Loss of heterozygosity of *MIR15A/MIR16-1*, negative regulators of the antiapoptotic gene *BCL2*, is not common in odontogenic keratocysts

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Objectives. The odontogenic keratocyst (OKC) is an aggressive odontogenic cyst that has a high recurrence rate. Apart from *PTCH1* mutations, few molecular alterations are described in OKCs. Low expression of microRNAs (miRNAs) miR-15a and/or miR-16-1 in association with increased expression of their target, Bcl-2, have been previously found in OKC. In humans, *MIR15A* and *MIR16-1* are clustered at chromosome position 13 q14, and loss of heterozygosity (LOH) at this locus occurs in different tumors. We aimed to determine whether deletion at 13 q14 is a potential mechanism leading to miR-15a/16-1 aberrant expression in OKC.

Methods. Genomic DNA was extracted from 15 formalin-fixed, paraffin-embedded microdissected OKC cases. The polymorphic DNA markers D13S272 and D13S273 on chromosome 13 q14.3, around *MIR15A/MIR16-1*, were amplified by polymerase chain reaction. LOH was examined by capillary electrophoresis DNA-fragment analysis.

Results. The D13S272 marker had no LOH in 12 informative cases, whereas 2 out of 9 informative cases (22%) had LOH at the D13S273 marker.

Conclusions. An LOH event at *MIR15A/MIR16-1* locus is not common in OKC. The mechanism underlying the regulation of miR-15a and miR-16-1 expression in OKC remains to be determined. (Oral Surg Oral Med Oral Pathol Oral Radiol 2018;125:313–316)

The odontogenic keratocyst (OKC) is an odontogenic cyst whose neoplastic nature is questionable. It has a high recurrence rate and affects mainly the posterior mandible.[1] OKC can occur sporadically or in association with nevoid basal cell carcinoma syndrome (OMIM [Online Mendelian Inheritance in Man] 109400), an inherited condition characterized by multiple basal cell carcinomas of the skin and OKCs in the jaws. Few genetic alterations are reported in OKCs,[2] and the molecular basis behind the clinical behavior of this lesion is poorly understood.

MicroRNAs (miRNAs) are small noncoding RNAs. They regulate gene expression post-transcriptionally, either by messenger RNA degradation or by translation inhibition.[3] Alteration of miRNAs regulating metastasis, cell proliferation, and differentiation genes are described in several neoplasms, including chronic lymphocytic leukemia, lung cancer, lymphoma, and bladder carcinoma.[4,5] In OKC, low expression of miR-15a and/or miR-16-1 together with increased expression of Bcl-2 have been reported.[6]

miR-15a and miR-16-1 are negative regulators of the antiapoptotic gene *BCL2*.[7] Bcl-2 overexpression results in increased cell survival, which, in turn, can lead to an aggressive pattern and recurrence of OKC.[8] Because loss of heterozygosity (LOH) of *MIR15A/MIR16-1* genes at 13 q14.3 is associated with downregulation of these miRNAs and Bcl-2 overexpression,[4] we aimed to determine whether OKC has deletion of *MIR15A/MIR16-1* locus. The understanding of the regulation mechanism of miRNAs expression is currently being used to tailor cancer treatment.[9] Therefore, the clarification of mechanisms associated with miR-15a and miR-16-1 low expression in OKC can serve in designing miRNA-based therapeutic strategies in the future.

MATERIALS AND METHODS

Samples

The ethics committee on human research at Universidade Federal de Minas Gerais, Belo Horizonte, Brazil,

Statement of Clinical Relevance

Bcl-2 overexpression may produce an increase in survival of epithelial cells, and this increased life span could, in turn, lead to the peculiar aggressive pattern of odontogenic keratocyst. Understanding the complex miRNA regulation of Bcl-2 expression may help to design new therapeutic approaches.

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approved this study. A convenience sample of 15 formalin-fixed, paraffin-embedded (FFPE) OKC were selected from the Oral Pathology Service files of Universidade Federal de Minas Gerais, including 13 sporadic lesions and 2 nevoid basal cell carcinoma syndrome–associated samples. Hematoxylin–eosin-stained slides and clinical information of all cases were reviewed, and only nonmarsupialized OKC cases with no histopathologic signs of inflammation were included in the study.

