Journal of Assisted Reproduction and Genetics (2019) 36:995–1002
https://doi.org/10.1007/s10815-019-01441-w

GENETICS

The role of *FAS*, *FAS-L*, *BAX*, and *BCL*-2 gene polymorphisms in determining susceptibility to unexplained recurrent pregnancy loss

Rafael Tomoya Michita[1] · Francis Maria Báo Zambra[1] · Lucas Rosa Fraga[1] · Maria Teresa Sanseverino[1,2,3] · Lavínia Schuler-Faccini[1,2,3] · José Artur Bogo Chies[1] · Priscila Vianna[1]

Received: 5 October 2018 / Accepted: 19 March 2019 / Published online: 2 April 2019

Abstract

Purpose Idiopathic recurrent pregnancy loss (RPL) is a multifactorial reproductive disorder where an impaired control of apoptosis is likely involved. Triggering the cell death mechanism occurs in a spatiotemporal manner and is strongly related to a healthy pregnancy. Single nucleotide polymorphisms (SNPs) at the regulatory regions of genes are known to influence the expression patterns of apoptosis-related molecules.

Methods A total of 296 unrelated female Brazilian patients were evaluated for clinical-demographic variables and genetic factors: 140 women who had experienced an unexplained RPL (with at least two consecutive abortions) and 156 healthy multiparous women. In all patients, six SNPs were evaluated in genes of apoptosis-related pathways: *FAS* (rs2234767, rs1800682), *FAS-L* (rs763110, rs5030772), *BAX* (rs4645878), and *BCL*-2 (rs2279115) by PCR followed by a restriction fragment length polymorphism (RFLP)-based analysis.

Results The *BAX*-248GA genotype is independently associated with idiopathic RPL [adjusted OR = 0.30, 95% CI 0.13–0.70, $P = 0.005$] susceptibility. In the same multivariate model, the variables ethnicity, smoking, and alcohol consumption were statistically associated with RPL susceptibility ($P < 0.05$). No association with RPL susceptibility was reported for the remaining SNPs.

Conclusion Our study is the first to evaluate the role of the main SNPs from both the extrinsic and intrinsic apoptosis pathways in RPL susceptibility. The association of *BAX*-248G/A with RPL susceptibility suggests that maternal predisposition for RPL has an essential contribution from genes involved in the delicate balance of endometrium cell turnover (cell death/proliferation). Therefore, apoptotic genes may represent promising targets for future studies on healthy pregnancies and the spectrum of pregnancy disorders.

Keywords Recurrent pregnancy loss · BAX · Intrinsic apoptosis · Extrinsic apoptosis · Polymorphism · Recurrent miscarriage

Introduction

Recurrent pregnancy loss (RPL) is a complex pregnancy disorder that is characterized by women who have experienced three or more miscarriages. Currently, some guidelines accept the threshold of having two or more consecutive pregnancy losses until 24 gestational weeks (GWs) [1]. RPL incidence may vary according to the diagnostic criteria. When defined by the presence of at least two consecutive miscarriages, RPL affects 2–5% of couples, and for three miscarriages, the incidence is ~ 1% [2, 3]. RPL can occur at two following subclinical levels: primary and secondary. Primary RPL refers to women who have never had a successful pregnancy; secondary RPL refers to women who have had a successful

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10815-019-01441-w) contains supplementary material, which is available to authorized users.

✉ José Artur Bogo Chies
jabchies@terra.com.br

[1] Department of Genetics, Institute of Biosciences, Universidade Federal do Rio Grande do Sul (UFRGS), Bento Gonçalves Avenue 9500, Campus do Vale, Porto Alegre, RS CEP 91501970, Brazil

[2] National Institute of Science and Technology in Population Medical Genetics (INAGEMP), Porto Alegre, RS, Brazil

[3] Medical Genetics Service, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil

pregnancy at term with a live newborn, followed by having a series of miscarriages [4]. Although some risk factors are associated with the disorder, the etiopathology of RPL is still unknown [2]. After the identification of the potential causes that lead to RPL, approximately 50% of recurrent miscarriage cases remain idiopathic [5].

The maintenance of normal pregnancy requires a complex network of interactions and the involvement of distinct, but correlated, biological processes [6, 7]. In this context, the differential expression of genes in the endometrial tissue and in the chorionic villi in normal pregnancies and RPL has been observed [8–12]. The variable results of the comparative gene expression that is seen in the literature reflect the heterogeneity of studies since the cellular composition of tissue samples from RPL and normal pregnancies may differ. Nevertheless, such studies provide important clues for potential biomarkers or therapeutic targets to improve the clinical management of women with RPL. In this sense, the higher expression of apoptosis-related genes, such as the pro-apoptotic genes *FAS*, *FAS-L*, *BAD*, *BAX*, *BID*, and the *CASPASE* family (3, 6, 7, 8, 9, 10, and 12), was observed in the chorionic samples from women with RPL compared with samples from normal pregnancies [8]. In contrast, the expression of antiapoptotic genes, such as *BCL*-2 and *BCL-XL*, was similar among chorionic samples from RPL and normal pregnancies, suggesting that apoptosis-related genes are directly involved in RPL [8]. In addition, it has been shown that apoptosis levels are higher in chorionic samples from women with RPL than in samples from electively terminated first-trimester pregnancies [13].

