DNA methylation polymerase chain reaction (PCR) array of apoptosis-related genes in pleomorphic adenomas of the salivary glands

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Objective. The aim of this study was to evaluate the DNA methylation profile in 22 apoptosis-related genes in pleomorphic adenomas (PAs) of the salivary glands, in comparison with normal salivary glands (NSGs), and to address the differences in methylation patterns between smaller and larger tumors. Additionally, we investigated if the hypermethylation of differentially methylated genes between NSGs and PAs impacted the messenger RNA (mRNA) transcription.

Design. Twenty-three fresh PA samples and 12 NSG samples were included. The PA samples were divided into 2 groups: PAs with clinical size larger than 2 cm (n = 12) and PAs with clinical size 2 cm or smaller (n = 11). DNA methylation at the promoter region of a panel of 22 genes involved in apoptosis was profiled by using a human apoptosis DNA methylation polymerase chain reaction array, and the transcriptional levels of genes showing differential methylation profiles between PAs and NSGs were assessed.

Results. *TNFRSF25* and *BCL2 L11* were highly methylated in PAs, in comparison with NSGs, irrespective of tumor size. However, no difference could be observed in the mRNA transcription between PAs and NSGs.

Conclusions. Hypermethylation of the proapoptotic genes *BCL2 L11* and *TNFRSF25* is observed in PA. However, this phenomenon did not impact mRNA transcription. (Oral Surg Oral Med Oral Pathol Oral Radiol 2017;124:554–560)

Pleomorphic adenomas (PAs) are the most common salivary gland neoplasms, accounting for about 60% of the salivary gland tumors.[1] Salivary gland PAs show great histologic variation and present 2 major proliferating cellular types: luminal and abluminal cells (with myoepithelial cell morphology).[2] Molecular heterogeneity in these tumors has also been proved.[3] PAs can recur or undergo malignant transformation,[4,5] and the clarification of their molecular pathogenesis may help improve the understanding of such phenomena.

Apoptosis is programed cell death involved in physiologic and pathologic processes. The balance between cell proliferation and cell death is altered in human diseases, including cancer.[6,7] Apoptosis has been extensively addressed in salivary gland PAs. Aoki et al.[8] correlated the expression of cyclooxygenase-2 (COX-2) with the expression of *BCL2* in PAs, suggesting that *COX-2* may inhibit apoptosis. A previous study by our group[9] pointed to an overall antiapoptotic gene transcriptional profile in these tumors. These results were further confirmed by another group, whose results pointed to an antiapoptotic profile, with higher expression of Bcl2 protein and lower expression of Bax in PAs.[10]

Epigenetic mechanisms are essential for the normal development and maintenance of tissue-specific gene expression patterns observed in mammals.[11] Disruption of epigenetic processes is one of the hallmarks of cancer and can lead to altered gene function and malignant cellular transformation.[12,13] DNA methylation is considered a "silencing" epigenetic mark, and such function of methylation was described in 1975.[14,15] DNA methylation consists of covalent addition of a methyl group (CH3) to the C-5 position of the cytosine ring, and several cancer types abnormally gain and lose DNA methylation.[12,16] Changes in DNA methylation patterns, especially in the promoter region of genes, can have profound effects on transcriptional silencing of their downstream genes.[13] These changes have been reported in different types of malignant and benign neoplasms, such as prostate and colon adenocarcinomas, bladder and head and neck carcinomas, adrenocortical adenomas, and PAs.[17-22]

DNA methylation is induced by DNA methyltransferases (DNMTs), and the immunoexpression of maintenance DNMT (DNMT1) and de novo DNA methylation DNMT (DNMT3a) in PAs has been previously

Statement of Clinical Relevance

DNA methylation of apoptosis-related genes may be used as targets for epigenetic drugs in pleomorphic adenomas of the salivary glands.

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assessed by our group.[18] Although all the PA samples that were evaluated exhibited nuclear DNMT1 immunostaining, less than 10% of the samples showed positive nuclear DNMT3a staining. The methylation status of small gene panels, including p16 and p14, among other tumor suppressor genes, has been evaluated in PAs of salivary glands.[23-26] Recently, Mariano et al.[27] assessed DNA methylation in 24 tumor suppressor genes in PAs and carcinoma ex-pleomorphic adenomas (CXPAs), showing hypermethylation of *RASSF1* in both the tumor types. Although the involvement of apoptosis has been previously studied in salivary gland neoplasms, the role of methylation of apoptosis-related genes in PA pathogenesis has not been addressed yet, and it is possible that these genes expressions are controlled by promoter DNA methylation.

