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ORIGINAL PAPER

Polymorphism in apoptotic *BAX* (-248G>A) gene but not in anti-apoptotic *BCL2* (-938C>A) gene and its protein and mRNA expression are associated with cervical intraepithelial neoplasia

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Abstract HPV is associated with cervical cancer and plays a crucial role in tumor formation. Apoptosis is regulated by different pathways involving genes that either promote (BCL2 gene) or inhibit (BAX gene) cell death. Our goal was to determine whether the *BCL2-938C>A* (rs2279115) and *BAX-248G>A* (rs4645878) single nucleotide polymorphisms (SNPs) are associated with squamous intraepithelial neoplasia (SIL) risk, and whether their phenotypic expression was impaired in these lesions. Two hundred and thirty-one cases showing SIL were classified as low SIL (LSIL, n = 101) or high SIL (HSIL, n = 130), and control subjects (n = 266) with no gynecologically proven SIL were recruited. No statistical difference in the genotype and allelic frequency of the *BCL-2-938C>A* polymorphism was observed among the groups. BCL2-938C/A and A/A homozygotes carriers had higher distribution of BCL-2-expressing cells in stroma in the SIL group. BCL2 mRNA-expression was not correlated with BCL2-938C>A SNPs in both groups. We did find a strong association of the BAX GG genotype and risk for SIL. No difference was observed between LSIL and HSIL groups. In BAX-248G/A and A/A homozygote carriers, the number of BAX-expressing cells was lower the epithelium area in SIL. However, mRNA expression was higher in SIL patients than in the control group. In conclusion, our data provide evidence that allele G carriers in the BAX-248G>A promoter SNP may influence the development of SIL. However, this genotype does not influence the SIL outcome. Additionally, we suggest a possible role of HPV infection in the inhibition of the expression of BAX protein, decreasing cell death, and favoring cervical carcinogenesis.

Keywords Cervical lesions · Apoptosis markers · Polymorphism · mRNA and protein expression

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Introduction

Cervical cancer was the 4th cause of cancer death in 2013 [1] and is the second most frequent cancer in women worldwide. Approximately 500,000 new cervical cancer cases and 273,505 cervical cancer deaths are reported per year [2]. Human papillomaviruses (HPVs) are associated with cervical cancer in most instances, and the early genes E6 and E7 from high-risk HPVs play a crucial role in tumor

formation, altering cell cycle control and downregulating the function of the tumor suppressors p53 and Rb [3]. Various lines of evidence indicate that HPV infection is necessary but not sufficient for induction of the malignant changes and that other factors are involved [4], such as ethnicity, age, age of menarche, age at first sexual intercourse, number of pregnancies, and tobacco use [5].

Apoptosis is regulated by different pathways involving a number of genes that either promote or inhibit cell death. The balance between death antagonists and agonists determines whether a cell will survive or undergo apoptosis. The best characterized apoptosis regulators include the anti-apoptotic B cell lymphoma 2 (BCL2) and the pro-apoptotic B-cell lymphoma 2-associated X (BAX) proteins. The protein products of these two genes physically interact with each other, and their relative levels are important determinants of the apoptosis rate [6, 7]. The HPV E6 oncoprotein has been associated with the modulation of a wide range of apoptosis regulators that belong to the extrinsic (CD95 or FADD) and intrinsic (BAX or BCL-2) pathways or are common regulators to both pathways, such as the anti-apoptotic c-IAP2 protein [8, 9]. The *BCL2* gene is located on chromosome 18q21.3, and a promoter gene single nucleotide polymorphism (SNP), the -938C>A genotype (rs2279115), is known to be correlated with high BCL2 protein expression and is associated with disease progression in chronic lymphocytic leukemia (CLL) [10], oropharyngeal squamous cell carcinoma [11], prostate carcinoma [12], and epithelial ovarian cancer [13]. The *BAX* gene has been mapped to chromosome 19q13.3. A SNP located within the 5′ untranslated region of the *BAX* promoter, -248G>A (rs4645878), has been described, and the A allele was reported to be associated with both reduced expression of BAX protein [14] and altered susceptibility to chronic lymphocytic leukemia [15–18].

To our knowledge, there is no report examining the association of *BAX* and *BCL2* polymorphisms with squamous intraepithelial neoplasia (SIL) and/or cancer risk. Our goal was to determine whether of *BAX*-248G>A and *BCL2*-938C>A promoter polymorphisms are associated with risk of SIL, and whether their phenotypic expression was impaired in HPV cervical lesions.

