

# BAX pro-apoptotic gene alterations in repeated pregnancy loss

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## Abstract

**Introduction:** Recurrent pregnancy loss (RPL) is a critical medical problem in about 0.5-2% of women. The molecular genetic background for spontaneous abortion is being increasingly understood, and some polymorphisms associated with it have been reported. This study investigates alterations of the Bax gene as a pro-apoptotic gene in women with idiopathic RPL.

**Material and methods:** The frequency of mutations in the Bax gene of 67 idiopathic RPL women was studied in comparison to a sample of 70 healthy women. The promoter and the entire coding regions (exons 1-7) were amplified using polymerase chain reaction (PCR). The purity of the PCR product was first verified by electrophoresis on a 2% agarose gel. The amplified fragment was then sequenced by automated DNA sequencing.

**Results:** A statistically significant difference was observed between patients and the control group regarding the frequency of alleles A(-179)G in the Bax promoter region ( $p = 0.013$ ). Also among patients, G90C and G95A transitions were found in the coding region of exon 1 that change amino acid glutamine (Q) to histidine (H) and arginine (R) to lysine (K), respectively. A statistically significant association was observed between H allele ( $p = 0.0001$ ) and K allele ( $p < 0.0001$ ) and the occurrence of RPL.

**Conclusions:** Our results indicate an association between A(-179)G mutation in the Bax promoter and RPL. Moreover, two polymorphisms, G90C and G95A in exon 1, found among our patients, could be considered as genetic factors making people susceptible to miscarriages. According to our findings, the Bax gene has an important role in pregnancy loss and the variations of this gene could help in the assessment of RPL.

**Key words:** BAX, repeated pregnancy loss, polymorphism, apoptosis.

## Introduction

Pregnancy loss is the most common complication in pregnancies and is defined as the unexpected and unplanned spontaneous loss of a pregnancy before the fetus is competent for extrauterine survival. Clinically recognized pregnancies terminate as a miscarriage in about 15% of cases [1]. Traditionally, recurrent abortion has been defined as the occurrence of three or more clinically recognized pregnancy losses before

20 weeks from the last menstrual period. About 1 in 300 couples and 0.5-2% of women are affected by repeated pregnancy loss (RPL) [2]. Assumed aetiological factors are chromosomal aberrations, uterine abnormalities, infectious disorders and endocrine dysfunctions such as hypothyroidism and luteal phase deficiency. These factors are present in about 50% of all women with RPL, but in the majority of cases the cause is unknown or idiopathic [3, 4]. For idiopathic RPL, recent studies propose that polymorphisms are susceptibility factors, which increase the chance of miscarriage [5-13].

In fact, biological processes supporting normal pregnancy are mediated by a series of differential gene expressions that can be affected by genomic variations. The pattern of fragmentation is one of these processes, and is a major predictor for developmental potential [14]. While some patterns of fragmentation have been ascribed to a process of oncogenesis [15], a series of experiments also suggested that a subset of embryo fragmentations results from apoptotic-like disintegration [16]. Human embryos often arrest and undergo fragmentation at the 4-8 cell stage, when the transition is supposed to happen from the maternal to embryonic genome [17].

It is evident that embryo viability depends on alterations in the expression of cell death regulatory molecules, although there are still controversies about apoptosis being a primary or secondary process in cellular fragmentation [16]. This is supported by reports on alteration in the ratio of Bcl-2 and Bax protein, as a very important apoptotic related protein, in bovine viable oocytes and embryos [18], as well as the elevated expression of Bcl-2 and Bax during compaction of the human embryo [19]. The modulated expression of Bax mRNA in human viable and non-viable embryos is another observation supporting this hypothesis [20]. The abnormal expression of apoptosis-related genes is seen in RPL [21]. In the internal apoptotic or mitochondrial pathway, one of the major genes of the Bcl-2 family is the Bax gene, which has an important role in fertilization and proliferation of the cells. Bax is a death-promoting protein shown to be a tumour suppressor and provoking cellular apoptosis in vivo [22-24]. Also lower expression of the Bax gene has been observed in some cases of uncontrolled cell proliferation such as breast adenocarcinoma [25]. The Bax gene is located on chromosome 19 and consists of seven exons and a promoter region with four p53 binding sites [26]. Five isoforms of Bax have been documented. In this study, we investigated Bax nucleotide alterations in women with a history of RPL and compared these data with the ones obtained from controls without a history of miscarriage.