The aim of this study was to evaluate the DNA methylation profile in the promoter region of 22 apoptosis-related genes in salivary gland PAs and to address the differences in methylation patterns between smaller and larger tumors. In addition, we examined whether the hypermethylation of differentially methylated genes of normal salivary glands and those with PA affected mRNA transcription.

MATERIALS AND METHODS

Tumor samples and experimental research strategy

This study was approved by the local ethics committee (Comitê de Ética da Universidade Federal de Minas Gerais, protocol etic 152/07), and the participants signed an informed consent form. The protocol was reviewed by the appropriate institutional review board and was in compliance with the Helsinki Declaration.

A convenience sample of 23 fresh cases of PAs of the salivary glands was included in this study. Fresh tumor samples were obtained from patients who had undergone surgical excision of PAs, and the sections were subsequently stored in liquid nitrogen. The hematoxylin and eosin (H&E)–stained tissue sections were obtained. The diagnosis of each case was reviewed by 2 oral pathologists (RSG and CCG). Twelve samples of normal salivary glands (NSGs) were collected during surgical procedures either from mucoceles (8 of 12) or from adjacent normal salivary gland tissues from patients with PAs (4 of 12) and used as controls. Frozen sections of these NSGs showed no evidence of inflammatory reaction. The PA samples were divided into 2 groups according to the clinical size of the tumor: PA with clinical size larger than 2 cm (n = 12) and PA with clinical size 2 cm or smaller (n = 11).

DNA isolation

Genomic DNA (gDNA) was extracted from frozen tissue samples by using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), according to manufacturer protocol. DNA was quantified by spectrophotometry (NanoDrop 2000, Thermo Fisher Scientific, Wilmington, MA). The samples were then combined into 3 pools: (1) NSGs, (2) PAs of size 2 cm or smaller, and (3) PAs of size larger than 2 cm. The pool's final concentrations were normalized to 1 μg of gDNA.

APAF1	*BAD*	*BAX*	*BCL2L11*	*BCLAF1*
BID	*BIK*	*BIRC2*	*BNIP3L*	*CASP3*
CASP9	*CIDEB*	*CRADD*	*DAPK1*	*DFFA*
FADD	*GADD45A*	*HRK*	*LTBR*	*TNFRSF21*
TNFRSF25	*TP53*			

Fig. 1. Panel of 22 apoptosis-related genes profiled by using the human apoptosis DNA methylation polymerase chain reaction (PCR) array.

DNA methylation analysis

DNA methylation at the promoter region of a panel of 22 genes involved in apoptosis was profiled by using a human apoptosis DNA methylation polymerase chain reaction (PCR) array (Apoptosis EpiTect Methyl qPCR array, EAHS-121 Z; Qiagen, Hilden, Germany).[28-30] The genes that were evaluated are shown in Figure 1. This array system is based on the differential cleavage of target sequences by 2 restriction enzymes, which depend on the presence or absence of methylated cytosine. Briefly, the 3 DNA pools were subjected to digestion using methylation-sensitive and/or a methylation-dependent restriction enzyme using the EpiTect Methyl DNA Restriction Kit (Qiagen, Germantown, MD), according to manufacturer protocol. Methylation-sensitive restriction enzyme selectively digests unmethylated (UM) and partially methylated DNA copies, whereas methylation-dependent restriction enzyme digests the completely methylated (M) DNA. The product of the double digestion measured the success of both enzymatic digestions and the background. After the digestion, each DNA pool was added to SYBR Green quantitative PCR (qPCR) Master Mix (Qiagen, Germantown, MD) into a PCR array plate containing prealiquoted gene-specific primers and submitted to quantitative real-time PCR (qPCR). Methylation-sensitive enzyme control (SEC) and methylation-dependent enzyme control (DEC) were used in the reaction. The qPCR quantifies the relative amount of (UM) and (M) DNA by the comparative cycle threshold method (ΔCT).