Materials and methods

Study population

From November 2008 to October 2010, 514 women were enrolled for this *BAX* and *BCL2* SNP genotypic study. Patients with histologically confirmed squamous intraepithelial lesion (SIL, n = 231) were enrolled consecutively from two cohorts followed at Fiocruz Clinical Care Units in Rio de Janeiro, RJ, Brazil: Fernandes Figueira Woman, Child and Adolescent National Institute (IFF, n = 182), and Evandro Chagas National Institute of Infectious Diseases (INI, n = 49). All patients received free appropriate clinical treatment [19] and provided written consent approved by the Institutional Review Board from INI and IFF/Fiocruz. HIV status was identified in all volunteers. According to the histopathological analysis at the baseline gynecological visit, two groups were defined as having-low (LSIL) and high (HSIL) grade squamous intraepithelial lesion. Control subjects with no proven lesions and who were genetically unrelated to the SIL cases were recruited from three Clinical Sites in Rio de Janeiro, RJ, Brazil: INI (n = 66); IFF (n = 17) and State University of Rio de Janeiro (UERJ), including the Piquet Carneiro Gynecology Ambulatory and Pedro Ernesto Hospital (n = 200). A standardized questionnaire was used to collect clinical and demographic data, including age, self determined ethnicity, age, tobacco use, age at first sexual intercourse, menarche and number of pregnancies. Case and control groups were matched by clinical and demographic data.

To demonstrate whether *BAX* and *BCL2* SNPs and/or gene expression are associated with cervical lesions, we analyzed both mRNA and protein expression and compared these to the genetic frequencies. To achieve this goal, 66 volunteers were selected from the genetic study described above. Additional identification of HPV E6 and E7 oncoproteins were evaluated in those volunteers.

BCL2 and BAX genotyping

Genomic DNA was isolated with the QIAGEN DNA Blood Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Restriction fragment length polymorphism–polymerase chain reaction (RFLP-PCR) was performed to identify *BCL2*-938C>A and *BAX*-248G>A polymorphisms in the promoter regions. Each PCR was performed in a 25 μL reaction mixture containing 50 ng of genomic DNA templates, 12.5 pmol of each primer, 0.1 mM of each deoxynucleoside triphosphate, 1X PCR buffer (50 mM KCl, 10 mM Tris–HCl, and 0.1 % Triton X-100), 1.5 mM $MgCl_2$, and 1.5 U Taq polymerase (Promega Corporation, Madison, WI). The PCR profile consisted of an initial melting step at 96 °C for 5 min followed by 35 cycles at 96 °C for 45 s, at 60 °C for 40 s, and at 72 °C for 30 s, and a final extension step at 72 °C for 10 min.

For the *BCL2*-938C>A polymorphism in the promoter, the primers (5′-CTGCCTTCATTTATCCAGCA-3′ and 5′-GGCGGCAGATGAATTACAA-3′) amplified a 300-bp DNA fragment. The PCR product was digested by *BccI* (New England Biolabs, Beverly, MA) overnight at 37 °C. The wild-type allele (CC) produced two bands of 189 and

111 bp; the wild-type/variant allele (CA) produced three bands of 111, 189, and 300 bp, and the variant allele (AA) produced a single 300-bp band [20].

For the *BAX*-248G>A polymorphism, the primers (5′-CATTAGAGCTGCGATTGGACCG-3′ and 5′-GCTCC CTCGGGAGGTTTGGT-3′) amplified a 109-bp DNA fragment. The PCR product was digested by *MspI* (New England Biolabs, Beverly, MA) overnight at 37 °C. The digested product was separated on a 2.5 % NuSieve 3:1 agarose (FMC BioProducts, Rockland, ME) gel with ethidium bromide. The homozygote (GG) wild-type allele produced two bands (89 and 20 bp), and the wild-type/variant allele (GA) produced 20, 89, and 109 bp bands; the variant allele (AA) lacks the *MspI* restriction site and therefore produced a single 109-bp band.

Expression of apoptotic markers in cervical lesions

Sixty-six (49 cases and 17 controls) volunteers were selected from the genetic study described above to perform the biological identification of BAX and BCL2 protein and mRNA expression and to further correlate its expression to the specific genotypes of *BAX* (*-248G<A*) and *BCL2* (*-938C<A*).