Apoptosis is fundamental to human reproduction because this phenomenon is directly involved in endometrial cell turnover, spiral artery remodeling, the development of the embryo, and in maternal-placental immunologic tolerance [4, 14]. Once apoptosis signaling is triggered by either the extrinsic (death receptor-mediated apoptosis) or the intrinsic (mitochondrial) pathways, it usually results in cell death by the activation of the caspase cascade. The main molecules of the extrinsic pathway are the death receptor FAS and its ligand FAS-L [15]. FAS is constitutively expressed in most cell types. FAS-L has a restricted tissue distribution, for example, in the testes, the eyes, activated T lymphocytes, and natural killer cells [16]. FAS/FAS-L signaling contributes to the maintenance of the immunologically privileged status of some tissues, including the maternal-fetal interface. It has been reported that extravillous trophoblast cells (EVTs) express FAS-L [17], thus implying that FAS-L expression in both maternal and fetal cells represents an additional mechanism related to the immunotolerance phenomenon in pregnancy [18]. Genetic variants in the regulatory region of *FAS* and *FAS-L* genes have been shown to impact gene expression patterns and the susceptibility of many immune-related disorders and pregnancy disorders, including RPL [19–27]. *FAS*-670A/G and *FAS*-1377G/A are single nucleotide polymorphisms (SNPs) that are located in the promoter region of *FAS*. They are in the binding site of critical transcription factors: the signal transducer and activator of transcription-1 (STAT-1) and specificity protein-1 (SP-1) [28, 29]. Two SNPs in the *FAS-L* have also been identified: *FAS-L* IVS2nt-124A/G, located in intron 2, and *FAS-L*-844T/C in the promoter region. The latter modifies the affinity for the CCAAT/enhancer-binding protein beta (C/EBPbeta). In addition, the *FAS-L*-844C allele confers higher *FAS-L* expression in lupus erythematosus patients [30].

The intrinsic pathway is under the control of proteins belonging to the BCL-2 family, with BCL-2 and BAX being the significant players of this pathway. Interestingly, apoptotic stimuli, such as oxidative stress, DNA damage, or hypoxia, lead to the activation of the downstream signaling of the intrinsic pathway [31]. BCL-2 has antiapoptotic activity and prevents cell death either by sequestering inactive forms of death-driving caspases or by regulating the release of mitochondrial apoptosis factors, such as cytochrome *c*, which binds to apoptotic protease-activating factor 1 (APAF-1) to form the apoptosome in the cytoplasm [32]. In contrast, when BAX is upregulated, it counteracts the negative effects of BCL-2 and promotes the release and activation of caspases through the regulation of mitochondrial permeability [33].

The *BCL-2* gene has two promoter regions, named promoter 1 (P1) and promoter 2 (P2). P1 has a stimulatory function, and P2 has an inhibitory function. If P2 activity is higher than that of P1, it decreases gene transcription, which is a negative regulatory element. The SNP located in P2-938C/A is reported to influence the binding of the transcription factor SP-1. Nuckel et al. [34] reported that in B cell chronic lymphocytic leukemia (CLL) patients, the P2-938AA genotype has a higher BCL-2 expression. Additionally, in transfected cells (specifically HEK293 and Karpas 422), the BCL-2 P2-938C allele results in the reduced transcriptional activity of the gene due to the increased activity of P2. However, the functional relevance of this SNP on endometrial tissue and pregnancy disorders remains to be investigated.

Currently, it is suggested that *BAX*-248G/A has no influence on cancer susceptibility [35, 36]. The effect of SNP *BAX*-248G/A on the expression levels of the protein in CLL patients is still controversial [37, 38]. However, initial reports observed that the *BAX*-248A allele is associated with low expression levels in untreated CLL patients [39]. In addition, this SNP has been suggested to influence drug (fludarabine) responses in CLL patients and provides insights for pharmacogenomic studies in other clinical settings [39, 40].

In pregnancy, previous studies have reported the high expression of apoptotic proteins in the chorionic villi from women with RPL [8, 41], thus suggesting that imbalances between the proapoptotic and antiapoptotic stimuli are likely involved in RPL pathogenesis. Currently, studies evaluating the role of

genetic variants in *FAS*, *FAS-L*, *BAX*, and *BCL-2* on RPL susceptibility [21, 22] are limited or even absent, and in most cases, a limited set of variants from a single apoptotic pathway was evaluated. Therefore, we evaluated whether SNPs in critical genes of the classical apoptosis pathway are associated with RPL risk in a Brazilian population.

Materials and methods

Selection and description of participants

We recruited patients at the Prenatal Diagnosis Clinic of the Medical Genetics Service at the *Hospital de Clínicas de Porto Alegre* (HCPA), Brazil. The current case-control study enrolled 140 idiopathic women with RPL: 101 (72.1%) primary RPL patients and 39 (27.9%) secondary RPL patients. The inclusion criteria comprised women reporting at least two pregnancy losses before 24 GWs with the same partner. A structured interview was performed to obtain the clinical and demographic data and the obstetric history. Before inclusion in the study, all patients underwent a standardized clinical evaluation, which included hysteroscopy, laparoscopy, ultrasound, and a full determination of hormonal status to identify the cause of the pregnancy losses. Karyotype examinations evaluated the chromosomal abnormalities (numerical and structural). Immunological risk factors were also investigated through the assessment of anticardiolipin, lupus anticoagulant, and antinuclear antibody levels; the patient medical history of autoimmune diseases was also reviewed. We considered the presence of any maternal clinical condition that could prevent a full-term pregnancy as exclusion criteria for this study. Alcohol consumption and cigarette smoking were defined as regular consumption/utilization (yes/no).

