DNA methylation profiles of 22 apoptosis-related genes in odontogenic keratocysts before and after marsupialization

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Objective. Odontogenic keratocysts (OKCs) are cystic lesions of the jaw and tend to recur after treatment. Marsupialization is an effective preliminary treatment for large OKCs. This procedure induces epithelial lining changes in association with reduction of Bcl-2 protein expression, but the underlying mechanisms remain unknown. The purpose of our study was to compare the methylation profile of the apoptosis-related genes of OKCs before and after marsupialization.

Study Design. We assessed the methylation percentages of the promoter region of 22 apoptosis-related genes in 13 OKCs, both marsupialized and nonmarsupIALIZED lesions, by using methylation quantitative polymerase chain reaction array. We validated the expression of genes that showed the greatest differences in methylation percentages between the 2 groups.

Results. LTBR and BCLAF1 showed higher DNA methylation percentages in the marsupialized OKCs, but this difference did not affect gene expression (P > .05). The other 20 genes showed similar DNA methylation in both OKC groups.

Conclusions. OKCs show a distinct methylation profile after marsupialization, but this is not followed by gene expression alterations. (Oral Surg Oral Med Oral Pathol Oral Radiol 2017;124:483–489)

Odontogenic keratocysts (OKCs) were first described by Philipsen in 1956.1 These cysts have a predilection for the posterior region of the mandible and a slight male predilection, and their peak incidence occurs in patients 10 to 30 years of age.1,2 Besides occurring sporadically, OKCs may occur in association with nevoid basal cell carcinoma syndrome (Gorlin syndrome).1,4 A number of studies have focused on different aspects of this lesion in an attempt to explain its biologic behavior.5 Mutations in the Patched (PTCH1) tumor suppressor gene were identified as the underlying genetic event in nevoid basal cell carcinoma syndrome,6 occurring in some sporadic OKCs.8,10

The most appropriate management for OKC remains controversial11 and may include marginal resection, enucleation with or without peripheral ostectomy, or use of the Carnoy solution, decompression, and marsupialization.11-15 The recurrence rate is approximately 30%,16 and may be associated with the presence of microscopic budding and epithelial islands in a cystic capsule, poor clinical response to decompression, and in-situ mutation of the lesion between the dental roots of the remaining tooth during a radiographic examination.3

Marsupialization involves converting a cyst into a pouch, and it is an effective preliminary treatment for large OKCs because it is highly successful in decreasing the size of a tumor before definitive surgery.15,17,18 With marsupialization, the fibrous capsule of the cyst becomes thicker and easier to enucleate and histologically appears to change and resemble normal oral mucosa.20,21 Studies of the mechanism behind the influence of marsupialization on the cystic capsule could provide molecular tools for the treatment of OKCs. Interestingly, apoptosis-involved protein expression in the cyst epithelium changes after marsupialization. Bcl-2 is an antiapoptotic protein whose prosurvival function was discovered by observing that it promotes hematopoietic cell survival.22 Bcl-2 protein expression is strictly limited to the basal layer of the initial biopsy of the OKC lining, but not in the histologic material obtained after marsupialization.23 Diniz et al.23 compared marsupialized OKCs with primary lesions and noticed an overall reduction in Bcl-2 immunoexpression after marsupialization.

Some studies have focused on the molecular aspects of OKCs, including epigenetic changes.9,23,28 A widely

Statement of Clinical Relevance

Marsupialization is considered an effective technique for preliminary treatment of large odontogenic keratocysts (OKCs). Some studies have focused on the molecular aspects of OKCs, but whether the impact of epigenetic mechanisms on the biologic changes in OKCs after marsupialization remains unclear.
DNA methylation is a postreplication modification that is predominantly found in the cytosines of the dinucleotide sequence CpG. This process is associated with gene inactivation during normal development and in a broad range of human diseases. Recently, our group found a hypomethylated profile of BCL2 L11 associated with increased expression of its messenger RNA (mRNA) in ameloblastoma compared with normal tissue, which suggests that the transcription of this apoptosis-related gene is possibly regulated by DNA methylation in the tumor.

The impact of epigenetic mechanisms on the biologic changes in OKCs after marsupialization remains unexplored. The purpose of this study was to compare the methylation profile of 22 apoptosis-related genes between marsupialized OKCs (m-OKCs) and nonmarsupialized OKCs.

MATERIALS AND METHODS

Patient recruitment and sample inclusion and processing

This study was approved by the Research Ethics Committee of the university (COEP-UFMG) (protocol number CAAE 30405514.5.0000.5149), and patients gave informed consent before sample collection. Patients were recruited at the Oral Medicine service at the School of Dentistry/UFMG (Belo Horizonte, Brazil). Patients with Gorlin syndrome were not invited to take part in the study.