Immunoperoxidase staining was performed as described previously by our group [21]. Sections were incubated overnight at 4 °C with specific antibodies against BAX (Zymed, South San Francisco, California, USA) and BCL2 (BD Pharmigen, Heidelberg, Germany). Positive stained cells were counted in twenty fields (×400) in the epithelium, basal layer of epithelium, stroma and perivascular fields of uterine cervix. Counts were performed using a grid (1 cm^2 divided into 10 × 10 mm^2) by two different observers.

Analysis of mRNA expression from paraffin-embedded cervical biopsies tissue Sects. (10 μm) were performed. Whole cervical tissue homogenates were prepared according to the procedure described in the QuantiGene Sample Processing Kit for FFPE Tissues (Panomics, Inc., Fremont, CA). BAX, BCL2 and E6 and E7 oncoprotein mRNA expression was analyzed using QuantiGene Plex 2.0 assay (Affymetrix, Santa Clara, CA) as described previously [22]. This methodology combines branched DNA (bDNA) signal amplification and multi-analyte profiling beads (xMAP®) technologies to enable the detection and quantitation of multiple RNA targets simultaneously. The bDNA assay is a hybridization-based method of target-specific RNA quantitation that amplifies signal rather than target RNA, using labeled DNA probes. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal positive control.

Statistical analysis

The deviation from Hardy–Weinberg equilibrium was determined for genotyping by the Chi square test (χ^2) in both SIL patients and control group. We used the χ^2 test to compare the differences in each allele and genotype of *BCL2* and *BAX* polymorphism frequency. Additionally, we used unconditional univariate and multivariate logistic regression analyses to examine the associations between the selected SNPs and SIL risk by estimating the odds ratios (ORs) and 95 % confidence intervals (CIs) with and without adjustment for age, self determined ethnicity, age, tobacco use, age at first sexual intercourse, menarche and number of pregnancies between SIL cases and control group. To evaluate interactions between *BCL2* and *BAX* genotypes in the risk of development of low and high SIL, logistic regression analysis was also used to assess potential interactions by evaluating departures from additive and multiplicative interaction models, comparing LSIL and HSIL subgroups among themselves and with controls.

Protein and mRNA apoptotic markers expression in LSIL and HSIL subgroups were compared with control group. For the mRNA analysis, the GAPDH housekeeping gene was used for normalization of BAX and BCL2 expression. Data analysis was carried out using the SPSS (version 16.0, September 2007). Student *t* test and Mann–Whitney test were used to compare means of positive cells in a particular area of tissue. Spearman's rho was used to investigate correlation among apoptotic markers expression.

All statistical tests were two-sided, a *p* value of 0.05 was considered significant, and analyses were performed using Epi Info 6 (Version 6.04, July 1996), SNPStats [29], and SPSS (Version 16, September 2007).

Results

Characteristics of the study population

A total of 514 women were enrolled for the genotyping study from Clinical Sites. Two hundred and thirty-one cases yielded SIL, classified as either LSIL (n = 102) or HSIL (n = 129). Two hundred eighty-three women showed no lesions upon gynecological evaluation and were enrolled as healthy control subjects. Table 1 describes clinical and behavior data on each study group. Patients (37.79 ± 10.14 years old) and controls (46.15 ± 6.19 years old) groups social and environmental data were identified such as age, self determined ethnicity, tobacco use, age at first sexual intercourse, menarche and number of pregnancies. No significant difference was found between SIL and control groups in all co-variables described, except in tobacco use ($p = 0.009$). In the LSIL and HSIL subgroups, the average age was 37.13 ± 10.48 and 38.31 ± 9.87 years,

Table 1 Clinical and environmental data

	Squamous Intraepithelial lesions			Control (n = 283)	*p*
	ALL (n = 231)	LSIL (n = 102)	HSIL (n = 129)		
Age years (average ± SD)	37.79 ± 10.14	37.13 ± 10.48	38.31 ± 9.87	46.15 ± 6.19	0.06[a]
Ethinicity (n, %)					
White	80 (34.6)	42 (41.2)	38 (29.5)	109 (38.5)	0.67[b]
Afro-Brazilian	145 (62.8)	59 (57.8)	86 (66.6)	169 (59.7)	
Indigen	2 (0.9)	1 (1)	1 (0.8)	2 (0.7)	
Asian-Brazilian	3 (1.3)	–	3 (2.3)	1 (0.4)	
No determinated	1 (0.4)	–	1 (0.8)	2 (0.7)	
Tobacoo use (n, %)					
Yes	121 (52.4)	42 (41.2)	79 (61.2)	113 (40.0)	0.009[c]
No	110 (47.6)	60 (58.8)	50 (38.8)	164 (57.9)	
Unknown	–	–	–	6 (2.1)	
Age at first sexual intercourse (average ± SD)	17.36 ± 3.88	17.9 ± 4.3	16.95 ± 3.48	19.85 ± 2.90	0.23[a]
Menarche (average ± SD)	12.72 ± 1.6	12.75 ± 1.69	12.71 ± 1.54	12.84 ± 1.68	0.32[a]
Number of pregnancies (average ± SD)	2.53 ± 1.91	2.32 ± 2.01	2.69 ± 1.83	2.53 ± 1.26	0.31[a]