The baseline and threshold values were manually adjusted. The ΔCT values generated were pasted into the Microsoft Excel data analysis spreadsheet by using an online tool (www.sabiosciences.com/dna_methylation_data_analysis.php). In the final results, UM represents the fraction of input gDNA containing no methylated CpG

Table 1. Clinical data of patients whose pleomorphic adenoma (PA) samples were evaluated

Sample	*Location*	*Size, mm*	*Tumor group*	*Age*	*Gender*
#01	Hard palate	15	1	67	F
#02*	Parotid	10	1	46	M
#03	Upper lip	10	1	41	F
#04	Parotid	13	1	58	F
#05	Parotid	20	1	22	F
#06*	Parotid	20	1	41	M
#07*	Parotid	20	1	28	M
#08	Submandibular gland	20	1	16	M
#09*	Submandibular gland	20	1	76	F
#10	Buccal mucosa	7	1	15	F
#11	Hard palate	20	1	26	M
#12	Buccal mucosa	70	2	19	M
#13*	Palate	30	2	28	M
#14	Hard palate	40	2	73	F
#15	Buccal mucosa	29	2	48	F
#16*	Parotid	30	2	25	F
#17*†	Parotid	40	2	39	M
#18*	Parotid	30	2	46	F
#19	Submandibular gland	50	2	35	F
#20	Hard palate	50	2	35	M
#21	Palate	40	2	34	M
#22	Buccal mucosa	30	2	35	F
#23	Submandibular gland	30	2	70	F

Tumor group 1 includes tumors ≤2 cm in size, and tumor group 2 includes tumors with clinical size >2 cm.
*Subjected to expression analysis.
†Recurrent PA.

sites in the amplified region of a gene, whereas M represents the fraction of gDNA containing 2 or more methylated CpG sites in the targeted sequence of the gene.

After these analyses, the genes that had higher levels of methylation (>60%) were submitted to the expression analysis. Additionally, we only validated genes expressions when the methylation levels of the gene showed a difference greater than 10% among the 3 groups.

RNA extraction and gene expression analysis

As gene promoter methylation directly impacts gene expression, the transcriptional levels of genes that show differential methylation profiles between tumors and NSGs were assessed. Total RNA from all available tumors, 8 PA (as indicated in Table 1), and 4 NSG tissue samples were extracted using TRIZOL reagent (Ambion, Carlsbad, CA), after rotor-stator homogenization, following manufacturer instructions. Because of the limited amount of tissue available from the tumors, total RNA could not be extracted from all the samples. Total RNA integrity of each individual sample was evaluated by using the 2100 Bioanalyzer instrument (Agilent technologies, Santa Clara, CA).[31] Complementary DNA (cDNA) was then synthesized using 1000 ng of the total RNA with Super Script III Reverse Transcriptase (Invitrogen, Carlsbad, CA).

The cDNA qPCR reactions were performed in duplicate, using SYBR Green power up qPCR master mix (Applied Biosystem, Austin, TX), using a Step One Plus (Applied Biosystems, Foster City, CA). Primer pairs sequences for *TNFRSF25*, *BCL2 L11*, and *28 S* were designed using the Primer Express software (Applied Biosystems, Foster City, CA); *18 s* and *HPRT1* have been described elsewhere.[32,33] Primer sequences were checked at Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast). All primers were tested and showed 90% to 100% amplification efficiency. Expressions of the 3 housekeeping genes, *18 s*, *28 s*, and *HPRT1*, were tested in the normal and tumor samples, and *18 s* presented the most homogeneous expression among the samples, which resulted in its selection as the endogenous gene. Primer sequences and amplicon sizes are shown in Table 2. The normalized relative quantification of the target genes expression was obtained using the 2-ΔΔCt method.[34]

Statistical analysis

Statistical analyses were carried out using the GraphPad prism software version 5.0. The methylation data were analyzed using an online tool to obtain the heat map (http://www.sabiosciences.com/dna_methylation_heatmap.php). The Kolmogorov-Smirnov test was performed to evaluate the normality of data distribution. Nonparametric Mann-Whitney or parametric *t* test was used to compare the 2 groups. The analysis of variance test was used when comparing more than 2 groups. *P* values <.05 were considered statistically significant.

