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ORIGINAL ARTICLE

DNA methylation pattern of apoptosis-related genes in ameloblastoma

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OBJECTIVES: DNA methylation is an important mechanism of gene control expression, and it has been poorly addressed in odontogenic tumours. On this basis, we aimed to assess the methylation pattern of 22 apoptosis-related genes in solid ameloblastomas.
MATERIALS AND METHODS: Ameloblastoma fresh samples (*n* = 10) and dental follicles (*n* = 8) were included in the study. The percentage fraction of methylated and unmethylated DNA promoter of 22 apoptosis-related genes was determined using enzymatic restriction digestion and quantitative real-time PCR (qPCR) array. The relative expressions of the genes that showed the most discrepant methylation profile between tumours and controls were analysed by reverse-transcription quantitative PCR (RT-qPCR).
RESULTS: Lower methylation percentages of *TNFRSF25* (47.2%) and *BCL2L11* (33.2%) were observed in ameloblastomas compared with dental follicles (79.3% and 59.5%, respectively). The RT-qPCR analysis showed increased expression of *BCL2L11* in ameloblastomas compared with dental follicles, in agreement with the methylation analysis results, while there was no difference between the expression levels of *TNFRSF25* between both groups.
CONCLUSIONS: On the basis of our results, the transcription of the apoptosis-related gene *BCL2L11* is possibly regulated by promoter DNA methylation in ameloblastoma. The biological significance of this finding in ameloblastoma pathobiology remains to be clarified.
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Keywords: ameloblastoma; DNA methylation; apoptosis; Bcl-2-like protein 11; *TNFRSF25*

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Introduction

Ameloblastoma is a benign epithelial odontogenic tumour characterised by aggressive biological behaviour and a high recurrence rate. These tumours are classified into solid/multicystic, unicystic, desmoplastic and peripheral variants according to their clinical and histopathological characteristics (Gardner *et al*, 2005). The multicystic type is the most prevalent type worldwide and in Brazil (Gardner *et al*, 2005; Fregnani *et al*, 2010; Dhanuthai *et al*, 2012; Hertog *et al*, 2012; Filizzola *et al*, 2014). The treatment of choice is surgery, varying from enucleation to wide resection, and is associated with significant morbidity (Mendenhall *et al*, 2007).

Several recent studies have identified molecular alterations in ameloblastoma (Gomes *et al*, 2010b; Amm and MacDougall, 2016; Diniz *et al*, 2016), being the most significant the BRAFV600E mutation in a high proportion of cases (Brown *et al*, 2014; Gomes *et al*, 2014; Kurppa *et al*, 2014; Sweeney *et al*, 2014; Diniz *et al*, 2015). Additionally, increased expression of apoptosis-related factors, such as Bcl-2 family proteins, cytochrome *c*, apoptotic protease-activating factor-1 (APAF-1), the inhibitor of apoptosis proteins (IAP), caspase-9, tumour necrosis factor (TNF-a) and their receptors was reported in ameloblastoma (Kumamoto *et al*, 2001; Kumamoto and Ooya, 2005a; Kumamoto and Ooya, 2005b; Rizzardi *et al*, 2009). These findings suggest that apoptosis may have a major role in oncogenesis, cytodifferentiation and malignant transformation of the odontogenic epithelium (Kumamoto *et al*, 2001; Kumamoto and Ooya, 2005a,b; Rizzardi *et al*, 2009).

Apoptosis can influence the kinetics of the tumour cells because it affects the balance between cell proliferation and death (Kerr *et al*, 1972). The inhibition of apoptosis contributes to the development and progression of tumour cells, and it may be controlled by different molecular mechanisms, including upregulation of anti-apoptotic genes and mutation or downregulation of pro-apoptotic genes (Elmore, 2007). Epigenetic modification changes gene function without altering DNA sequence or structure, and it has an impact on the regulation of many genes involved in different cellular pathways including apoptosis (Spruijt and Vermeulen, 2014).