## Material and methods

### Patients and samples

A total of 335 couples suffering from RPL were referred to the Research and Clinical Center for Infertility (Iran, Yazd) between September 2006 and June 2008. Among these people, 67 women were screened as idiopathic at reproductive age. Diagnosis of RPL was based on a documented history of at least three consecutive spontaneous abortions before 20 weeks of gestation with the same partner. In this study pregnancy loss was confirmed mainly by observing the empty sac. The aborted material was also examined by the pathologist from some other aspects. Neither these examinations nor the gynaecologist's referral documents reported any case of hydatidiform moles. RPL women underwent a standard diagnostic work-up to rule out any known cause of RPL. The diagnostic procedure included paternal and maternal karyotype, uterine sonography, TORCH infection study (*Toxoplasmosis, Rubella, Cytomegalovirus, Herpes Simplex Virus* type II and *Listeria*), assessment of hormonal status, and IgM/IgG anticardiolipin, antiphospholipid tests. All of the cases were suffering from primary RPL and had no history of child delivery. The control group consisted of 70 women with at least two live births and no history of pregnancy loss. All the controls were at reproductive age and underwent sampling in the labour room during their second or later normal delivery. All of the participants were informed about research and signed the consent form approved by the ethical committee.

### DNA extraction

Blood was collected from the women with RPL and the control individuals in tubes containing potassium-EDTA. Genomic DNA was isolated from the blood samples using a Flexigene blood DNA kit (QIAGEN, Germany) according to the manufacturer's protocol. The isolated DNA was stored at 4°C until use.

### PCR/Sequencing analysis

Four pairs of primers were designed using the 5' and 3' flanking sequences to amplify the promoter region and the exons of the Bax gene (Table I).

The PCR reactions were performed in four series according to the conditions detailed in Table II. The reaction mixture for PCR contained 10 pM of each primer, 1 unit of Taq polymerase (Cinnagene, Iran), each dNTP at a final concentration of 200 µM, and 2.5 µl PCR buffer at a final volume of 25 µl. 1.5 µl of DMSO was also used in the reaction mixture for the promoter and exon 1 fragment.

**Table I.** Oligonucleotide primers used in the mutational screening of BAX gene

Name of primers	Sequence	Tm	Product size [bp]
BAX-PE1-F	5'-CGG GGT TAT CTC TTG GGC-3'	58.2	663
BAX-PE1-R	5'-TGC TTG GAG ATC GCA CAG-3'	56.0	
BAX-E2,3,4-F	5'-TCT TCC CTT CCT TTC TCC-3'	53.7	811
BAX-E2,3,4-R	5'-ACC TTG AGC ACC AGT TTG-3'	53.7	
BAX-E5,6-F	5'-GAT TCT TGC TCT ATT GTC C-3'	52.4	710
BAX-E5,6-R	5'-TGA GGA GTC TCA CCC AAC-3'	56.0	
BAX-E7-F	5'-ACT GAT GTC CCT GTC TCC-3'	56.0	303
BAX-E7-R	5'-GAC TCA GAT GCC GAA GTG-3'	56.0	

**Table II.** PCR programs for amplification of promoter and exons regions of BAX gene

Fragments	Initial denaturation	Number of cycles	Denaturation	Annealing	Extension	Final extension
Promoter and Exone 1	95°C – 5 min	35	95°C – 1 min	55°C – 1 min	72°C – 60 sec	72°C – 5 min
Exone 2, 3, 4	95°C – 5 min	35	94°C – 1 min	54°C – 1 min	72°C – 50 sec	72°C – 5 min
Exone 5, 6	95°C – 1 min	35	94°C – 1 min	58°C – 1 min	72°C – 50 sec	72°C – 5 min
Exone 7	95°C – 5 min	35	94°C – 1 min	56°C – 1 min	72°C – 30 sec	72°C – 5 min

The PCR reactions were performed in a thermal cycler (TECHNE) and the amplified fragments were detected on 2% agarose gels (Figure 1).

The PCR-amplified fragments were purified and then sequenced by a 3730XL ABI DNA analyzer (Macrogen, Korea). DNA sequences were determined using the same PCR primers. The results of DNA sequence analysis were compared with the published sequences in the NCBI database using the Chromas and Clustal-X programs. Sequence variants not found in the NCBI database were identified as novel alterations.

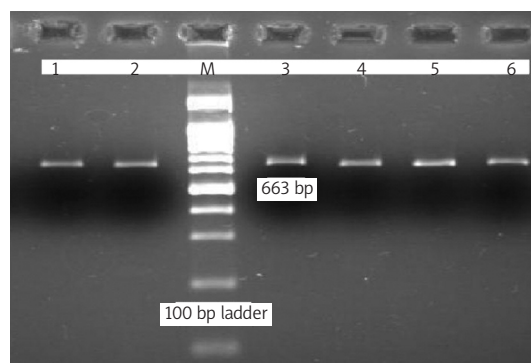
### Statistical analysis

All statistical analyses were carried out with SPSS software package 16.0 (SPSS Inc., USA). Using Chi-square and Fisher's exact tests, the prevalence of sequence variation was compared between case and control groups. The odds ratio (OR) was used as a measure of the strength of the association between allele frequencies and idiopathic recurrent miscarriage.  $P < 0.05$  was considered statistically significant.