Table 2. Primer pair sequences and amplicon sizes

cDNA	*Primers*	*Sequence 5'-3'*	*Amplicon size*
BCL2 L11	Forward	CACAAACCCCAAGTCCTCCTT	60 bp
	Reverse	TGGAAGCCATTGCACTGAGA	
TNFRSF25	Forward	GCCACCCTGACCTACACATACC	68 bp
	Reverse	CCAGCTTCATCTGCAGTAACCA	
18 S	Forward	ATCCCTGAAAAGTTCCAGCA	154 bp
	Reverse	CCCTCTTGGTGAGGTCAATG	

bp, Base pairs; *cDNA,* complementary DNA.

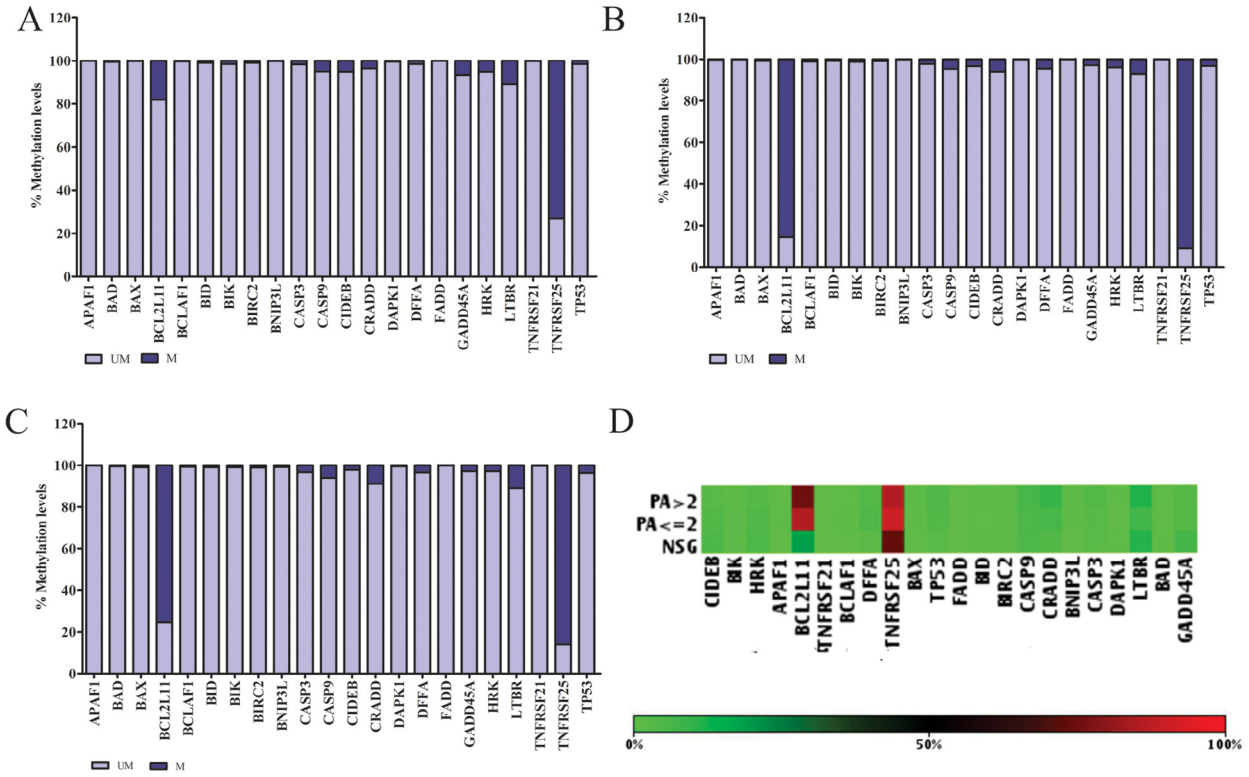

Fig. 2. DNA methylation levels in the salivary gland pleomorphic adenomas (PAs) and normal salivary glands (NSGs). These figures show the methylation profile of each of the 22 genes evaluated in NSGs **(A)**, PAs ≤2 cm **(B)**, PAs >2 cm **(C)**. For each gene, the percentage of methylated DNA (M) and unmethylated DNA (UM) are represented (A, B, C). In **(D)**, a heat map shows the methylation levels in both PA groups and in NSGs. Methylation levels vary according to the color scale, ranging from light green (low) to red (high). *BCL2 L11* showed a marked methylation percentage difference between normal glands and tumors, being highly methylated in tumors (A, B, C, D). Difference of *TNFRSF25* methylation levels for normal glands and tumors was discrete, but it was higher than 10% (A, B, C, D).