DNA isolation

All samples were evaluated microscopically, and the epithelium (tumor) and fibrous capsule (reference tissue) were manually microdissected. A microtome and sterile surgical blade were used to obtain a series of 20-µm-thick FFPE tissue sections. Genomic DNA of the epithelium and capsule tissues of each sample was extracted with an QIAamp DNA FFPE Tissue Kit (Qiagen, Redwood City, CA, USA), following the manufacturer's protocol. Concentration and purity of DNA were evaluated with Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA), and an absorbance ratio of 260/280 of 1.8 and 260/230 between 1.8 and 2.2 were considered.

Loss of heterozygosity analysis

LOH was examined by automatic DNA fragment analysis using the polymorphic DNA markers D13S272 and D13S273, flanking the *MIR15A/MIR16-1* locus[4] (Figure 1A). Fluorescently labeled forward primers and unlabeled reverse primers were used. Polymerase chain reaction products were subjected to capillary electrophoresis in an ABI PRISM 3130 (Applied Biosystems, Foster City, CA, USA). Results were analyzed using GeneMapper software version 4.0 (Applied Biosystems). LOH analysis was done as previously reported.[10] Briefly, the ratio between short-allele/long-allele (reference tissue) and short-allele/long allele (tumor) was calculated. Ratio values <0.66 or >1.5 were interpreted as LOH. Homozygous cases were considered noninformative. Unclear or noninterpretable electropherograms were considered as not possible to be analyzed. Whenever necessary, stutter correction was done as reported elsewhere.[11] The frequency of allelic loss in percentage was calculated for each marker, according to the ratio between LOH cases and informative cases.

RESULTS

The main clinical characteristics of the patients and the results of LOH analysis are given in Table I. Patient age ranged from 13 to 62 years (median: 36 years). The female-to-male ratio was 1.5:1, and the most affected site was the posterior mandible (80%), followed by the posterior maxilla (13.3%).

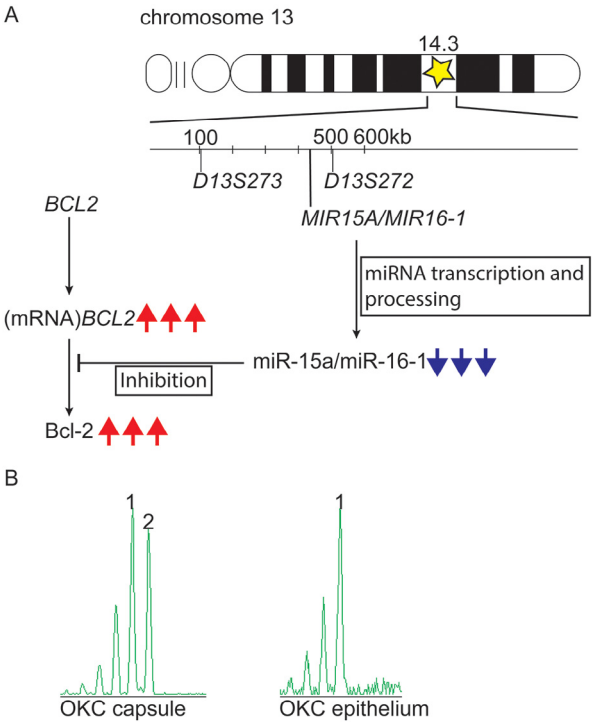

Fig. 1. Map of chromosome 13, scheme of microRNA (miRNA) action, and electropherogram of DNA fragment analysis. **(A)** The map shows the localization of D13S273 and D13S272 markers and *MIR15A/MIR16-1* genes at the long arm of chromosome 13, band 14.3. The miRNA genes encode miR-15a and miR-16-1. These miRNAs negatively regulate *BLC2* expression at a posttranscriptional level by inhibiting BCL2 mRNA translation. This translation inhibition leads to decreased expression of the antiapoptotic protein Bcl-2. In the odontogenic keratocyst (OKC), there is super-expression of *BCL2* at mRNA and protein levels *(up arrows in red)* and reduction of miR-15a and/or miR-16-1 *(down arrows in blue)*.[6] The mechanisms underlying changes in miRNA expression in neoplastic cells may involve LOH *(yellow star),* as well as promote hypermethylation and changes in miRNA processing. **(B)** The electropherogram shows loss of heterozygosity (LOH) in 1 OKC sample. The capsule (reference tissue) presents 2 alleles in heterozygosis, whereas the epithelium (lesion) shows one allele, exhibiting allelic loss (LOH).