Methylation is the most common DNA epigenetic modification. Abnormal DNA methylation is associated with gene reactivation or repression and chromosomal instabilities (Gopisetty *et al*, 2006). DNA methylation involves the addition of a methyl (CH_3) group to the 5-position of the cytosine ring (Delpu *et al*, 2013). Methylation of CpG islands (CGIs) in promoter regions results in transcriptional silencing of downstream genes because the presence of methyl groups promotes the remodelling of chromatin, which makes it less accessible to transcription (Khojasteh *et al*, 2013).

DNA methylation may represent a fundamental step in the pathway by which normal tissue undergoes tumour transformation (Delpu *et al*, 2013; Dong *et al*, 2014). Studies indicate that aberrant DNA methylation of the promoter region of specific genes, such as tumour suppressor genes, is related to tumourigenesis in different types of the neoplastic diseases, including head and neck cancer (Esteller, 2007; Demokan and Dalay, 2011). Further, alterations in DNA methylation patterns between tumour and normal cells, as well as specific DNA methylation changes present in many tumours, can be used as biomarkers (Ushijima, 2005; Dong *et al*, 2014).

To date, studies on the role of genes methylation in epithelial odontogenic tumours pathogenesis are scarce in the literature. Our research group showed an increased expression of a DNA methyltransferase (DNMT3a) and a distinct methylation profile of cell-cycle-associated genes in odontogenic tumours (Moreira *et al*, 2009; Gomes *et al*, 2010a). Moreover, methylation of the *P16* gene has been associated with ameloblastoma malignant transformation (Abiko *et al*, 2007; Khojasteh *et al*, 2013). The purpose of the present study was to investigate the possible role of changes in the methylation status of a panel of apoptosis-related genes in the pathobiology of ameloblastoma.

Materials and methods

Subjects and samples

The study was approved by the University Human Research Ethics Committee (protocol number 664.383/2014) and all subjects provided a written informed consent. Ten fresh samples of ameloblastomas were obtained during surgical procedures between 2008 and 2015. All the cases were classified as multicystic ameloblastoma based on the World Health Organization Classification of Odontogenic Tumours (Gardner *et al*, 2005). The age of the subjects with ameloblastoma ranged from 21–50 years (33.5 ± 10.47), and the male to female ratio was 1:2.33. Additionally, eight dental follicles samples obtained from asymptomatic impacted third molars extracted from healthy individuals were used as controls. The control group was composed of 02 male and 06 female subjects (1:3) with age ranging from 19 to 26 years (20 ± 4.54). Dental follicles were chosen as controls because of the similarity of the gene expression profile of ameloblastoma with dental epithelium (Heikinheimo *et al*, 2015). All samples were divided into two or three fragments: one was fixed in formalin, paraffin-embedded, submitted to routine histologic processing and sectioned for histopathological analysis, while the others were stored in Tissue-Tek® (Sakura Finetek, Torrance, CA, USA) and/or RNA*holder*® (BioAgency Biotecnologia, São Paulo, SP, Brazil) for genomic DNA and RNA extraction, respectively. All samples were stored at −80°C. The clinical and histopathological data of the patients with ameloblastoma are summarised in Table 1.

DNA isolation

The presence of tumour epithelium was guaranteed by cryosectioning each sample. Genomic DNA (gDNA) was isolated from fresh frozen tissue using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The DNA concentration and purity were determined by spectrophotometry using NanoDrop™ 2000 (Thermo Fisher Scientific, Waltham, MA, USA). For DNA methylation status analysis, the samples were combined into two pools (multicystic ameloblastomas and dental follicles) containing 1 μg of gDNA each.

DNA methylation status

The DNA methylation pattern of a panel of 22 apoptosis-related genes (*BAD*, *BAX*, *BCL2L11*, *BCLAF1*, *BID*, *BIK*, *BNIP3L*, *CASP3*, *CASP9*, *CIDEB*, *CRADD*, *DAPK1*, *DFFA*, *FADD*, *GADD45A*, *HRK*, *LTBR*, *TNFRSF21*, *TNFRSF25*, *TP53*, *BIRC2* and *APAF1*) was assessed by using the Human Apoptosis DNA Methylation PCR Array, Signature Panel EAHS-121Z (Qiagen, Maryland, USA) – a EpiTect Methyl II PCR Array System (Qiagen). This technology is based on the detection by quantitative real-time polymerase chain reaction (qPCR) of remaining input gDNA after cleavage with methylation-dependent and methylation-sensitive restriction enzymes. The restriction digestions were performed using EpiTect Methyl II DNA Restriction Kit (Qiagen, Maryland, USA) following the manufacturer's recommendations, and the reactions were run on a StepOnePlus instrument (Applied Biosystems, Foster City, CA, USA). Data analysis was performed using a specific EpiTect Methyl II PCR Array Microsoft Excel-based template (available at www.sabiosciences.com/