### Results

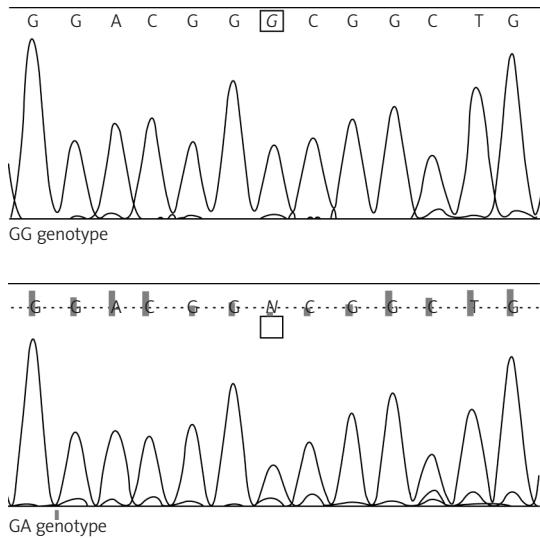
Our data show that the mean  $\pm$  SD age of the women with RPL at the time of blood sampling was 27.54  $\pm$  5.08 and the mean  $\pm$  SD number of pregnancy losses in this group was 3.84  $\pm$  1.62 (Table III). There was a consanguineous marriage in 27 (40.3%) women with RPL and 23 (32.9%) healthy women. The difference between the two groups was not significant ( $P = 0.366$ ).

Sequencing of the Bax gene showed three alterations in the promoter region and exon 1. The promoter genotype of 67 RPL and 70 healthy women was determined from 400 nucleotides before exon 1. An A(-179)G transition was observed

**Figure 1.** Demonstration of amplified 663-base-pair promoter and exon 1. Lanes 1-6 show the amplified fragment from samples and controls, and M shows the 100-base-pair DNA ladder**Table III.** Characteristics of RPL and healthy women

	RPL women	Healthy women
Mean age $\pm$ SD at miscarriage study [years]	27.54 $\pm$ 5.08	30.20 $\pm$ 4.11
Age at miscarriage study [years]*	27 (19-40)	30 (22-39)
No. of live birth*	0	2 (2-4)
No. of miscarriage*	3 (3-11)	0
Mean $\pm$ SD of miscarriage	3.84 $\pm$ 1.62	0

\*Median (range)



**Figure 2.** Diagrams of BAX promoter variations in women

in the promoter region. The frequencies of the GG and AG genotypes were 95.52% and 4.48% among the RPL group and 81.43% and 18.57% among the controls, respectively. This difference was significant ( $P = 0.01$ ). Also, the allele frequencies among the women with RPL and the controls were 2.24% and 9.29% for allele A (wild type), and 97.76% and 90.71% for allele G (mutant type),

respectively. Therefore, an association could be suggested between allele G and the occurrence of RPL ( $P = 0.012$ , odds ratio = 0.22;  $0.05 < OR < 0.87$ ; Table V).

The substitution of guanine with cytosine ( $G > C$ ) at nucleotide 90 in the coding region of exon 1 changes the amino acid glutamine to histidine at codon 7 (Q7H). This allele alteration was seen in 33 (24.62%) of the RPL and 11 (7.86%) of the healthy women ( $P = 0.0001$ , odds ratio = 3.83,  $1.76 < OR < 8.51$ ). Also, transition of guanine to adenine ( $G > A$ ) was seen at nucleotide 95. In this alteration, the amino acid arginine is changed to lysine at location 9 (R9K) in 93 (69.40%) of the RPL and 20 (14.29%) of the healthy women ( $P < 0.0000$ , odds ratio = 13.61,  $7.19 < OR < 26$ ). The genotype and the allele frequency of these genotypes are shown in Tables IV and V.

For exons 2, 3, 4, 5, 6, and 7, however, no nucleotide alterations were seen in the women with RPL

### Discussion

In this study, Bax gene nucleotide alterations were investigated in RPL women. Also, the association between the polymorphic sites in the promoter as well as the coding regions of the Bax gene and the occurrence of RPL was studied. One polymorphism

**Table IV.** Genotypes of Bax variations among RPL and healthy women

Polymorphisms		Genotype frequency			
			Women with RPL (n = 67) <sup>a</sup>	Healthy women (n = 70) <sup>a</sup>	P-value <sup>b</sup>
A(-179)G	Genotypes	GG	64 (95.5)	57 (81.4)	0.010
		GA	3 (4.5)	13 (18.6)	
G90C	Genotypes	CC	11 (16.4)	4 (5.7)	0.005
		CG	11 (16.4)	3 (4.3)	
		GG	45 (67.2)	63 (90)	
G95A	Genotypes	AA	39 (58.2)	6 (8.6)	< 0.001
		AG	15 (22.4)	8 (11.4)	
		GG	13 (19.4)	56 (80)	

