°C to prepare for polymerase chain reaction (PCR) experiments. Another part was fixed at room temperature with 10% formalin and embedded with paraffin. The section thickness was $5 \mu\text{m}$, and was placed on a glass slide with APES for immunohistochemistry and apoptotic staining.

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Apoptosis rate as detected with terminal deoxynucleotidyl-transferase-mediated dUTP nick end labelling (TUNEL) method

Reagents and equipment. DeadEnd™ Fluorometric TUNEL system kit (Promega, Madison, WI, USA), proteinase K (Biotechnology Engineering and Technical Services Co., Shanghai, China), propidium iodide (Sigma-Aldrich, St. Louis, MO, USA), and Slowfade® Gold Antifade reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) were used to determine the apoptotic rate. Inverted fluorescence microscope (Olympus, Tokyo, Japan) was used for imaging.

Procedure. Preprocessing of the tissue sections was performed before paraffin embedding [dewaxing with xylene, graded alcohol and phosphate-buffered saline (PBS) washing]. The tissues were incubated in polyformaldehyde solution at room temperature for 15 min, followed by washing with PBS. The proteinase K solution (20 µg/ml) of 100 µl was added and incubation was carried out at room temperature for 8 min, followed by another PBS washing. The poly-formaldehyde solution was incubated at room temperature for 15 min and then washed with PBS. For apoptosis detection, each sample was covered using 100 µl equilibrium buffer to cover the cells. The nucleoside mixture was thawed at room temperature for 10 min and soaked with bibulous paper. Fifty microliters of rTdT incubation buffer was added to every 5 cm² area of the cells, followed by covering with a cover glass. The sections were incubated in a humidified chamber to avoid light for incubation at 37°C for 60 min for the tailing reaction. The 20X SSC was diluted with deionized water to 1:10, and added to cover the dye vat. Subsequently, the reaction was stopped by placing at room temperature for 15 min. The PBS was used again to remove the redundant fluorescein-deoxyuridine triphosphate. Two drops of SlowFade® Gold Antifade reagent with DAPI was added to the sample. Finally, transparent nail polish was used for slide mounting.

To assess the staining, green staining of the nucleus was indicative of an apoptotic cell under fluorescence microscopy. Three fields were selected for each sample (100x magnification). Each field was photographed under a fluorescence microscope at 520-nm wavelength (green TUNEL) and at 430-nm wavelength (bluish violet DAPI). Photoshop CS5 was used to merge the two images for the final fluorescence analysis with ImagePro Plus 6.0.

Detection of TP53 protein with immunohistochemistry (IHC)

Reagents and equipment. Mouse anti-human TP53 monoclonal antibody (1:300; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), goat anti-mouse antibody (GBI Labs, Mukilteo, WA, USA) and DAB (ZSGB-BIO, Beijing, China) were used for IHC. Leica RM2245 paraffin slicing machine (Leica, Shanghai, China) was utilized.

Procedure. The procedure for IHC was followed as described elsewhere (6). The positive tissue in the positive control group was used as the control group. The PBS buffer was used instead of the primary antibody in the negative control with other operating procedures unchanged. Each sample was observed by taking images from three horizons at 200x magnification. The images in each field were calculated from the gray value

by using ImageJ 1.44 software, and then the average value of three horizons was taken.

Detection of the relative expression of CDKN1A and BAX mRNA as determined by real-time quantitative PCR method

Main reagents and instruments. TRIzol (Invitrogen Life Technologies), reverse transcription kit (Takara, Dalian, China), DNAMarker Marker III, 50-bp ladder, 1-kb ladder, Lambda-HindIII fragment (Fermentas International, Inc., Burlington, ON, Canada), SYBR-Green Mix (Roche, Basel, Switzerland). PTC-220 PCR instrument (MJ Research Inc., Waltham, MA, USA), GIS-1000 Gel imaging system (Shanghai Quanray Electronics Co., Shanghai, China), real-time PCR instrument (Applied Biosystems Life Technologies, Foster City, CA, USA), Model 650-60 fluorescence spectrophotometer (Hitachi, Ltd., Tokyo, Japan), ZF-90 Multifunctional box type UV transilluminators (Gu-cun, Shanghai, CN) and a 96-well culture dish (Applied Biosystems Life Technologies) were used for real-time quantitative PCR method.