LSIL low squamous intraepithelial lesion, *HSIL* high squamous intraepithelial lesion

[a] *p* value, *t* student

[b] Two sided, from χ^2 test for trend

[c] *p* value, two sided, from χ^2 test

respectively, and co-variables were not significant in both subgroups (data not shown).

BCL2-938C>A and BAX-248G>A gene frequency and their associations with SIL presence

The genotypes and allele distributions of *BCL2-938C>A* and *BAX-248G>A* in the SIL patients and control group are summarized in Table 2. The observed genotype frequencies of these SNPs were in agreement with Hardy–Weinberg equilibrium in the case and control groups in *BCL2* ($p = 0.6$ and 0.9, respectively) and *BAX* ($p = 0.9$ and 0.06, respectively). No association in genotype and allelic frequencies of *BCL2*-938C>A polymorphism was observed between SIL and control groups (Table 2), even when the SIL group was stratified into LSIL and HSIL subgroups (Table 3). No association was identified between LSIL and HSIL subgroups ($p > 0.05$), in any genetic model tested. There was no significant overall difference when the OR was adjusted for univariate model including age, ethnicity, tobacco use, age at first sexual intercourse, menarche, and number of pregnancies or in multivariate analysis. These variables did not change the risk for SIL development.

BAX-248G/G genotype was identified as a strong factor [$p < 0.001$, *OR* 3.50 (2.39–5.23)] for SIL development, with G allele carriers showing a 3.38-fold ($p < 0.001$) higher risk for SIL development compared to homozygote wild-type allele carriers (Table 2). There was no significant difference overall when the OR was adjusted for univariate or multivariate analysis. When women with LSIL or HSIL were compared, there were no differences on genotypic or allelic frequencies. However, when those subgroups were compared with the control group, a strong increase in risk was identified in LSIL and in HSIL women; both GG genotype [$p < 0.001$, OR 4.15 (2.48–7.20); $p < 0.001$, OR 3.14 (1.96–5.03), respectively] and the G allele [$p < 0.001$, 3.74 (2.30–6.08); $p < 0.001$, 3.14 (2.07–4.77), respectively] were associated with risk for SIL (Table 3). These results indicate that GG genotype and G allele may influence the risk of SIL development, but not its severity. When the analysis was adjusted by ethnicity, age, menarche, age at first sexual intercourse, number of pregnancies, and tobacco use, no interference was found between the variant genotype and risk of SIL among groups.

BAX-expressing cells were present in all groups and were rarely expressed in keratinocytes in the epithelium in LSIL and control groups, but were notably decreased in the HSIL group. However, the frequency of BAX-expression was enhanced in the basal layer of the epithelium mainly in the LSIL group. BCL2-expressing cells in the epithelium and the stroma were enhanced in the HSIL group when compared with LSIL group.

Table 2 Logistic regression analysis of associations between *BAX* (-248G<A) and *BCL-2* (-938C<A) polymorphisms and risk of squamous intraepithelial lesions

Polymorphisms	SIL n = 231 (%)	Control n = 283 (%)	*p*	χ^2	OR
Bcl2 (-938 C<A)[c]					
CC	67 (29)	83 (29.3)	0.90[a]	0.014	1
CA	111 (48)	132 (46.6)			1.04
AA	53 (23)	68 (24.1)			0.97
CC	67 (29)	83 (29.3)	0.94[b]	0.01	0.98 (0.67–1.44)
AA + CA	164 (71)	200 (70.7)			
AA	53 (22.9)	68 (24.1)	0.77[b]	0.08	0.94 (0.62–1.42)
CC + CA	178 (77.1)	215 (75.9)			
Alleles					
C	245 (53)	298 (52.6)	0.9[b]	0.01	1.01 (0.79–1.30)
A	217 (47)	268 (47.4)			
Bax (-248G<A)[d]					
GG	182 (78.8)	145 (51.2)	0.000001[a]	44.5	1
GA	46 (19.9)	106 (37.5)			0.35
AA	3 (1.3)	32 (11.3)			0.08
GG	182 (78.8)	145 (51.2)	0.00000001[b]	41.71	3.5 (2.39–5.23)
AA + GA	49 (21.2)	138 (48.8)			
AA	3 (1.3)	32 (11.3)	0.000001[b]	20.08	0.1 (0.03–0.34)
GG + GA	228 (98.7)	251 (88.7)			
Alleles					
G	410 (88.7)	396 (69.9)	0.00000001[b]	52.98	3.38 (2.41–4.75)
A	52 (11.3)	170 (30.1)			