The control group included 156 women with two or more successful pregnancies (healthy multiparous women) and no history of pregnancy loss or infertility. These women were randomly selected to participate in the study during blood collection for routine laboratory analyses at the *Hospital de Clínicas* in Porto Alegre (HCPA). These women verbally answered a standardized questionnaire about demographics, consanguinity, family history, and the number of pregnancies. The results were reported and analyzed following the previous recommendation by STREGA [42].

Genomic DNA extraction

Biological samples consisted of blood or saliva. The genomic DNA from the blood samples was extracted from leukocytes according to the methods by Lahiri and Numberger [43] and was maintained at − 20 °C. The DNA from the saliva samples was obtained using the Oragene® DNA collection kit (DNA Genotek Inc., Canada) according to the manufacturer's protocol.

Polymorphism genotyping

The primer sequences, polymerase chain reaction conditions, and the genotyping methods for the polymorphisms of *FAS-L*-844T/C (rs763110), *FAS*-670A/G (rs1800682), and *FAS*-1377G/A (rs2234767) have been previously described by Sun et al. [44]. The amplification reaction and the genotyping conditions for *FAS-L* IVS2nt-124A/G (rs5030772) were previously described by Zhang et al. [45]. The *BAX*-248G/A (rs4645878) and *BCL*-2-938C/A (rs2279115) variants were analyzed as described, respectively, in Moshynska et al. [38] and Zhang et al. [46]. Briefly, the PCR products were digested with the *Fok*I, *Bsr*DI, *Scr*FI, *Bst*UI, *Aci*I, and *Bcc*I restriction enzymes (New England Biolabs, Beverly, MA) to distinguish the *FAS-L* IVS2nt-124A/G, *FAS-L*-844T/C, *FAS*-670A/G, *FAS*-1377G/A, *BAX*-248G/A, and *BCL*-2-938C/A polymorphisms, respectively. All genotypes were determined by electrophoresis with an 8% polyacrylamide gel stained with ethidium bromide. Finally, we randomly selected 10% of the samples for an independent repetition of the genotyping procedure, and the results were 100% concordant with the initial data.

Statistical analyses

Chi-squared and Mann-Whitney tests were used to compare categorical and continuous variables, respectively. The median and the 25th–75th percentiles describe asymmetric distributions. The Hardy-Weinberg (HWE) equilibrium was calculated in the control samples using web-based software: http://www.oege.org/software/hwe-mr-calc.shtml [47]. The expectation-maximization algorithm determined the presence of linkage disequilibrium in the *FAS* and *FAS-L* genetic variants using Haploview software [48]. Analyses were stratified according to the RPL subclinical types and were compared with the control group. Binary logistic regression analyses were performed to assess the strength of the association between the genetic markers and the incidence of RPL by evaluating the odds ratio (OR) and the confidence interval (CI). Covariates were included in the multiple regression models based on the cutoff values for significance [association with the study factor (genotype) and with the final significance of $P < 0.20$]. The *Bonferroni* correction was applied for multiple tests, and the alpha was set at 0.0083 ($\alpha_{\text{Bonferroni}} = 0.05/6$ SNPs tested). For all other nonindicated instances, the nominal P value < 0.05 was considered to be statistically significant. All statistical analyses were performed with standard software (SPSS for the Social Science, v.18.0 for Windows).

Results

In total, we evaluated 140 cases: 101 (72.1%) were diagnosed with primary RPL, and 39 (27.9%) were diagnosed with secondary RPL. The demographic and clinical characteristics of the individuals, as well as the pairwise comparisons between the RPL clinical subtypes and the controls, are described in Table 1. Smoking prevalence was not different among the studied groups ($P > 0.05$). In addition, the prevalence of smoking in controls (21.8%) was similar to the general estimates for pregnant women (17.5%) from the major cities of Brazil [49]. A high frequency of European ancestry (77.6%) was observed in the control group. This observation is in accordance with a sampling of the Brazilian population (82%), and European ancestry is the most frequent ethnic group in the southern region of Brazil [50]. Alcohol consumption was more frequent in women with primary and secondary RPL than in healthy multiparous women ($P < 0.001$ and $P = 0.043$, respectively).

The control group adheres to HWE expectations for all SNPs evaluated. Linkage disequilibrium (LD) was evaluated for *FAS* (-670A/G and -1377G/A; $D' = 1.0$, $r^2 = 0.111$) and *FAS-L* (FAS-844T/C and IVS2nt-124A/G; $D' = 0.89$, $r^2 = 0.10$). No differences in the haplotype frequencies between RPL and control patients were observed (Supplementary Table 1).

Multiple logistic regression models were performed to assess the effect of individual genotypes on primary RPL risk. A regression model adjusting for the effects of covariates and *BAX*-248G/A genotypes is presented in Table 2. The *BAX*-248GA genotype (OR = 0.30, 95% CI 0.13–0.70, $P = 0.005$) was independently associated with protection for primary RPL. The covariates ethnicity (OR = 0.42, 95% CI 0.23–0.75, $P = 0.004$), smoking (OR = 0.45, 95% CI 0.21–0.99, $P = 0.049$), and alcohol consumption (OR = 3.87, 95% CI 2.08–7.16, $P < 0.001$) were also statistically significant in the model. Additionally, we highlight the fact that the *BAX*-248AA homozygous genotype was exclusively observed in women with primary RPL.