Thirteen fresh samples of sporadic OKCs were collected from 13 patients; 9 post-m-OKCs and 4 first biopsy (nonmarsupialized) OKCs were included in this study. The m-OKC and primary OKC samples were obtained from different patients. The age of the patients ranged from 11 to 54 years, and the male/female ratio was 1.16:1. In all cases, the OKCs occurred in the mandible. In the m-OKC group, samples included tissue fragments obtained 8 to 13 months after marsupialization. Histologically, m-OKCs presented moderate to severe inflammatory infiltrate, whereas nonmarsupialized OKCs did not show inflammatory infiltrate. A sample of dental follicles collected from asymptomatic unerupted impacted third molars extracted from healthy individuals was used as the reaction calibrator in transcription validation.

All samples were divided into 3 portions; one was immediately included in Tissue-Tek (Sakura Finetek, Torrance, CA) stored at −80°C, another was stored in RNAholder (BioAgency, SP, Brazil) at −80°C, and the largest fragment was formalin fixed, paraffin embedded, and submitted for histopathologic analysis to confirm the diagnosis.

DNA methylation analysis

Each tumor sample was cryosected to guarantee the presence of epithelium. Genomic DNA (gDNA) was extracted using the DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA) according to the manufacturer’s protocol. DNA concentration and purity were determined using a spectrophotometer with Nanodrop 2000 (ThermoFisher Scientific, Wilmington, DE), and then samples from each group were combined into pools that contain 1 μg of gDNA each.

The methylation status of the promoter region of a panel of 22 apoptosis-related genes was analyzed by using the human methylation signature panel EpiTect Methyl II PCR Array System: EAHS-121 Z (Qiagen, Germantown, MD). The genes included in this panel are listed in Table I. This method is based on the quantitative detection of remaining target DNA molecules after treatment with methylation-sensitive and methylation-dependent restriction enzymes that digest unmethylated and methylated DNA, respectively. The restriction digestions were performed using the EpiTect Methyl II DNA Restriction Kit (Qiagen, Chatsworth, CA) according to the manufacturer’s instructions. Four digestion reactions, namely, no-enzyme mock (Mo), methylation-sensitive (Ms), methylation-dependent (Md), and methylation-sensitive plus methylation-dependent double (Msd) were set up. The quantitative polymerase chain reaction array was performed in a StepOne Plus Real-Time PCR System (Applied Biosystems, Foster City, CA). Data outputs representing DNA methylation levels were determined and analyzed by using EpiTect Methyl DNA PCR Data Analysis (Qiagen, Chatsworth, CA).

mRNA transcription analysis

The genes that showed the most striking methylation profile differences among the OKC groups were selected for the transcription analysis by reverse transcription quantitative polymerase chain reaction. Three OKC samples were not included in the mRNA transcription analysis because of the limited amount of available fresh tissue for RNA extraction. Therefore, we used all the available samples that comprised 6 m-OKCs and 4 nonmarsupialized OKCs. Total RNA was extracted from 50 mg to 100 mg of the frozen sample by using TRIzol Reagent (Ambion, Foster City, CA) according to the manufacturer’s specifications. Total RNA was treated with DNase I (Invitrogen Life Technologies, Carlsbad, CA) to avoid gDNA contamination. Reverse transcription from 1 μg of treated RNA to complementary DNA was performed using the SuperScript III First-Strand-Synthesis System (Invitrogen, Carlsbad, CA).

Primers for BCLAF1 and LTBR complementary DNA were designed using the Primer Express software; LTBR, forward: 5’ GAGATGTCAGGAACCATGCTGAT 3’ and reverse: 5’ GGTGGCAAGGAGCAGAAAGA3’.
DNA methylation analyses

LTBR and BCLAF1 showed different DNA methylation patterns between the m-OKCs and the nonmarsupialized OKCs. The methylation results are shown in Table I and Figure 1. LTBR methylation frequency in the m-OKC group (57.1%) was higher than that in the OKC group (11.1%). BCLAF1 was also more highly methylated in the m-OKC (53.6%) group compared with the OKC group (1.2%) (Table I; Figure 1).

The methylation percentage of the other gene promoters that were studied was low and similar between the 2 OKC groups (Table I; Figure 1).

<table>
<thead>
<tr>
<th>Genes</th>
<th>OKC (M)</th>
<th>OKC (UN)</th>
<th>m-OKC (M)</th>
<th>m-OKC (UN)</th>
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<tr>
<td>APAF1</td>
<td>0.58%</td>
<td>99.42%</td>
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<td>BAD</td>
<td>0.36%</td>
<td>99.64%</td>
<td>0.09%</td>
<td>99.91%</td>
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<tr>
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<td>BCLAF1*</td>
<td><strong>1.16%</strong></td>
<td><strong>98.84%</strong></td>
<td><strong>53.61%</strong></td>
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<td>BIRC2</td>
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<td>CASP9</td>
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<td>LTBR*</td>
<td><strong>11.09%</strong></td>
<td><strong>88.91%</strong></td>
<td><strong>57.05%</strong></td>
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</table>

*M, methylated DNA; PCR, polymerase chain reaction; UN, unmethylated DNA.