RPL – Repeated pregnancy loss, <sup>a</sup>Values in parenthesis are percentages, <sup>b</sup>Pearson chi square test

**Table V.** Allele frequencies of Bax variations among RPL and healthy women

Polymorphisms		Allele frequency			
		Women with RPL (134 alleles) <sup>a</sup>	Healthy women (140 alleles) <sup>a</sup>	P-value <sup>b</sup>	Odds ratio 95% CI
A(-179)G	Allele A	3 (2.24)	13 (9.29)	0.0128	0.22
	Allele G	131 (97.76)	127 (90.71)		
G90C	Allele C	33 (24.62)	11 (7.86)	0.0001	3.83
	Allele G	101 (75.37)	129 (92.14)		
G95A	Allele A	93 (69.40)	20 (14.29)	< 0.0001	13.61
	Allele G	41 (30.60)	120 (85.71)		

RPL – Repeated pregnancy loss, <sup>a</sup>Values in parenthesis are percentages, <sup>b</sup>Pearson chi square test, CI – confidence interval

in the promoter region, A(-179)G, and two mutations in exon 1, G90C and G95A, were identified as novel variations in the Bax gene (Table IV).

There was a significant A(-179)G polymorphism in the promoter region of the patients, which suggests its involvement in spontaneous miscarriages. The G(-248)A promoter polymorphism, which is associated with increased lifespan of neutrophils in osteomyelitis, has been previously reported [27]. Also, this variation results in a significantly lower survival rate in patients with chronic lymphocytic leukaemia [28]. Sequence variations in the Bax gene promoter can affect its expression and its pro-apoptotic function [24, 29]. It is known that changes in the 5' untranslated region sequence can inhibit the initiation of translation. It can alter the expression of the Bax protein and most likely implicates a post-transcriptional mechanism [30]. Thus, the polymorphism in its promoter region can cause declined expression, activation and ultimately apoptosis. However, according to our data the frequency of promoter polymorphism in RPL women is lower than in control women, which supports this hypothesis. On the other hand, quantitative PCR and Western blot are suggested for future studies to investigate whether the observed mutation in the promoter region really affects the expression of Bax protein in the tissues.

The changes found in exon 1 lead to amino acid alterations and finally changed properties of Bax protein. Based on the bioinformatic analysis, the Q7H and R9K alterations lead to increased final total energy and decreased Bax protein stability (<http://swiss-model.expasy.org>). These polymorphisms can raise the pro-apoptotic activity of the Bax protein in relation to other proteins or independently. Therefore, an increased tendency of the cell to death or apoptosis will result. This mechanism could underlie the role of these variations in inducing pregnancy loss. However, to support this hypothesis, site-directed mutagenesis and in vitro expression studies are needed to provide more functional data on the role of these mutations.

Apoptosis, or programmed cell death, is responsible for a number of normal developmental processes [31]. Since the regulation of cell death and proliferation is required for successful human pregnancy, apoptosis and its precise regulation are crucial in a normal pregnancy [32, 33]. Therefore, the aberrant expression of apoptosis-related genes during development is also detrimental and can lead to RPL. Interestingly, higher expression levels of apoptosis-related genes, including the Bax gene, were observed in chorionic villi of RPL patients compared to normal people [34]. Bax induces apoptosis, and the ratio of its homo- and heterodimers to Bcl2 determines the fate of the cell [35]. Different constitutive expressions of Bax are related to the Bcl2/Bax ratio, leading to the failure

of mitochondrial disruption associated with conformational changes. Dysfunction and dysregulation of the apoptotic programme is involved in some pathological conditions such as cancers, neurodegenerative disorders and autoimmune diseases [36]. Also, apoptosis of villous trophoblasts is upregulated in common pregnancy diseases related to the placenta, intrauterine growth retardation (IUGR), and pre-eclampsia [37, 38].

The Bax gene is also a primary transcriptional target after p53 activation [26, 39, 40]. It is possible that the other role of A(-179)G or G90C and G95A polymorphisms is blunting of the cellular response to p53 activation via both transcription-dependent and independent factors. More research is needed to clarify how this pro-apoptotic effect of Bax polymorphisms may cause susceptibility to RPL.

Because of the difficulties in obtaining aborted materials, in the current study we focused solely on the parents.

Based on the studies conducted so far, more than 40 gene products are differentially expressed in the RPL compared to the normal women. These genes may have regulatory roles in establishing or maintaining normal pregnancy [21]. Therefore, any nucleotide alterations in such genes may lead to different expression and activity which endangers the general health during pregnancy. A better understanding of the genetic background and the physiological significance of these polymorphisms may help to manage subsequent pregnancies and prevent pregnancy complications.

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