SIL squamous intraepithelial lesion, *OR* odds ratio

[a] *p* value, two sided, from χ^2 test for trend

[b] *p* value, two sided, from χ^2 test)

[c] *BCL2* Hardy–Weinberg equilibrium ($p = 0.43$ for SIL and $p = 0.27$ for control)

[d] *BAX* Hardy–Weinberg equilibrium ($p = 0.96$ for SIL and $p = 0.07$ for control)

Functional *BCL2* and *BAX expression and SNPs association*

BCL2-938C>A and *BAX*-248G>A SNPS were both reported to be associated with reduced BCL2 and BAX protein expression in cancer [14–18]. To our knowledge there is no previous report relating those SNPs and their protein and mRNA expression to SIL. Here, sixty-six samples were selected, based on the availability of cervical tissue to analyze the association of genotype frequencies and both protein and mRNA expression.

BCL-2-expressing intralesional inflammatory cells were not influenced by the any of these genotypes. However, *BCL2-938C/A* and *A/A* homozygotes had higher distribution of BCL-2-expressing cells, only in the cervical stroma area in the SIL group, compared with control group ($p = 0.044$, Fig. 1a, c). BCL2-expressing cells had a morphology consistent with that of inflammatory and fibroblast cells (Fig. 1a). BCL2 mRNA-expression was not correlated with BCL2-938C>A SNPs in either group (Fig. 1e). These results suggest that the increased BCL2 expressing-cells seen in SIL patients inhibit apoptosis and activate the relase of pro-angiogenic factors, which are crucial for carcinogenic process.

In *BAX*-248G/A and in A/A homozygote carriers, fewer BAX-expressing cells were identified in the intralesional epithelium area in SIL group than in the control group ($p = 0.002$, Fig. 1b, d). However, BAX mRNA expression was more pronounced found in those SIL patients than in the control group ($p = 0.0039$, Fig. 1f), suggesting that HPV infection might influence the BAX protein down-regulation and it is genetically correlated, since there was no difference in *BAX* G/G homozygotes carriers.

Discussion

In this case–control study, we assessed the effects of *BAX-248G>A* and *BCL2-938C>A* polymorphisms on risk of cervical intraepithelial lesions. It is now well known that

Table 3 Logistic regression analysis of associations between *BAX* (-248G<A) and *BCL-2* (-838C<A) polymorphisms and risk of squamous intraepithelial lesions