Twelve samples were informative for the D13S272 marker, and all had allelic retention. Nine samples were informative for D13S273 marker, and 2 cases presented LOH (frequency of allelic loss = 22%). A representative example of LOH at D13S273 is shown in Figure 1B.

DISCUSSION

miRNAs are a class of master regulators of gene expression with diverse roles in cellular processes, including proliferation, differentiation, apoptosis, and cell cycle regulation. miR-15a and miR-16-1 are a natural antisense

Table I. Clinical information and LOH results per odontogenic keratocyst samples

						LOH markers	
Sample	*Sex*	*Age (y)*	*Location*	*Type*	*Recurrence*	*D13S272*	*D13S273*
1	M	14	Left posterior maxilla, extending from 27 to 28	NBCCS	Primary	Allelic Retention	Noninformative
2	M	34	Right posterior mandible, extending from mandibular rami to 48	NBCCS	—	Noninformative	NA
3	F	36	Posterior mandible	Sporadic	Primary	Allelic Retention	Noninformative
4	F	13	Left posterior mandible, extending from mandibular angle to 47 and 48	Sporadic	Primary	Allelic Retention	NA
5	F	54	Right maxillary tuber	Sporadic	Primary	Noninformative	LOH
6	F	29	Left mandibular rami, surrounding a molar tooth	Sporadic	Primary	Allelic retention	Allelic retention
7	F	62	Right mandibular rami	Sporadic	Primary	Allelic retention	Allelic retention
8	M	39	Right mandibular rami, surrounding 48	Sporadic	Primary	Allelic retention	Allelic retention
9	F	48	Left mandibular body and rami	Sporadic	Primary	Allelic retention	Allelic retention
10	M	38	Left posterior mandible	Sporadic	Primary	Allelic retention	Allelic retention
11	M	57	Left mandibular body, angle and rami	Sporadic	—	Allelic retention	Allelic retention
12	F	15	Left mandibular rami, surrounding 38	Sporadic	Primary	Allelic retention	Allelic retention
13	F	26	Left mandibular angle	Sporadic	Primary	NA	Noninformative
14	M	38	Right posterior mandible, surrounding 48	Sporadic	Primary	Allelic retention	Noninformative
15	F	34	Mandibular body, extending from 34 to 35	Sporadic	Recurrent	Allelic retention	LOH

M, male; *F,* female; *NBCCS,* nevoid basal cell carcinoma syndrome; *LOH,* loss of heterozygosity; *NA,* not possible to be analyzed.

transcript regulating *BCL2*; previously, we reported on miR-15a and/or miR-16-1 downregulation together with high *BCL2* mRNA expression in OKC samples.[6] Now, in an attempt to shed light on the causative factor, we searched for allelic loss at the *MIR15A/MIR16-1* locus in OKC. Deletion of *MIR15A/MIR16-1* genes leads to downregulation of miR-15a/miR-16-1, resulting in overexpression of Bcl-2 in diseases.[7] However, our results indicated that this is not true for OKC.

Of note, OKC basal cells consistently express Bcl-2, which may lead to increased cell survival.[8] Moreover, in vitro overexpression of miR-15a/16-1 resulted in a decreased level of this protein.[6] Therefore, a clear understanding of the complex miRNA regulation of *BCL2*-expression and its impacts on OKC tumor cells may help in the design of new therapeutic approaches. Our LOH analysis indicated that most OKCs harbored the 2 alleles of the *MIR15A/MIR16-1* genes, despite having low expression of them. However, other than LOH, changes in miRNA expression in neoplastic cells may be caused by other molecular mechanism. These mechanisms include promoter hypermethylation and changes in miRNA processing[12] (Figure 1A). Whether such a mechanism is associated with the aberrant miR-15a/16-1 expression in OKC remains to be tested.

Interestingly, the conversion of the OKC cyst into a pouch (i.e., marsupialization) was associated with increased miR-15a expression in 5 OKC cases investigated,[6] and with negative Bcl-2 immunoexpression.[13] In the present study, we excluded marsupilized OKC samples from our analysis. Genomic and proteomic profiling of OKCs before and after marsupialization might reveal important cell signaling pathways associated with miRNAs regulation and the impact of this clinical procedure on OKC behavior.

In conclusion, LOH at *MIR15A/MIR16-1* locus is not commonly found in OKC. The mechanism underlying the regulation of miR-15a and miR-16-1 expression in OKC remains to be determined.

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