When the *FAS*, *FAS-L*, and *BCL*-2 polymorphisms and primary RPL were evaluated individually, there was no correlation even when the effects of genotypes were adjusted for covariates or in dominant and additive genetic models (Supplementary Table 2). In addition, no significant differences in polymorphisms between the secondary RPL and control cases were observed (Supplementary Table 3). Additionally, no significant difference in the pooled analysis of RPL clinical subtypes compared with the control subtypes was observed (data not shown).

Discussion

Few studies have evaluated the effects of *FAS* and *FAS-L* SNPs on the risk of RPL [21, 22, 27]. In an Indian population, Nair et al. (2012) assessed the impact of *FAS*-670A/G and

Table 1 Comparison of clinical and demographic characteristics among idiopathic RPL women and healthy multiparous women

Variables	Primary RPL ($n = 101$)	Secondary RPL ($n = 39$)	Healthy multiparous women ($n = 156$)	Primary RPL vs. secondary RPL	Primary RPL vs. healthy multiparous women	Secondary RPL vs. healthy multiparous women
				P value		
Age at first pregnancy, median (25–75%)	23 (18.0–29.0)	20 (18.0–25.0)	22 (20.0–25.0)	0.113	0.224	0.212
BMI, median (25–75%)	25 (22.5–28.5)	26 (24.0–29.0)	24.0 (21.0–28.0)	0.259	0.226	0.057
European-derived, *n* (%)	59 (58.4)	29 (76.3)[a]	121 (77.6)[b]	0.051	< 0.001*	0.763
Alcohol consumption, *n* (%)	46 (45.5)	14 (35.9)	32 (20.5)	0.301	< 0.001*	0.043*
Smoking, *n* (%)	14 (13.9)	7 (17.9)	34 (21.8)	0.544	0.111	0.598
Alcohol and smoking habit, *n* (%)	11 (10.9)	4 (10.3)	9 (5.8)	1.000	0.134	0.297
Number of pregnancies, mean (±SD)	3.4 (1.8)	4.41 (1.39)	2.87 (1.15)	N/A	N/A	N/A
Miscarriages, mean (±SD)	3.4 (1.8)	2.97 (1.06)	N/A	N/A	N/A	N/A
2 miscarriages, *n* (%)	36 (35.6)	14 (35.9)	N/A	0.151[c]	N/A	N/A
3 miscarriages, *n* (%)	32 (31.7)	18 (46.2)	N/A		N/A	N/A
> 4 miscarriages, *n* (%)	33 (32.7)	7 (17.9)	N/A		N/A	N/A

RPL recurrent pregnancy loss, *BMI* body mass index, *SD* standard deviation, *N/A* not applicable

[a] One patient has missing data

[b] Two patients have missing data

[c] *P* value of categorical miscarriages between primary vs. secondary RPL

*Statistically significant

Table 2 Multivariate logistic regression for the effect of BAX-248G/A genotypes in idiopathic primary RPL corrected for covariates

SNP	Primary RPL, *n* (%)[a]	Healthy multiparous women, *n* (%)[b]	OR	95% CI	*P*
BAX-248 G/A[c]					
GG	87 (87.0)	123 (79.4)	Ref.		
GA	10 (10.0)	32 (20.6)	0.30	0.13–0.70	0.005*
AA	3 (3.0)	–			
Ethnicity	59 (58.4)	121 (78.6)	2.39	1.32–4.34	0.004*
Smoking	14 (13.9)	34 (21.8)	0.46	0.21–0.99	0.049*
Alcohol consumption	46 (45.5)	32 (20.5)	3.87	2.08–7.16	< 0.001*

Genotypes were not available for one patient in RPL and one in control. Reference categories were European-derived (ethnicity), "no" (smoking) and "no" (alcohol consumption)

OR odds ratio, *CI* confidence interval, *Ref.* reference category, *P P* value

[a] Primary RPL, $n = 101$

[b] Healthy multiparous women, $n = 156$

[c] OR for BAX-248AA genotype is absent due to a low count AA genotype in controls

*Statistically significant values

FAS-1377G/A SNPs on RPL risk. In this study, they observed an association of *FAS*-1377A allele carriers with RPL risk. Nevertheless, the combined genotype of *FAS*-670GG/-1377AA conferred the highest risk of RPL. However, the *FAS*-670A/G variant had no impact per se on RPL risk, thus implying that gene-gene interaction is a significant factor to take into account for RPL susceptibility [21, 51]. No association with RPL was reported with *FAS*-1377G/A in our study. The differences in the RPL diagnostic criteria (≥ 3 pregnancy loss, ≤ 12 GWs) and in the genetic population background may account for these conflicting results. Further studies are needed to confirm whether the findings of our study apply to other definitions of RPL.

In agreement with a previous study with Brazilian and Asian populations [22, 27], our findings suggest that the *FAS*-670A/G SNP does not affect the risk of RPL in our population. Nevertheless, this SNP seems to play a role in other pregnancy disorders, such as preterm premature rupture of fetal membranes [52], preeclampsia [20, 23, 26], and fetal growth restriction [19]. Additionally, *FAS*-670A/G has been reported to play a role in nonrelated pregnancy conditions, such as cancer and autoimmune diseases [30, 44, 45, 53].

Regarding *FAS-L* variants, no association with RPL risk was observed. In contrast with our findings, Banzato et al. studied a Brazilian population and reported statistically significant differences in the *FAS-L*-844C/T genotype and allele distributions between the RPL and control patients. Additionally, among women with RPL, they observed higher levels of *FAS-L* mRNA expression, but no correlation with the *FAS-L*-844C/T genotypes was observed. Once again, conflicting results possibly reflect the RPL definition criteria adopted by different studies. Additionally, it is important to note that the RPL definition varies regarding the number of previous miscarriages among different international guidelines. Nevertheless, studies suggest that women who report two or more miscarriages should undergo clinical evaluation, as the risk factors and the chance of a miscarriage are similar to those with three or more miscarriages [54–56].