	SIL (n = 231)									
	LSIL n = 101(%)	Control n = 283 (%)	*p*	χ2	OR	HSIL n = 129(%)	Control n = 283 (%)	*p*	χ2	OR
Bcl2										
CC	27 (26.4)	83 (29.3)	0.51[a]	0.44	1	40 (31)	83 (29.3)	0.36[a]	0.8	1
CA	47 (46.1.5)	132 (46.6)			1.09	64 (49.6)	132 (46.6)			1.01
AA	28 (27.5)	68 (24.1)			1.27	25 (19.4)	68 (24.1)			0.76
CC	27 (26.4)	83 (29.3)	0.58[b]	0.30	0.86 (0.52–1.44)	40 (31)	83 (29.3)	0.73[b]	0.12	1.08 (0.69–1.70)
CA + AA	75 (73.6)	200 (70.7)				89 (69)	200 (70.7)			
AA	28 (27.5)	68 (24.1)	0.49[b]	0.47	1.19 (0.71–2.00)	25 (19.4)	68 (24.1)	0.29[b]	1.1	0.76 (0.45–1.27)
CC + CA	74 (72.5)	215 (75.9)				104 (80.6)	215 (75.9)			
Alleles										
C	101 (49.5)	298 (51.1)	0.44[b]	0.59	0.88 (0.64–1.21)	144 (55.8)	298 (51.1)	0.39[b]	0.71	1.13 (0.84–1.52)
A	103 (50.5)	268 (48.9)				114 (44.2)	268 (48.9)			
Bax										
GG	83 (81.4)	145 (51.2)	0.000001[a]	23.75	1	99 (76.7)	145 (51.2)	0.000001[a]	28.88	1
GA	17 (16.7)	106 (37.5)			0.28	29 (22.5)	106 (37.5)			0.4
AA	2 (1.9)	32 (11.3)			0.16	1 (0.8)	32 (11.3)			0.05
GG	83 (81.4)	145 (51.2)	0.0000001[b]	28.20	4.15 (2.48–7.20)	99 (76.7)	145 (51.2)	0.00000001[b]	23.87	3.14 (1.96–5.03)
GA + AA	19 (18.6)	138 (48.8)				30 (23.3)	138 (48.8)			
AA	2 (2.0)	32 (11.3)	0.004[b]	8.13	0.15 (0.04–6.67)	1 (0.8)	32 (11.3)	0.0002[b]	13.34	0.06 (0.003–0.33)
GG + GA	100 (98)	251 (88.7)				128 (99.2)	251 (88.7)			
Alleles										
G	183 (89.7)	396 (69.9)	0.00000001[b]	32.62	3.74 (2.30–6.08)	227 (88)	396 (69.9)	0.00000001[b]	32.6	3.14 (2.07–4.77)
A	21 (10.3)	170 (30.1)				31 (12)	170 (30.1)			

SIL squamous intraepithelial lesion, *LSIL* and *HSIL* low and high squamous intraepithelial lesion, *OR* odds ratio