RESULTS

Clinical data and DNA methylation analysis

Clinical data of patients with salivary gland PAs are shown in Table 1. The NSG group consisted of samples from 12 patients with mean age of 34.8 years (70 ± 11 years); 2 patients (16.7%) were male and 10 (83.3%) were female. Four NSG samples were from major salivary glands, whereas 8 were from minor glands.

The methylation results of 22 apoptosis-related genes analyzed in PAs and in NSGs are shown in Figure 2. DNA from most of the evaluated genes promoter regions (20 of 22) was highly unmethylated in normal glands as well as in tumors. Conversely, *TNFRSF25* and *BCL2 L11* were highly methylated in tumors. *BCL2 L11* showed marked difference in methylation levels for normal glands and tumors, with methylation percentage of 75.4% in PAs larger than 2 cm, 85.5% in PAs 2 cm or smaller, and 17.9% in NSGs. In addition, *TNFRSF25* presented an increased level of methylation in all 3 groups: PAs larger than 2 cm (86.05%), PAs 2 cm or smaller (90.85%), and NSGs (73.12%). These 2 genes met our inclusion criteria for expression validation.

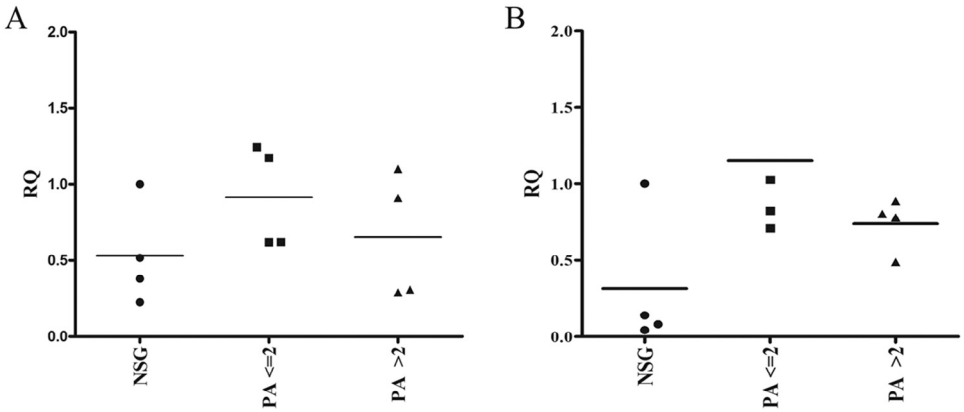

Fig. 3. *BCL2 L11* and *TNFRSF25* genes expressions in normal salivary glands (NSGs) and pleomorphic adenomas (PAs). There was no difference in the expression of the 2 genes among the NSGs, small PAs (≤2 cm), or larger tumors (>2 cm). **A,** *BCL2 L11* gene expression. **B,** *TNFRSF25* gene expression. *RQ,* Relative quantification.

Gene expression analysis

RNA integrity numbers (RINs) of the samples, before cDNA synthesis, ranged from 2.6 to 8.6. The transcriptional levels of *BCL2 L11* and *TNFRSF25* were quantified by qPCR, as these genes presented higher methylation levels. There was no difference in the expression of these genes among the 3 groups or among the tumors (grouping both PA groups) versus NSGs ($P > .05$) (Figure 3).

DISCUSSION

One of the hallmarks of cancer is tumor resistance to cell death, and thus, apoptosis has been studied in several tumor types.[3,8-10] Another hallmark of cancer is the global changes in the epigenetic landscape.[13] Although these cancer hallmarks have been proposed on the basis of the behavior of malignant neoplasms, it is possible that benign neoplasms, such as PAs, share these hallmarks. Interestingly, new treatment options are focusing on the epigenome (including DNA methylation),[35] and DNA methylation has also been proposed as a prognostic marker.[36] Promoter hypermethylation of *RASSF1 A*, for example, was identified as an independent predictor of disease-free survival in patients with salivary gland adenoid cystic carcinomas.[36]

The DNA methylation profile of apoptosis-related genes has been assessed in neoplasms, such as colon, prostate, and bladder cancers.[17,19,21] In PAs, some authors studied methylation, focusing mainly on tumor suppressor genes.[23-27] In this study, we evaluated the methylation status of the promoters of 22 apoptosis-related genes in PAs. Among these 22 genes, 2 proapoptotic genes were highly methylated in the tumor samples (*BCL2 L11* and *TNFRSF25*), irrespective of tumor size groups.