Table 1 Clinical data of the multicystic ameloblastoma samples included in the methylation assay

Sample	*Histopathological type*	*Age (years)*	*Gender*	*Location*
01	Follicular	33	M	Mandible
02[a]	Plexiform	24	F	Mandible
03	Plexiform	22	F	Mandible
04	Follicular	50	F	Mandible
05[a]	Follicular	28	M	Mandible
06	Follicular	33	F	Mandible
07[a]	Follicular	39	F	Mandible
08	Follicular	35	F	Mandible
09	Follicular	50	M	Mandible
10[a]	Plexiform	21	F	Mandible

[a]Samples #02, 05, 07 and 10 were included in the qPCR for the evaluation of the transcriptional levels of *TNFRSF25* and *BCL2L11*. All samples, but #04, were BRAFV600E mutation-positive (tested by TaqMan qPCR, data not shown). F = female; M = male.

dna_methylation_data_analysis.php), according to manufacturer's instructions. The Excel Template normalises the cycle threshold (Ct) values of both digests with the mock digestion values to calculate and report the percentage of the gDNA that is methylated and unmethylated.

RNA isolation and reverse transcription
Total RNA was isolated from frozen tissue samples using TRIzol® reagent (Ambion, Carlsbad, CA, USA) following the manufacturer's instructions. The RNA concentration and purity were analysed by spectrophotometry using NanoDrop 2000™ (Thermo Fisher Scientific, Waltham, MA, USA) and integrity was checked using Agilent 2100 Bioanalyzer instrument, with the Agilent RNA 6000 Nano Kit (Agilent, Waldbronn, Germany). The isolated RNA was treated with DNase I (Invitrogen, Carlsbad, CA, USA) and converted into complementary DNA (cDNA) using SuperScript® VILO™ cDNA System Kit (Invitrogen, Carlsbad, CA, USA) as outlined by the manufacturer.

Reverse-transcription quantitative polymerase chain reaction (RT-qPCR)
The relative expressions of the genes that showed the highest differential methylation status were investigated by RT-qPCR. Four multicystic ameloblastomas and four dental follicles were included in this transcription experiment. Due to the limited amount of tissue available from the tumours and dental follicles, the total RNA could not be extracted from all samples. Power Up SYBR® Green Master Mix (Applied Biosystems, Austin, TX, USA) and the following primers were used: *TNFRSF25* forward: 5' GCC ACC CTG ACC TAC ACA TAC C 3' and reverse: 5' CCA GCT TCA TCT GCA GTA ACC A 3' (68-bp amplicon); *BCL2L11* forward: 5' CAC AAA CCC CAA GTC CTC CTT 3' and reverse: 5' TGG AAG CCA TTG CAC TGA GA 3' (60-bp amplicon) and *28S* forward 5' TTG AAA ATC CGG GGG AGA G 3' and reverse 5' ACA TTG TTC CAA CAT GCC AG 3' (100-bp amplicon). The primer pairs PCR showed ~100% efficiency. The *28S* was selected as the reference gene, as it showed stable expression among tissue samples (Diniz *et al*, 2016). The normal oral mucosa was used as calibrator. All experiments were performed in duplicate and run on a StepOnePlus instrument (Applied Biosystems, Foster City, CA, USA). Analysis of relative gene expression was performed with the 2(-Delta Delta CT) method (Pfaff, 2001).

Statistical analysis
The Kolmogorov–Smirnov was performed to evaluate the normality of the data's distribution. The Mann–Whitney tests were used to compare gene expression between the groups, and it was performed using GraphPad Prism software, version 6.0 (GraphPad Software, CA, USA). *P* values <0.05 were considered statistically significant.

