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Methylation frequencies of cell-cycle associated genes in epithelial odontogenic tumours

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ABSTRACT

Objective: The benign epithelial odontogenic tumours constitute a group of lesions derived from epithelial elements of the tooth-forming apparatus. This group includes lesions of different biological behaviour, such as ameloblastoma, calcifying cystic odontogenic tumour (CCOT) and adenomatoid odontogenic tumour (AOT). The pathogenesis of these neoplasms remains uncertain and the occurrence of methylation in cell-cycle related genes may be involved in their development. The aim of this study was to investigate the methylation status of *P16*, *P21*, *P27*, *P53* and *RB1* genes in epithelial odontogenic tumours.
Design: Methylation-specific polymerase chain reaction (MSP) was used to evaluate the presence of methylation in 13 samples of ameloblastoma, six samples of CCOT, three samples of AOT and 14 samples of dental follicles, included as control.
Results: Our results showed a distinct methylation profile in each group. In ameloblastoma, the highest methylated genes were *P16* and *P21*, while in CCOT the *P21* and *RB1* genes were the most commonly methylated genes. Only the *P16* and *P21* genes were methylated in the AOT samples. In the dental follicle samples, *P16*, *P27* and *RB1* genes were commonly methylated. A high percentage of the odontogenic tumours analysed showed methylation of the *P21* gene, in contrast to dental follicles.
Conclusions: Epithelial odontogenic tumours show a distinct methylation profile in cell-cycle associated genes. In addition to this, the current findings show that epigenetic alterations are common events in epithelial odontogenic tumours.

1. Introduction

The benign epithelial odontogenic tumours constitute a group of lesions derived from epithelial elements of the tooth-forming apparatus[1] and comprise a diverse group of lesions presenting different biological behaviour. The ameloblastoma, the adenomatoid odontogenic tumour (AOT) and the calcifying cystic odontogenic tumour (CCOT) are examples of tumours included in this group and they display highly heterogeneous histopathologic features and clinical manifestations.

The ameloblastoma is the most common benign odontogenic tumour and it is characterised by a locally invasive behaviour and a high rate of recurrence.[2] Histologically, the

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ameloblastoma consists of either anastomosing epithelial strands (the plexiform type) or discrete epithelial islands (the follicular type) with columnar cells at the border with the adjacent fibrous stroma, exhibiting nuclei usually in the apical half of the cell body away from the basement membrane.[3] The AOT and the CCOT are composed of odontogenic epithelium in a variety of histoarquitectural patterns, embedded in mature fibrous stroma, without odontogenic ectomesenchyme.[1] The CCOT presents an epithelium similar to the ameloblastoma's epithelium, together with ghost cells that may calcify.[4] Although benign, the ameloblastoma, the AOT and the CCOT are locally destructive.[1]

Although recent studies demonstrated molecular alterations in epithelial odontogenic tumours, the pathogenesis of these lesions remains uncertain.[2,5,6] Mutations on the β-catenin gene were found in the CCOT.[7] Also, mutations in the ameloblastin gene were recently described in ameloblastoma, AOT and squamous odontogenic tumour.[5] Alterations in the expression of several genes, including some cell-cycle related genes were detected by microarray in ameloblastoma samples.[2] In addition, it has been suggested that tumour suppressor genes are involved in the development of odontogenic tumours via alteration of cellular proliferation control.[8]

Epigenetic factors related to the regulation of genes involved in the cell-cycle control have been described.[9] DNA methylation is an efficient epigenetic mechanism of transcriptional repression that occurs in cytosines within CpG dinucleotides.[10] The presence or absence of methyl groups in cytosines promote the remodelling of chromatin, making it less or more accessible to transcription.[11–13] DNA methylation is an event considered important in the pathogenesis of benign and malignant tumours of head and neck[14] and may be involved in the development of the epithelial odontogenic tumours.

The investigations of the methylation patterns in cyclin dependent kinase inhibitors (CDKIs) genes, as *P16 (CDKN2A)*, *P21 (CDKN1A)* and *P27 (CDKN1B)*, and in genes of regulatory proteins, as *RB1* and *P53 (TP53)*, have offered insights in a wide spectrum of diseases.[15–18] On the other hand, there is a paucity of information regarding epigenetics alterations in odontogenic tumours.[19,20] The aim of present study was to investigate the presence of methylation in *P16*, *P21*, *P27*, *P53* and *RB1* genes in ameloblastoma, AOT and CCOT.

2. Materials and methods

2.1. Sample collection

A total of 36 samples were included in the study, comprising: 13 samples of ameloblastoma (11 solid/multicystic and two unicystic), six CCOT, three AOT, and 14 cases of dental follicles (DF). The main clinical characteristics of the study group are listed in Table 1.

Fresh samples and paraffin-embedded tissues of the epithelial odontogenic tumours were collected during incisional biopsy, enucleation, or resection of the lesion. Dental follicle samples obtained from asymptomatic third molar impacted teeth extracted from healthy volunteers were used as control. The fresh samples were immediately included in Tissue-Tek (Sakura Finetek, CA, USA) and stored at −80 °C. Paraffin-embedded tissues were previously fixed in 10% formalin. This study was approved by the local ethics committee.

Table 1 – Clinical characteristics of the epithelial odontogenic tumours included in the study.

		Ameloblastoma	CCOT	AOT	DF
n (total)		13	6	3	14
Gender	Male	4	2	0	6
	Female	9	4	3	8
Age	Median	24	23.5	21	22.5
	Range	8–64	12–32	17–25	13–35

SD: standard deviation; CCOT, calcifying cystic odontogenic tumour; AOT, adenomatoid odontogenic tumour; DF, dental follicle.

2.2. DNA extraction

Twenty serial slices of 10 μm from each sample were used to DNA extraction. The genomic DNA from tissue sections was extracted with the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The concentration of DNA was determined with a spectrophotometer.

2.3. Bisulfite modification

The methylation pattern of the genes was assessed using DNA modification by bisulfite treatment, similar to that reported by Goldenberg et al.[21] The bisulfite treatment leads to a chemical conversion of unmethylated cytosines to uracil, while methylated cytosines remain unmodified. The bisulfite conversion was carried out with 1 μg of DNA, which was denatured by incubation with 2 μl of NaOH 3 M for 20 min at 50 °C. Then the samples were treated with sodium bisulfite (2.5 M) and hydroquinone (1 M) for 3 h at 70 °C. Modified DNA samples were purified with Wizard DNA purification resin according to the manufacturer's instructions (Promega, Madison, WI, USA) and eluted in distilled H_2O. NaOH (3 M) was added to complete the modification and this was followed by ethanol precipitation. The pellets were resuspended and 100 ng of DNA was used for the PCR assay.

2.4. Methylation-specific polymerase chain reaction (MSP)

The MSP distinguishes the presence of methylation in a given gene using specific primers for methylated or unmethylated DNA sequences. The primer sequences and amplification conditions used to evaluate *P16*, *P21*, *P27*, *P53* and *RB1* genes were previously reported.[19,22–24] The reactions were performed in a thermocyler (Eppendorf AG, Germany) and the PCR products were visualised in silver stained 6.5% polyacrylamide gel.

Genomic DNA treated with SssI methylase (New England Biolabs, Ipswich, Mass, USA) was used as positive control in the specific reactions for the methylated DNA. DNA treated