In line with our results, hypermethylation of *BCL2 L11* and *TNFRSF25* was reported in other neoplasms. Cho et al.[21] evaluated the DNA methylation profile of apoptosis-related genes in colon cancer cells and found 6 genes that were highly methylated, including *BCL2 L11* and *TNFRSF25*. These authors observed a possible demethylating effect in cells treated with a combination of docosahexaenoic acid and butyrate, compared with untreated control cells. Whether such an effect could also be achieved in human neoplasms remains an unanswered question. *BCL2 L11*, also known as Bim (Bcl-2-interacting mediator of cell death), is a BH3-only proapoptotic member of the family. It directly activates the proapoptotic effectors Bax and Bak, or it can act in neutralizing antiapoptotic Bcl2 proteins.[37-39] *BCL2 L11* silencing has been described in cancers, such as lymphomas, leukemia, and renal cell carcinoma, but the molecular mechanisms driving this phenomenon are poorly understood.[40-43] Bell et al.[44] evaluated the DNA methylation profile in salivary gland adenoid cystic carcinoma, and *BCL2 L11* was among the hypermethylated genes in this salivary gland tumor. However, the impact of methylation in the transcription of this gene was not evaluated in their study.

TNFRSF25 is a member of the tumor necrosis factor receptor (TNFR) superfamily, also known as DR3 (death receptor-3). *TNFRSF25* expression is mostly restricted to lymphocytes.[45] TNFRSF25 has a ligand (TL1 A) and a high degree of homology with TNFR-1, allowing it to activate downstream pathways and, in turn, initiating apoptosis. In addition, its overexpression in in vitro experiments induced nuclear factor-kB activation, in turn, triggering apoptosis.[46-48] Anglim et al.[49] examined the methylation profile of 42 loci in a panel of 45 lung squamous cell cancer samples and adjacent nontumor lung tissues from the same patients, describing higher DNA methylation levels of *TNFRSF25* in tumors, compared with normal tissue. A previous study evaluated 17 gene promoters, suspected to be associated with tumor progression in 96 malignant and 30 normal urothelial samples and reported that *TNFRSF25* methylation was associated with tumor progression.[50] In agreement with this finding, another study showed a

significant increase in *TNFRSF25* methylation levels in bladder carcinoma compared with the control group.[17]

Both highly methylated genes identified in PAs in our study are proapoptotic, and we speculate that the reversion of their methylation status in tumors can result in apoptosis induction and tumor size reduction. On the basis of their hypermethylation, we would expect their mRNA expressions to be decreased. However, the expression levels of both genes were similar in NSGs and PAs (grouped or separated, according to tumor sizes). The difference of methylation percentage of *BCL2 L11* among PAs and NSGs was greater than the difference of the methylation percentage of *TNFRSF25* and was therefore expected to result in lower transcription levels. However, this lack of concordance between the methylation and transcription data may have resulted from the small number of PA samples available for gene transcription validation by qPCR and also from the known molecular heterogeneity of PA,[3] once DNA and RNA have been isolated separately from different tumor portions. Additionally, the relationship between DNA methylation and expression is more complex than previously thought, and negative correlation between methylation levels and gene expression has been previously reported.[51-55] As an example of this complex interaction, the relationships among sequence variation, DNA methylation, and gene expression were studied in untransformed adult human fibroblast cells, and CpG sites with positive and negative correlations to gene expression showed distinctive patterns with respect to the histone marks and chromatin accessibility seen in their genomic region.[52] Wagner et al. also showed interindividual variation and correlation between DNA methylation and gene expression in fibroblast cells, even when removed from the same location in the body.[52]

CONCLUSIONS

Our results show that hypermethylation of the proapoptotic genes *BCL2 L11* and *TNFRSF25* occurs in PAs. However, this phenomenon did not impact mRNA transcription of these genes in the samples that were evaluated.

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