Results

DNA methylation status
Two genes (*TNFRSF25* and *BCL2L11*) presented the most discrepant methylation status. *TNFRSF25* methylation frequency in ameloblastomas (47.2%) was lower than in dental follicles (79.3%). The *BCL2L11* gene promoter also showed lower methylation levels in ameloblastomas (33.2%) compared with control (58.3%) (Figure 1).

Gene expression profiling
Higher *BCL2L11* expression levels were found in ameloblastomas compared with dental follicles ($P < 0.05$) (Figure 2). No difference was observed among *TNFRSF25* expression levels in ameloblastoma and dental follicle group ($P > 0.05$).

Discussion

Apoptosis is known to be involved in tumourigenesis because the insufficiency of cell death may result in

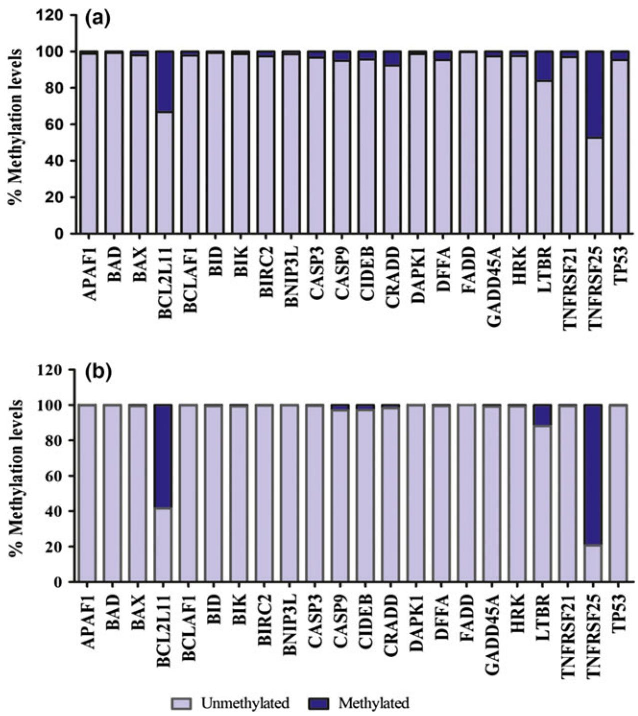

Figure 1 Methylation levels percentage in (a) ameloblastoma and (b) dental follicle pools. Note that *BCL2L11* and *TNFRSF25* showed lower methylation rates in the tumours (a) than in the control group (b) [Colour figure can be viewed at wileyonlinelibrary.com]

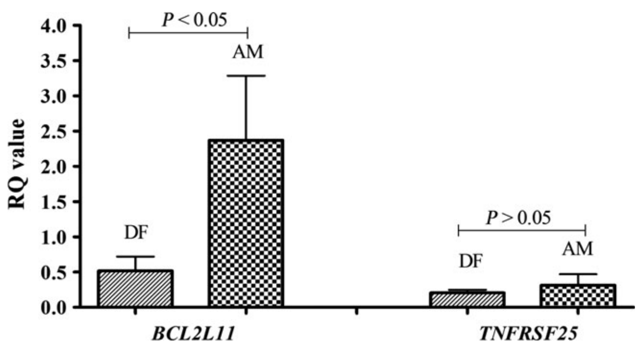

Figure 2 Gene expression of *BCL2L11* and *TNFRSF25* in dental follicles (DF) and ameloblastomas (AM). Increased expression of *BCL2L11* was observed in ameloblastomas, while there was no difference in the mRNA transcriptional levels of *TNFRSF5* between the two groups

tumour growth (Danial and Korsmeyer, 2004). Epigenetic alterations may lead to suppression of apoptosis-related genes, as well as suppression or activation of a variety of other genes related to critical cell processes. Up to now, DNA methylation is the most investigated epigenetic change in tumours. However, there are few studies addressing DNA methylation changes in odontogenic tumours (Moreira *et al*, 2009; Gomes *et al*, 2010a; Guimarães *et al*, 2015) and to date, no study addressed the relevance of DNA methylation profile of apoptosis-related genes in ameloblastomas.