Procedure. The conventional TRIzol method was used to extract RNA. The concentration and purity of the RNA were determined by UV spectrophotometer, and cDNA was synthesized with the kit. The primers were designed and synthesized by Takara and were: CDKN1A forward, 5'-GCAGCGGAACAAGGAGT, 3'-GGAGAAACGGGAACCAG; BAX forward, 5'-CCCCCGAGAGGTCTTTTTC, 3'-TGTCCAGCCCATGATGGTTC; internal reference GAPDH forward, 5'-GGCTACAGCAACAGGGTG, 3'-TTTGGTTGAGCACAGGGT.

The PCR reaction system consists of SYBR® Premix Ex Taq™ [2x (12.5 µl)], primers 10 µM (1 µl), ddH₂O (10.5 µl) to make a final volume of 25 µl. The reaction conditions consisted of denaturation at 95°C for 30 sec, 95°C for 3 sec, annealing at 60°C for 30 sec, extension at 84°C for 1 sec for up to 40 cycles. The fluorescence intensity was measured at 84°C to construct the dissolution curve. The relative expression of mRNA was calculated using the 2^{-ΔΔCt} method.

Statistical analysis. SPSS statistical software was used for data input and analysis. The quantitative data are represented as mean ± standard deviation, and t-test was applied for the inter-group comparison. Qualitative data are expressed as number or percentage (%), and the comparison between groups was carried out using the χ² test. p<0.05 indicates a statistically significant difference.

Results

Comparison of the apoptosis rate. The apoptosis rate in the spontaneous abortion group was significantly increased as compared to the rate noted in the healthy control group (Fig. 1).

Comparison of TP53 protein expression. TP53 protein was mainly expressed in the nucleus, shown as brown or brownish yellow granules. The TP53 protein expression level in the recurrent abortion group was significantly increased as compared to the expression noted in the healthy control group (Fig. 2).

Comparison of the relative expression of CDKN1A and BAX mRNA. The expression levels of CDKN1A and BAX mRNA

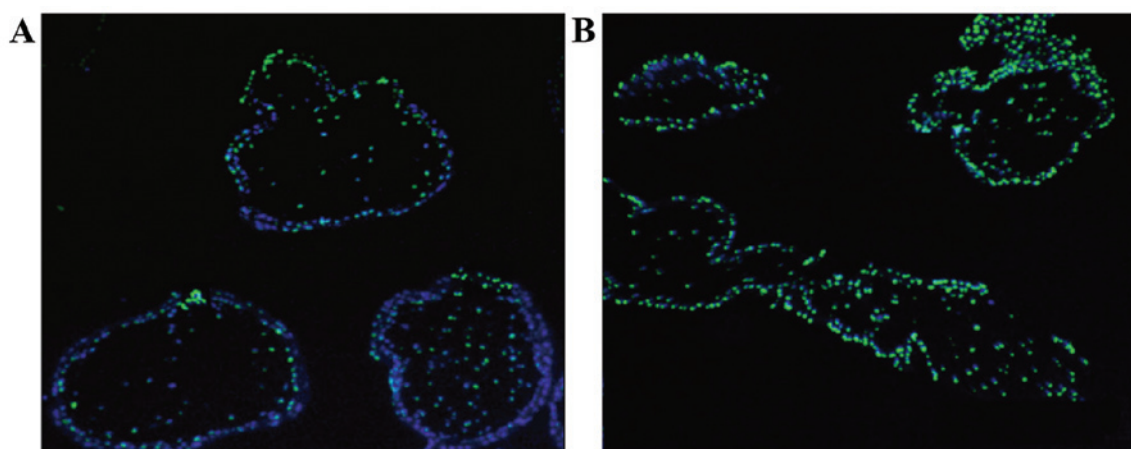


Figure 1. Apoptosis rate as detected by TUNEL method (magnification, 100x). (A) Control group and (B) recurrent abortion group.

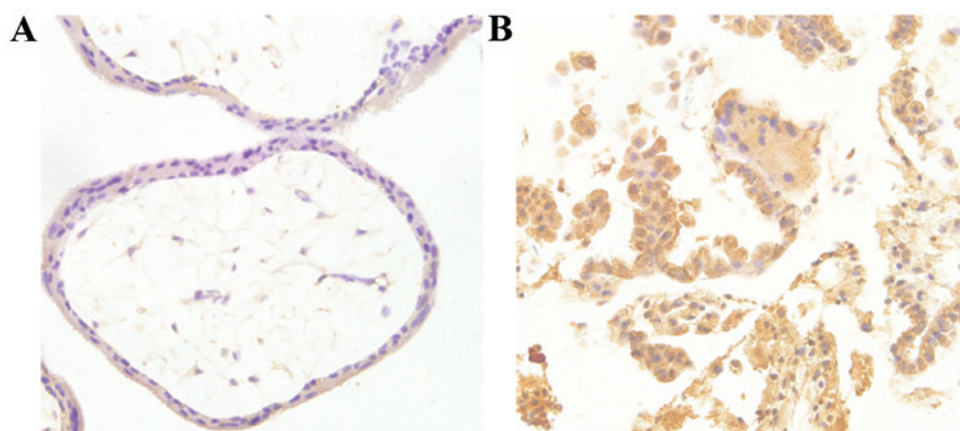


Figure 2. Immunohistochemical staining for TP53 protein (magnification, 200x). (A) Control group and (B) recurrent abortion group.