[a] *p* value, two sided, from χ^2 test for trend

[b] *p* value, two sided, from χ^2 test

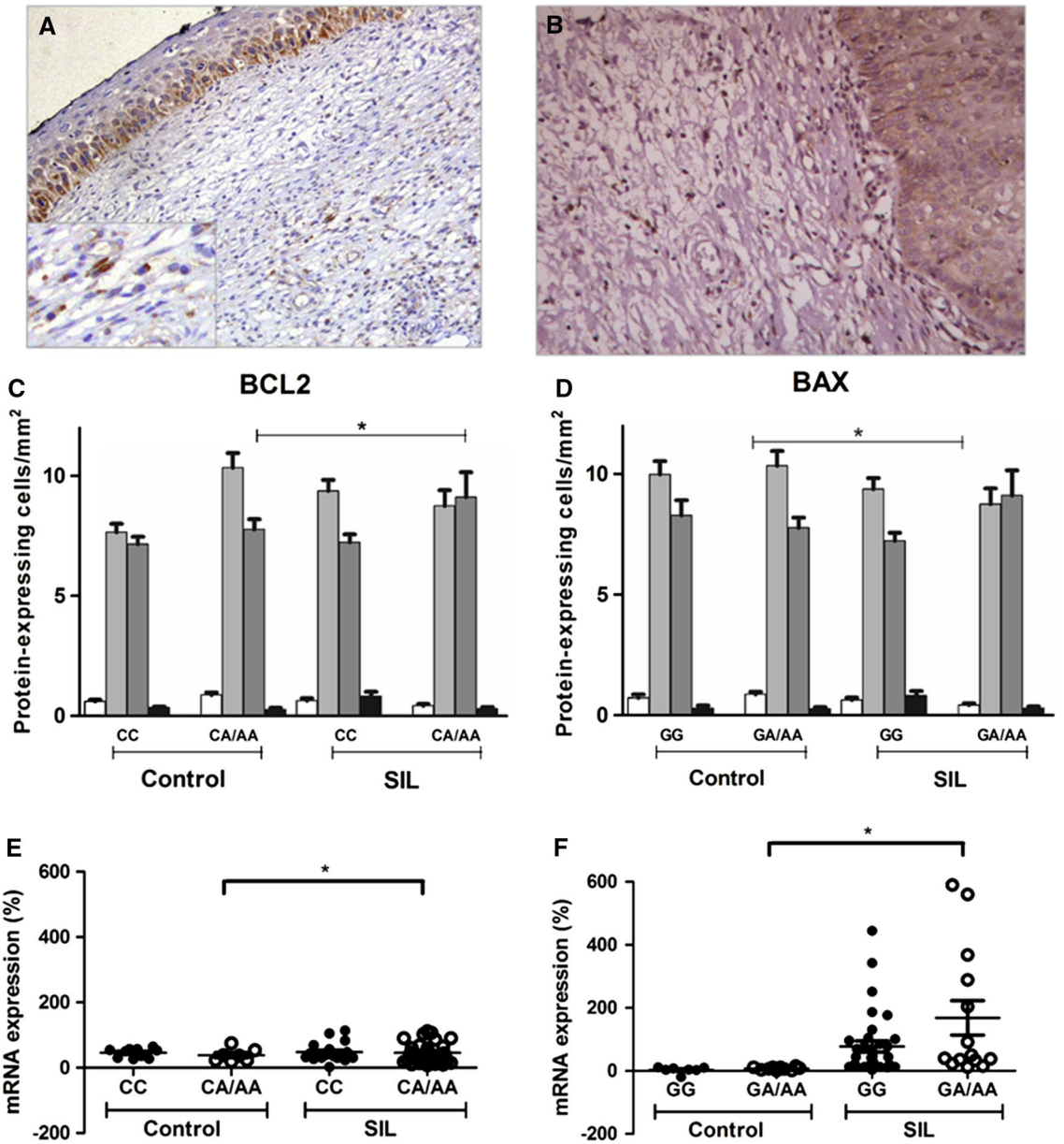

Fig. 1 Distribution of BCL2 (**a**, **c**) and BAX (**b**, **d**) expressing cells in uterine cervix from squamous intraepithelial lesions (SIL) and control groups carrying *BCL2*-938C>A or *BAX*-248G>A polymorphism was detected by immunohistochemistry in epithelium (*white bars*), basal layer of epithelium (*light gray bars*), stroma (*dark gray bars*) and perivascular area (*black bars*); and by mRNA expression (**e**, **f**). Images in **a** (200×, inset 400×) and **b** (200×) are representative from patients with SIL

both neoplastic events and cancer initiation are influenced by genetic background. Control of cell proliferation is achieved by balance between the regulation of apoptosis and cell cycle genes.

When social and environmental data were analyzed, no significant association was found between SIL and control groups in all co-variables described, except in tobacco use. The effects of smoking have been examined in many case–control studies, and tobacco smoking has been seen to increases the risk of developing HSIL and cervical cancer [23]. Nicotine and its major metabolite (cotinine) were increased 4- and 40-fold in the cervical mucus of healthy female smokers and women with CIN, respectively [24]. Moreover, DNA repair defects related to carcinogenesis and DNA damage were found in the cervical tissue of smokers. Benzo[a]pyrene (BaP), identified as a prime carcinogen in cigarette smoke, was detected in cervical tissue, and DNA adducts were present in smokers twice as often as in noncurrent smokers. Further, cell growth and DNA damage induced by BaP were higher in

HPV-16 immortalized cervical cells than in normal tissue [25].

BCL2 was initially identified as an anti-apoptotic regulatory protein [26], but it also serves as an inhibitor of proliferation [27]. The functional implication of BCL2 in tumorigenesis is ambiguous, as the anti-apoptotic effect may be oncogenic while the anti-proliferative effect appears tumor-suppressive. Which of the dual functions predominates seems to be tissue specific. This may explain why the prognostic implication of BCL2 expression depends on cancer type. In a previous study [21], we evaluated the presence of BCL2 and BAX-expressing cells in cervical lesions, according the degree of lesions, and BCL2 expression was observed mainly in HSIL women when compared with LSIL and control groups, favoring carcinogenesis. In accordance, BCL2 expression was associated with decreased survival in leukemia and prostate cancer [28, 29], whereas in colorectal [30, 31] and breast cancer, it was related to favorable outcome.

Moreover, the association of BCL2 polymorphisms to cancer and other diseases is still controversial. In this study, the single-locus analysis for *BCL2*-938C>A polymorphism did not reveal any significant association with SIL development. No study has been reported regarding the association of *BCL2* polymorphism with SIL, cervical cancer or HPV infection. Kuhlmann et al. [32] and Chen et al. [20] did not identify any association between *BCL2-938C>A* polymorphism and risk of multiple sclerosis or squamous cell carcinoma of the head and neck. Nevertheless, the *BCL2*-938A/A genotype has been associated with increased BCL-2 expression and identified as a novel unfavorable genetic marker in patients with B-chronic lymphocytic leukemia (CLL) and lung cancer [10, 33]. Hirata et al. [34] observed that *BCL2*-938C/C carriers showed higher biochemical recurrence in prostate cancer than -938C/A and A/A genotypes carriers. Additionally, in oropharyngeal squamous cell carcinoma, the *BCL2*-938C/A genotype was associated with relapse and survival [11].