In the present study, we investigated the methylation status of 22 apoptosis-related genes in multicystic ameloblastoma and two of them, *TNFRSF25* and *BCL2L11,* showed important differences in the methylation levels compared with dental follicles. A hypomethylated profile of both genes was observed in ameloblastoma compared with the dental follicle. Noteworthy, gene expression results for *BCL2L11* were consistent with this finding as we found increased expression levels in ameloblastomas. On the other hand, no difference was observed in *TNFRSF25* between both groups. Such discrepancy may be explained by the molecular heterogeneity of the tumoral tissues included in the analysis, which is a well-described feature in malignant and benign neoplasias (Gomes *et al*, 2016).

The *BCL2L11* gene, also known as *BIM,* codifies a BH3-only pro-apoptotic member of the Bcl-2 family, which constitutes one of the most relevant classes of regulators of apoptosis (Czabotar *et al*, 2014). The Bcl2-L-11 protein can bind to and neutralise pro-survival Bcl-2 proteins or directly activate pro-apoptotic effectors Bax and Bak (Czabotar *et al*, 2014). Activation of Bax and Bak leads to oligomerization to form pores in the mitochondrial outer membrane (MOM) followed by MOM permeabilization, cytochrome *c* release and consequent activation of effector caspases leading to apoptosis (Sionov *et al*, 2015).

Balance in the intracellular expression levels of pro-apoptotic and anti-apoptotic proteins is crucial for regulating apoptosis. Many studies showed that ameloblastoma has two relatively distinct patterns of Bcl-2 family proteins: anti-apoptotic members are predominantly expressed in the outer layer, which is composed of columnar cells with reverse polarity. On the other hand, the pro-apoptotic members are mainly expressed in the inner layer, which is formed by loosely arranged angular cells (Sandra *et al*, 2001; Luo *et al*, 2006). However, Kumamoto and Ooya (2008) detected immunohistochemical expression of BH3-only proteins, including Bcl2-L-11, in the outer layers of ameloblastic tumours. These authors suggested that interactions with other anti-apoptotic proteins may suppress apoptosis initiated by the BH3-only proteins. This study also observed that benign and malignant ameloblastic tumours, as well as tooth germs, present similar immunohistochemical expression of BH-only proteins, including Bcl2-L-11, indicating that these proteins may have a role in apoptosis of normal and neoplastic odontogenic epithelium. Further, it was observed a distinct expression pattern between histopathological subtypes of ameloblastoma, suggesting that BH3-only proteins, such as Bcl2-L-11, may be involved in the tumour cell differentiation (Kumamoto and Ooya, 2008).

Alterations of Bcl2-L-11 isoforms are reported in several diseases, including cancer, but its role in the apoptosis of neoplastic cells can be more complicated. Bcl2-L-11 downregulation is involved in cell transformation and reduced the sensitivity of malignant neoplastic cells to various chemotherapeutic drugs (Sionov *et al*, 2015). In contrast, a recent study showed that it is highly expressed in prostate and breast cancer cells, which could be mediated by the transcription factor E2F1. Additionally, Bcl2-L-11 silencing caused cell apoptosis. These findings suggest that Bcl2-L-11 can promote cell survival in addition to its apoptosis-inducing function (Gogada *et al*, 2013).

Previous studies agree that pro-apoptotic genes, such as CD95 and PUMA, can also promote the growth of tumours depending on context and specific cell type. This non-apoptotic activity is mediated by different pathways, such as c-Jun N-terminal kinases (JNK), leading to expression of proteins related to tumour initiation (Chen *et al*, 2010; Tang *et al*, 2011). Furthermore, the presence of inhibitory proteins can repress the major function of pro-apoptotic members (Tang *et al*, 2011). The available data do not allow us to conclude that the same mechanism is true for BCL2L11 in ameloblastoma.

On the basis of our results, the transcription of the apoptosis-related gene *BCL2L11* is possibly regulated by promoter DNA methylation in ameloblastoma. The biological significance of this finding in ameloblastoma pathobiology remains to be clarified.

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Author contribution

RSG, CCG, and MGD conceived and designed the study. SFSC, NBP, KMA, WHC and KC performed the experiments. RSG, CCG and SFSC drafted the paper. NBP and SFSC performed statistical analysis. All authors revised the final version of the manuscript. Authors also confirm to have no conflict of interest.

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