DNA methylation levels (%) in marsupialized OKCs (m-OKCs) and nonmarsupialized OKCs.

*LTBR and BCLAF1 were the 2 genes whose methylation percentages showed striking differences between m-OKCs and OKCs. All the other genes showed similar methylation percentage status in the 2 groups.

DISCUSSION

Epigenetic changes are nongenetic alterations that can affect regulation of gene expression without changing DNA sequence.31,32 DNA methylation is the most extensively studied epigenetic phenomenon, and abnormalities of DNA methylation patterns are observed in different diseases, including odontogenic tumors34 and cancers.35 These abnormalities include promoter CpG island DNA hypermethylation, which is related to transcriptional silencing of genes, such as tumor suppressor genes.30,36 DNA hypomethylation has also been found in many types of cancers, but its biologic significance is not understood.37,38 Few published data exist on the
epigenetic changes in OKCs. DNA methylation depends on the addition of methyl groups to cytosine nucleotides in specific areas of the gene by DNA methyltransferases enzymes. Increased expression of this enzyme was found in OKCs. Moreover, methylation of the P21 gene was reported in OKCs, but protein expression analysis was not performed. The promoter of PTCH1 gene in OKCs was not hypermethylated. Analyses of global LINE-1 (long interspersed nuclear element-1) methylation profile showed differences between OKCs and ameloblastomas.

Diverse cellular functions, including regulation of inflammatory gene expression, DNA repair, and cell proliferation, are regulated by changes in DNA methylation and post-translational modifications of histones. DNA methylation may silence gene expression, including expression of apoptosis-related genes. Apoptosis (programmed cell death) is crucial to the efficiency of cellular homeostasis. Disruption of the balance between cell proliferation and cell death as a result of modifications in the apoptotic pathway leads to various human diseases, including cancer. With regard to apoptotic proteins in OKCs, a number of studies have observed immunoregulation of apoptosis-related factors in the epithelial lining of this lesion. Expression of the antiapoptotic protein Bcl-2 has been described in OKCs. However, the relevance of this finding has not been discussed in detail. Cells use a variety of molecular mechanisms, such as epigenetic alterations in the apoptotic genes and oncogenic mutations, to acquire resistance to apoptosis.

To our knowledge, this study is the first to describe methylation in apoptosis-related genes in OKCs. Our results showed higher methylation percentages at the genes BCLAF1 and LTBR in marsupialized lesions compared with nonmarsupialized samples. These genes are inducers of apoptosis, and their overexpression has been shown in different tumor types. BCLAF1 (bcl-2-associated transcription factor 1), which is a proapoptotic member of the Bcl-2 family, interacts with antiapoptotic members and can induce apoptosis and autophagy. Increased BCLAF1 expression was reported in rectal cancer, and upregulation of BCLAF1 was found in patients with recurrent esophageal squamous cell carcinoma. LTBR (lymphotoxin-beta receptor) is a member of the tumor necrosis factor (TNF) receptor family, and its ligand is TNFSF14/LIGHT. The TNFSF14/LTBR signaling pathway induces cell death via caspase-dependent and caspase-independent pathways. Various studies have suggested the involvement of the LTBR signaling pathway in malignant tumors, such as non-Hodgkin lymphoma, hepatocellular carcinoma, and prostate cancer. An agonistic anti-LTBR antibody inhibited tumor growth in xenograft models of colon carcinoma, suggesting a novel approach to the treatment of this cancer.

Considering that we found hypermethylation of both BCLAF1 and LTBR in m-OKCs, we would expect them to be underexpressed in the lesions. However, despite the differences in the methylation patterns in OKC lesions before and after the marsupialization, no change in gene expression was found. The relationship between methylation and gene expression is a complex interaction involving multiple factors.
phenomenon,[30,60,61] which can be influenced by many factors, including the presence of inflammation[62,63] and tumor heterogeneity. Therefore, the inflammatory infiltrate could have contributed to the epigenetic changes in m-OKCs. This hypothesis is supported by a previous study by Sreedhar et al., which showed that inflamed OKCs exhibit altered expression of factors related to cellular proliferation and apoptosis compared with noninflamed OKCs.[30] Furthermore, even well-described methylation events, such as promoter CpG methylation, do not always result in gene silencing, especially when these promoters are characterized by a low CpG density.[30]

The low number of samples is one limitation of our study. Another limitation was that samples of cysts before and after marsupialization were obtained from different patients because the amount of tissue available in each biopsy was not sufficient to perform all the experiments.

CONCLUSIONS
OKCs show a distinct methylation profile after marsupialization, but this is not followed by gene expression alterations.

REFERENCES


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