Our study is the first to evaluate the role of *BAX*-248G/A and *BCL-2*-938C/A SNPs in RPL susceptibility, and consequently, our study is the first description to suggest that protection is conferred by the *BAX*-248GA genotype in idiopathic primary RPL. Studies evaluating these SNPs in pregnancy disorders are scarce. However, the *BAX*-248G/A SNP has attracted much attention to malignant disorders [36, 40]. In this context, low levels of BAX expression in untreated CLL patients are associated with *BAX*-248GA/AA genotype carriers [39, 40]. Furthermore, the *BAX*-248GA genotype and the *BAX*-248A allele confer a decreased risk for systemic lupus erythematosus development [24]. A defective triggering of apoptosis characterizes both disorders previously cited (i.e., CLL and systemic lupus erythematosus). Although we were not able to investigate the association of *BAX*-248A/G SNP with the apoptosis index in endometrial biopsies, it is tempting to speculate that *BAX*-248GA heterozygotes have a better control of *BAX* expression and, thus, have a well-balanced proapoptotic and antiapoptotic ratio in comparison with the homozygous state. Nevertheless, the homozygous state of the *BAX*-248A allele was found only in women with primary RPL in our study. Additional studies are necessary to confirm our findings and to elucidate the functional relevance of *BAX*-248A/G on pregnancy outcome. Notably, the association of the *BAX*-248GA genotype with a decreased risk of primary RPL was significant even after the adjustment for potential confounding factors (ethnicity, smoking, and alcohol consumption). However, we highlight that external factors (i.e., psychological stress, endocrine disruptors, and air pollution) that were not covered in this study may influence women's

reproductive health and should also be further investigated [2, 57, 58]. The association of ethnicity with RPL susceptibility in black and Asian women has been reported [59, 60].

In the current study, we were not able to measure smoking and alcohol intake. We considered that even at a low dosage, alcohol consumption is a risk factor for adverse pregnancy outcomes, including miscarriage [2, 61]. Additionally, the observed effect of smoking on RPL susceptibility is paradoxical since smoking affects reproductive health, and the risk of miscarriage is dose-dependent [2, 62]. On the other hand, it has been suggested that smoking during the latter half of pregnancy may be protective against preeclampsia [63–65]. Thus, the association of smoking and the *BAX*-248GA genotype with RPL protection should be further investigated. Interestingly, a high expression of *PGF*, *HIF1A*, and the proapoptotic genes *TP53* and *BAX* in villous samples from electively terminated, early pregnancies has been associated with maternal smoking [66]. In the same study, the authors speculate that maternal smoking influences placental morphological changes by inducing angiogenesis via PGF and apoptosis via the mitochondrial pathway (TP53 and BAX/BCL2) [66]. Since *BAX* expression is regulated by the TP53 protein [67], which is a transcription factor, it is likely that the effect of combined SNPs (epistasis) in both genes modifies apoptosis signaling and disease susceptibility. For example, the joint effect of the genotypes *TP53*, Arg72pro (rs1042522), and BAX-248AA is associated with the risk of head and neck squamous cell carcinoma [68]. Notably, a previous study from our group reported that genetic variation in the genes of the *TP53* network influences RPL susceptibility, suggesting that impaired post-implantation mechanisms, such as p53-mediated embryo selection through apoptosis, contribute to recurrent pregnancy loss [69].

The distinction of RPL subclinical types is hardly made. Therefore, women with secondary RPL represent a poorly understood group and might represent a different entity of a characteristic phenotype. We observed an association of the *BCL-2*-938CA+CC genotypes with secondary RPL, although it lost its statistical significance after *Bonferroni* correction, and we highlight the need for further validation in more extensive studies. Previous studies that evaluated healthy endometrial tissues have reported that BCL-2 expression is high in the proliferative phase and gradually decreases toward the menstrual phase, with low or even absent expression after the middle secretory phase [70]. However, BCL-2 expression in the placenta is generally low throughout the gestational period, whereas BAX expression gradually increases by the end of pregnancy, thus implying that the regulation of both expression and the balance between BCL-2/BAX is spatio-temporal during pregnancy [71]. Therefore, imbalances between the proapoptotic and antiapoptotic proteins from the BCL-2 family might impair the delicate balance between programmed cell death and proliferation, thus increasing the risk for pregnancy disorders. In summary, data from the current study suggest that *BAX*-248AG is a protective genotype for primary RPL and that the BCL-2-938CC and C genotypes are risk factors for secondary RPL, but these findings will require confirmation in more extensive studies due to the low frequency of some genotypes.

In conclusion, our study is the first to evaluate the role of the main SNPs in both extrinsic and intrinsic apoptosis pathways in RPL susceptibility. Our data suggest that the regulatory variants in apoptosis-related genes contribute to the RPL risk in a Brazilian population. The association of the *BAX*-248G/A polymorphism with RPL risk implies that maternal genetic susceptibility for RPL has a potential contribution from genetic variants in regulatory regions that are involved in the delicate balance of endometrium cell turnover (cell death and proliferation). Therefore, apoptosis-related genes are potential candidates for future studies about healthy pregnancies and the spectrum of pregnancy disorders, as well as potential molecular biomarkers/targets for identifying women with fertility issues.