The functional BCL2-938 C>A promoter polymorphism is located in the inhibitory P2 promoter region. C and A alleles differentially bind transcription factors and affect promoter activity. The C allele shows altered binding of transcription factors, increased activity of inhibitory promoter P2 and therefore decreased expression of BCL2 [35]. In this study, we evaluated the possible correlations between BCL2 polymorphism and its phenotypical expression (protein and mRNA) in SIL patients. A prior publication [12] demonstrated that significantly higher BCL2 mRNA expression in prostate carcinoma patients harboring the AA genotype. However, this study did not quantify BCL2 expressing cells or determine their distribution in cancer tissue. Our study is the first to identify the correlation between BCL2 protein and mRNA expression in cervical lesions. A higher distribution of BCL2-expressing cells was observed, only in C/A + A/A genotypes carriers, in cervical stroma, when SIL patients were compared with control subjects, without association with mRNA expression. Two possible explanations may justify this discrepancy. First, the mRNA expression was performed in whole cervical tissue homogenates and not specifically in stroma. Additionally, a high concentration of a particular protein does not necessarily mean that the corresponding gene is being highly expressed at the moment of measurement. Second, the epigenetic DNA-modification induced by methylation still not clearly defined in cervical lesions induced by HPV- infection. Epigenetic gene silencing via dense DNA methylation within CpG islands has been demonstrated in HPV-associated cervical cancer. Tumor suppressor genes (TSGs), that have an important role in the pathogenesis of cervical cancer, are common targets for gene silencing in this disease [36]. The identification of a panel of aberrantly methylated TSGs represent a wide spectrum of tumor suppressive functions, as cell signaling, gene transcription, cell cycle, apoptosis, and cell adhesion, and has great promise to provide a powerful set of DNA methylation biomarkers for use in disease diagnosis and/or prognosis [37]. To clarify this inconclusive association, other studies may be addressed to correlate the higher BCL2 expression in cervical stroma area instead in the epithelial area where HPV infection occurs, including the role of methylation in BCL2 gene.

BAX is a pro-apoptotic protein and controls apoptosis by regulating mitochondrial outer membrane permeabilization [38]. Increased BAX expression may contribute to better prognosis to apoptosis in several cancers, such as hepatocellular carcinoma [39], colorectal cancer [40] and nasopharyngeal carcinoma [41]. In cervical cancer, it is still controversial. One study showed no prognostic significance [42] and two showed a negative correlation [43]. In a previous study from our group [21], we observed decreased *BAX* expression in HSIL, compared with LSIL and control groups, suggesting that this protein has a pivotal role in the carcinogenic process.

In relation to *BAX-238G>A* polymorphism, we identified a strong association ($p < 0.001$) between GG genotype and G allele with a risk for SIL development. There is no study in the literature correlating *BAX-248G>A* polymorphism and development of SIL or cervical cancer. However, its role in cancer is controversial. The G allele of this SNP has the same profile in lung cancer [44]. On the other hand, *BAX-248A/A* genotype was related to cancer progression and failure to achieve complete response to therapy in B-CLL [15] and squamous cell carcinoma of the head and neck [45].

The functional study comparing the *BAX-248G>A* genotypes with the phenotypic profile (protein and mRNA)

showed an evident BAX mRNA, and a decreased expression of BAX protein, in SIL patients carrying *BAX-238* G/G and G/A genotypes. These results may suggest that HPV can inhibit the BAX protein expression in cervical lesion as reported by others. Oh et al. [46] demonstrated that HPV-E5 decreased BAX expression through the stimulation of protein degradation by an ubiquitin-proteosome-dependent pathway without decreased mRNA levels. Besides, Li et al. [47] observed that the levels of the microRNA miR-886-5p were overexpressed in human cervical squamous cell carcinoma (CSCC) cell lines, SiHa, and lowered BAX expression knockdown of this miRNA increased BAX protein and apoptotic cell death in cells of the CSCC. Additionally, HPV infection has been reported to play an anti-apoptotic role by the inhibition of BAX activity post-transcriptionally, which is caused by the stimulation of p53 degradation [48].

In conclusion, our data from a relatively large case–control study provides evidence that G allele carriers in the promoter regions of BAX-248G>A is related to risk of SIL development, but not to the disease severity. Additionally, we suggest a possible role for HPV infection in BAX protein expression inhibition, decreasing cell death, and favoring cervical carcinogenesis.

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Compliance with ethical standards

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

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