Table I. Comparison of the relative expression of CDKN1A and BAX mRNA.

Group	CDKN1A	BAX
Control	0.42±0.09	0.46±0.08
Recurrent abortion	0.13±0.05	0.15±0.06
T	7.528	7.629
P-value	0.009	0.006

were significantly upregulated in the recurrent abortion group ($p < 0.05$) as compared with levels in the healthy control group (Table I).

Discussion

Currently, treatment for recurrent spontaneous abortion includes progesterone and hCG supplementation therapy, immune therapy for blocking antibody-negative patients, antibody therapy, coagulopathy for those with anti-phospholipid antibody syndrome, aspirin and heparin treatment for those with coagulation disorder. The methods are unsatisfactory,

and the mechanisms of recurrent spontaneous abortion remain unclear.

The apoptosis and proliferation of placenta villi and decidua during cell normal pregnancy is in a relative state of balance, and the apoptosis rates of decidual cells in women with repeated abortion are significantly higher than those of normal pregnant women. Excessive apoptosis can lead to a series of cellular dysfunction and eventually threaten the entire process of pregnancy (7). During a study of the mechanism of spontaneous abortion in pregnant women exposed to PCBs, it was found that PCBs induced abnormal gC1qR expression resulting in trophoblast cell apoptosis eventually leading to the occurrence of spontaneous abortion (8). The apoptotic phenomena of villus trophoblast cells and decidual cells were noted in both patients with recurrent abortion and those with healthy pregnancy and the apoptosis rate in the recurrent abortion group was significantly higher. The expression levels of TP53, CDKN1A and Bax mRNA were significantly increased. Whether the high expression level of the TP53 protein was caused by the high level of upregulation of the transcription level or TP53 post-transcriptional modification warrants further study. TP53 protein is a typical inhibitor protein of the G1 phase of the cell cycle (9). When cells are damaged, TP53 encodes transcription activated protein that can be quickly assembled in the DNA damaged location, resulting in

termination of the cell cycle in the G1 phase and DNA replication. The damaged DNA is then repaired. If the cell repair cannot be completed, the cell tends to undergo apoptosis mediated by TP53 protein (10). When the cells are damaged and cannot complete the TP53-mediated apoptosis, the genetic material of the cell may change and ultimately lead to malignant transformation. The TP53 gene is highly correlated with human tumors (11).

The cell signal transduction pathway mediated by TP53 plays an important role in the regulation of normal cell activities, and cell contact between other signal transduction pathways is very complex. Thus, TP53 is included in the regulation for more than 160 genes (12). CDKN1A protein is the downstream transcription protein of the TP53 gene. The rpl3 gene was found to regulate TP53-CDKN1A so as to block cell cycle and promote cell apoptosis (13). *Rabdosia rubescens* was found to increase CDKN1A expression and promote autophagy and apoptosis in prostate cancer cells (14). BAX has a pro-apoptotic effect that can directly activate the mitochondrial apoptosis pathway activated by TP53 (15). BCL2 and BAX are two important members of the BCL2 family, and BCL2 is an anti-apoptotic molecule. The Bax expression levels in trophoblast cells, endometrial cells, stroma and decidua cells in patients with early spontaneous abortion were found to be significantly higher than those in women with voluntary terminated pregnancy (16). The expression of Bax in placenta of preeclampsia was also higher than normal term placenta and Bax expression was lower in placenta of patients with diabetes than that of the normal term placenta. In addition, melatonin was found to decrease the BCL2 expression level and increase the expression level of Bax in a study of *in vitro* mouse xenograft melatonin therapy, so as to be therapeutic through apoptosis and necrosis and also to prove the BCL2/Bax balance relationship (17).

In conclusion, the apoptosis rate of human decidua cells and cytotrophoblasts in patients with recurrent abortion was increased, which may be related to the abnormal expression of CDKN1A and BAX genes in the downstream mediated by TP53 protein.

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