Funding information This study was supported by Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (grant no. 14/2253-2 PqG) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (grant nos. 473115/2011-5 and 305839/2015-2).

Compliance with ethical standards

This study was approved by the Research Ethics Committee of the HCPA (HCPA-CEP-CPPG) under protocol number #11-242. We obtained written, informed consent according to the Declaration of Helsinki from all individual participants included in this study.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Practice Committee of the American Society for Reproductive Medicine. Evaluation and treatment of recurrent pregnancy loss: a committee opinion. Fertil Steril. 2012;98:1103–11.
2. Rai R, Regan L. Recurrent miscarriage. Lancet. 2006;368:601–11.
3. Christiansen OB. Research methodology in recurrent pregnancy loss. Obstet Gynecol Clin N Am. 2014;41:19–39.
4. Toth B, Jeschke U, Rogenhofer N, Scholz C, Würfel W, Thaler CJ, et al. Recurrent miscarriage: current concepts in diagnosis and treatment. J Reprod Immunol. 2010;85:25–32.
5. Shahine L, Lathi R. Recurrent pregnancy loss. Obstet Gynecol Clin N Am. 2015;42:117–34.
6. Aghaeepour N, Ganio EA, Mcilwain D, Tsai AS, Tingle M, Van Gassen S, et al. An immune clock of human pregnancy. Sci Immunol. 2017;1:2.
7. Diemert A, Arck PC. Pregnancy around the clock. Trends Mol Med. 2018;24:1–3.
8. Choi H-K, Choi BC, Lee S-H, Kim JW, Cha KY, Baek K-H. Expression of angiogenesis- and apoptosis-related genes in chorionic villi derived from recurrent pregnancy loss patients. Mol Reprod Dev. 2003;66:24–31.

9. Baek K-H. Aberrant gene expression associated with recurrent pregnancy loss. Mol Hum Reprod. 2004;10:291–7.
10. Sõber S, Rull K, Reiman M, Ilisson P, Mattila P, Laan M. RNA sequencing of chorionic villi from recurrent pregnancy loss patients reveals impaired function of basic nuclear and cellular machinery. Sci Rep. 2016;6:38439.
11. Lee J, Oh J, Choi E, Park I, Han C, Kim DH, et al. Differentially expressed genes implicated in unexplained recurrent spontaneous abortion. Int J Biochem Cell Biol. 2007;39:2265–77.
12. Krieg SA, Fan X, Hong Y, Sang Q-X, Giaccia A, Westphal LM, et al. Global alteration in gene expression profiles of deciduas from women with idiopathic recurrent pregnancy loss. Mol Hum Reprod. 2012;18:442–50.
13. Wei D, Wu Q, Shi H. Apoptosis and p53 expression in the placental villi of females with unexplained recurrent spontaneous abortion. Exp Ther Med. 2014;7:191–4.
14. Oreshkova T, Dimitrov R, Mourdjeva M. A cross-talk of decidual stromal cells, trophoblast, and immune cells: a prerequisite for the success of pregnancy. Am J Reprod Immunol. 2012;68:366–73.
15. Galluzzi L, Vitale I, Aaronson SA, Abrams JM, Adam D, Agostinis P, et al. Molecular mechanisms of cell death: recommendations of the nomenclature committee on cell death 2018. Cell Death Differ. 2018;25:486–541.
16. Peter ME, Hadji A, Murmann AE, Brockway S, Putzbach W, Pattanayak A, et al. The role of CD95 and CD95 ligand in cancer. Cell Death Differ. 2015;22:549–59.
17. Murakoshi H, Matsuo H, Laoag-Fernandez JB, Samoto T, Maruo T. Expression of Fas/Fas-ligand, Bcl-2 protein and apoptosis in extravillous trophoblast along invasion to the decidua in human term placenta. Endocr J. 2003;50:199–207.
18. Niederkorn JY. See no evil, hear no evil, do no evil: the lessons of immune privilege. Nat Immunol. 2006;7:354–9.
19. Robinson R, Hsu CD, Chesebro AL, Nguyen J, Ali N, Maramreddy H, et al. A single-nucleotide polymorphism (-670) of the maternal Fas gene is associated with intrauterine growth restriction. Am J Obstet Gynecol. 2009;201:620.e1–4.
20. Ciarmela P, Boschi S, Bloise E, Marozio L, Benedetto C, Castellucci M, et al. Polymorphisms of FAS and FAS ligand genes in preeclamptic women. Eur J Obstet Gynecol Reprod Biol. 2010;148:144–6.
21. Nair RR, Khanna A, Singh K. Association of FAS-1377 G>A and FAS-670 A>G functional polymorphisms of FAS gene of cell death pathway with recurrent early pregnancy loss risk. J Reprod Immunol. 2012;93:114–8.
22. Banzato PCA, Daher S, Traina É, Torloni MR, Gueuvoghlanian-Silva BY, Puccini RF, et al. FAS and FAS-L genotype and expression in patients with recurrent pregnancy loss. Reprod Sci. 2013;20:1111–5.
23. Nasr AS, Abdel Aal AA, Soliman A, Setohy KAAEL, Shehata MF. FAS and FAS ligand gene polymorphisms in Egyptian females with preeclampsia. J Reprod Immunol. 2014;104–105:63–7.
24. Glesse N, Vianna P, Paim LMG, Matte MCC, Aguiar AKK, Palhano PL, et al. Evaluation of polymorphic variants in apoptotic genes and their role in susceptibility and clinical progression to systemic lupus erythematosus. Lupus. 2017;26:746–55.
25. Raguema N, Zitouni H, Ben Ali Gannoun M, Benletaifa D, Almawi W, Mahjoub T, et al. FAS A-670G and Fas ligand IVS2nt A 124G polymorphisms are significantly increased in women with pre-eclampsia and may contribute to HELLP syndrome: a case-controlled study. BJOG. 2018;125:1758–64.
26. Masoumi E, Tavakkol-Afshari J, Nikpoor AR, Ghaffari-Nazari H, Tahaghoghi-hajghorbani S, Jalali SA. Relationship between Fas and Fas ligand gene polymorphisms and pre-eclampsia. J Obstet Gynaecol Res. 2016;42:1272–8.
27. Han AR, Choi YM, Hong MA, Kim JJ, Lee SK, Yang KM, et al. Fas and FasL genetic polymorphisms in women with recurrent pregnancy loss: a case-control study. Hum Fertil. 2018:1–6.
28. Huang QR, Morris D, Manolios N. Identification and characterisation of polymorphisms in the promoter region of the human Apo-1/Fas (CD95) gene. Mol Immunol. 1997;34:577–82.
29. Sibley K, Rollinson S, Allan JM, Smith AG, Law GR, Roddam PL, et al. Functional FAS promoter polymorphisms are associated with increased risk of acute myeloid leukemia. Cancer Res. 2003;63:4327–30.
30. Wu J, Metz C, Xu X, Abe R, Gibson AW, Edberg JC, et al. A novel polymorphic CAAT/enhancer-binding protein beta element in the FasL gene promoter alters Fas ligand expression: a candidate background gene in African American systemic lupus erythematosus patients. J Immunol. 2003;170:132–8.
31. Galluzzi L, Kepp O, Trojel-Hansen C, Kroemer G. Mitochondrial control of cellular life, stress, and death. Circ Res. 2012;111:1198–207.
32. Hao Z, Duncan GS, Chang CC, Elia A, Fang M, Wakeham A, et al. Specific ablation of the apoptotic functions of cytochrome c reveals a differential requirement for cytochrome c and Apaf-1 in apoptosis. Cell. 2005;121:579–91.
33. Marie Hardwick J, Soane L. Multiple functions of BCL-2 family proteins. Cold Spring Harb Perspect Biol. 2013;5:a008722.
34. Nückel H, Frey UH, Bau M, Sellmann L, Stanelle J, Dürig J, et al. Association of a novel regulatory polymorphism (-938C>A) in the BCL2 gene promoter with disease progression and survival in chronic lymphocytic leukemia. Blood. 2007;109:290–7.
35. Sahu SK, Choudhuri T. Lack of association between Bax promoter (-248G>A) single nucleotide polymorphism and susceptibility towards cancer: evidence from a meta-analysis. PLoS One. 2013;8(10):e77534.
36. Feng Y, Chen X, Zheng Y, Liu Q, Chen H, Cai Y, et al. Prognostic value and susceptibility of BAX rs4645878 polymorphism in cancer. Medicine (Baltimore). 2018;97:e11591.
37. Skogsberg Å, Tobin G, Kröber A, Kienle D, Thunberg U, Åleskog A, et al. The G(-248)A polymorphism in the promoter region of the Bax gene does not correlate with prognostic markers or overall survival in chronic lymphocytic leukemia. Leukemia. 2006;20:77–81.
38. Moshynska O, Sankaran K, Saxena A. Molecular detection of the G(-248)A BAX promoter nucleotide change in B cell chronic lymphocytic leukaemia. Mol Pathol. 2003;56:205–9.
39. Starczynski J, Pepper C, Pratt G, Hooper L, Thomas A, Milligan D, et al. Common polymorphism G(-248)A in the promoter region of the bax gene results in significantly shorter survival in patients with chronic lymphocytic leukemia once treatment is initiated. J Clin Oncol. 2005;23:1514–21.
40. Fegan C, Starczynski J, Pratt G, Pepper C. The role of the bax gene polymorphism G(-248)A in chronic lymphocytic leukemia. Leukemia. 2006;20:1460–1.
41. Shang W, Shu M-M, Liu M, Wang A-M, Lv L-B, Zhao Y, et al. Elevated expressions of p53, CDKNA1, and Bax in placental villi from patients with recurrent spontaneous abortion. Eur Rev Med Pharmacol Sci. 2013;17:3376–80.
42. Little J, Higgins JP, Ioannidis JP, Moher D, Gagnon F, von Elm E, et al. STrengthening the REporting of Genetic Association Studies (STREGA)—an extension of the STROBE statement. PLoS Med. 2009;3:6:e22.
43. Lahiri DK, Numberger JI. A rapid non-enzymatic method for the preparation of HMW DNA from blood for RFLP studies. Nucleic Acids Res. 1991;19:5444.
44. Sun T, Miao X, Zhang X, Tan W, Xiong P, Lin D. Polymorphisms of death pathway genes FAS and FASL in esophageal squamous-cell carcinoma. J Natl Cancer Inst. 2004;96:1030–6.

45. Zhang Z, Wang L-E, Sturgis EM, El-Naggar AK, Hong WK, Amos CI, et al. Polymorphisms of FAS and FAS ligand genes involved in the death pathway and risk and progression of squamous cell carcinoma of the head and neck. Clin Cancer Res. 2006;12:5596–602.
46. Zhang N, Li X, Tao K, Jiang L, Ma T, Yan S, et al. BCL-2 (-938C > A) polymorphism is associated with breast cancer susceptibility. BMC Med Genet. 2011;12:48.
47. Rodriguez S, Gaunt TR, Day INM. Hardy-Weinberg equilibrium testing of biological ascertainment for Mendelian randomization studies. Am J Epidemiol. 2009;169:505–14.
48. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics. 2005;21:263–5.
49. Kroeff LR, Mengue SS, Schmidt MI, Duncan BB, Favaretto ALF, Nucci LB. Fatores associados ao fumo em gestantes avaliadas em cidades brasileiras. Rev Saude Publica. 2004;38:261–7.
50. Ruiz-Linares A, Adhikari K, Acuña-Alonzo V, Quinto-Sanchez M, Jaramillo C, Arias W, et al. Admixture in Latin America: geographic structure, phenotypic diversity and self-perception of ancestry based on 7,342 individuals. PLoS Genet. 2014;10:e1004572.
51. Gilbert-Diamond D, Moore JH. Analysis of gene-gene interactions. Curr Protoc Hum Genet. 2011;1:Unit1.14.
52. Kalish RB, Nguyen DP, Vardhana S, Gupta M, Perni SC, Witkin SS. A single nucleotide A>G polymorphism at position -670 in the Fas gene promoter: relationship to preterm premature rupture of fetal membranes in multifetal pregnancies. Am J Obstet Gynecol. 2005;192:208–12.
53. Sezgin M, Barlas IÖ, Yildir S, Türköz G, Ankarali HÇ, Şahin G, et al. Apoptosis-related Fas and FasL gene polymorphisms' associations with knee osteoarthritis. Rheumatol Int. 2013;33:2039–43.
54. Jaslow CR, Carney JL, Kutteh WH. Diagnostic factors identified in 1020 women with two versus three or more recurrent pregnancy losses. Fertil Steril. 2010;93:1234–43.
55. Bhattacharya S, Townend J, Bhattacharya S. Recurrent miscarriage: are three miscarriages one too many? Analysis of a Scottish population-based database of 151,021 pregnancies. Eur J Obstet Gynecol Reprod Biol. 2010;150:24–7.
56. Rull K, Nagirnaja L, Laan M. Genetics of recurrent miscarriage: challenges, current knowledge, future directions. Front Genet. 2012;3:34.
57. Krieg SA, Shahine LK, Lathi RB. Environmental exposure to endocrine-disrupting chemicals and miscarriage. Fertil Steril. 2016;106:941–7.
58. Conforti A, Mascia M, Cioffi G, De Angelis C, Coppola G, De Rosa P, et al. Air pollution and female fertility: a systematic review of literature. Reprod Biol Endocrinol. 2018;16:117.
59. Mukherjee S, Velez Edwards DR, Baird DD, Savitz DA, Hartmann KE. Risk of miscarriage among black women and white women in a U.S. Prospective Cohort Study. Am J Epidemiol. 2013;177:1271–8.
60. Harb HM, Al-rshoud F, Dhillon R, Harb M, Coomarasamy A. Ethnicity and miscarriage: a large prospective observational study and meta-analysis. Fertil Steril. 2014;102:e81.
61. Kesmodel U, Wisborg K, Olsen SF, Henriksen TB, Secher NJ. Moderate alcohol intake in pregnancy and the risk of spontaneous abortion. Alcohol Alcohol. 2002;37:87–92.
62. Lindbohm M-L, Sallmén M, Taskinen H. Effects of exposure to environmental tobacco smoke on reproductive health. Scand J Work Environ Health. 2002;28(Suppl 2):84–96.
63. Wikström A-K, Stephansson O, Cnattingius S. Tobacco use during pregnancy and preeclampsia risk. Hypertension. 2010;55:1254–9.
64. Jeyabalan A, Powers RW, Durica AR, Harger GF, Roberts JM, Ness RB. Cigarette smoke exposure and angiogenic factors in pregnancy and preeclampsia. Am J Hypertens. 2008;21:943–7.
65. Karumanchi SA, Levine RJ. How does smoking reduce the risk of preeclampsia? Hypertension. 2010;55:1100–1.
66. Kawashima A, Koide K, Ventura W, Hori K, Takenaka S, Maruyama D, et al. Effects of maternal smoking on the placental expression of genes related to angiogenesis and apoptosis during the first trimester. Zenclussen AC, editor. PLoS One. 2014;9:e106140.
67. Miyashita T, Reed JC. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell. 1995;80:293–9.
68. Chen K, Hu Z, Wang LE, Sturgis EM, El-naggar AK, Zhang W, et al. Single-nucleotide polymorphisms at the TP53-binding or responsive promoter regions of BAX and BCL2 genes and risk of squamous cell carcinoma of the head and neck. Carcinogenesis. 2007;28:2008–12.
69. Fraga LR, Dutra CG, Boquett JA, Vianna FSL, Gonçalves RO, Paskulin DD, et al. p53 signaling pathway polymorphisms associated to recurrent pregnancy loss. Mol Biol Rep. 2014;41:1871–7.
70. Vaskivuo TE, Stenbäck F, Karhumaa P, Risteli J, Dunkel L, Tapanainen JS. Apoptosis and apoptosis-related proteins in human endometrium. Mol Cell Endocrinol. 2000;165:75–83.
71. De Falco M, De Luca L, Acanfora F, Cavallotti I, Cottone G, Laforgia V, et al. Alteration of the Bcl-2:Bax ratio in the placenta as pregnancy proceeds. Histochem J. 2001;33:421–5.