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Assessing pathogenic mutations in dental follicles as an attempt to identify early events in odontogenic tumours tumourigenesis

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ABSTRACT

Objective: Driver oncogenic mutations have been reported in several benign neoplasms. While ameloblastomas show *BRAF* p.V600E mutations, adenomatoid odontogenic tumours harbour either *KRAS* p.G12R or p.G12 V. The lack of understanding of the core molecular changes involved in tumour initiation and progression represents a critical barrier to developing new strategies for cancer detection and prevention. Considering the fact that ameloblastoma and adenomatoid odontogenic tumours can originate from dental follicles, we hypothesized that the *BRAF* and *KRAS* mutations might be early events in odontogenic tumours tumourigenesis. We aimed to assess *BRAF* and *KRAS* mutations in dental follicles associated with asymptomatic impacted teeth.

Design: Forty-eight dental follicles containing odontogenic epithelial remnants were included in the study. As ameloblastomas most often occur in the posterior mandible and adenomatoid odontogenic tumours have a predilection for the anterior jaws, we assessed by allele-specific qPCR the presence of *BRAF* p.V600E in 32 dental follicles associated with impacted 3rd mandibular molar teeth and *KRAS* p.G12 V and *KRAS* p.G12R mutations in 16 dental follicle specimens obtained from around impacted anterior teeth. Sanger sequencing was used as an additional method.

Results: None of the dental follicle cases tested positive for the mutations.

Conclusion: In conclusion, we tried to detect the early genetic events associated with odontogenic tumours development in dental follicles, but we were unable to showcase that *BRAF* p.V600E and *KRAS* p.G12R or p.G12 V mutations are the early genetic events associated with odontogenic tumours development.

1. Introduction

Driver oncogenic mutations have been reported in several benign neoplasms, including odontogenic tumours (Diniz et al., 2017; Kato et al., 2016; Marino-Enriquez & Fletcher, 2014). Recurrent pathogenic mutations in *BRAF* and *KRAS* occur in a high proportion of two epithelial odontogenic tumours, ameloblastomas and adenomatoid odontogenic tumours, respectively. While ameloblastomas show *BRAF* p.V600E mutations (Diniz et al., 2015; Kurppa et al., 2014), adenomatoid odontogenic tumours harbour either *KRAS* p.G12R or p.G12 V (Coura et al., 2019; Gomes et al., 2016).

KRAS mutations occur in 70% of adenomatoid odontogenic tumours (Coura et al., 2019). The frequency of the *BRAF* mutation in ameloblastomas ranges from approximately 60 to 80% (Diniz et al., 2015; Kurppa et al., 2014), whereas in ameloblastic carcinomas, their malignant counterpart, this frequency is 38% (Diniz et al., 2015). The higher frequency of the *BRAF* mutation in the benign tumour in comparison to the malignant counterpart is in line with the scenario described in other organs, illustrated by a higher frequency of this *BRAF* mutation in benign nevi (80%) than in dysplastic nevi (60%) or melanoma (40–50%) (Kato et al., 2016).

While ameloblastomas more frequently occur in the posterior region of the jaws, adenomatoid odontogenic tumours most often develop in the anterior jaws. Ameloblastomas represent an interesting model to

Abbreviations: qPCR, [2]quantitative polymerase chain reaction; TCGA, [3]the Cancer Genome Atlas; FFPE, [4]formalin-fixed, paraffin embedded; gDNA, [5]genomic DNA

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study the tumourigenic process, as there is an encapsulated form of the tumour (unicystic), as well as an infiltrative form (solid), in addition to a malignant counterpart (ameloblastic carcinoma), all of them showing the presence of the *BRAF* p.V600E mutation (Diniz et al., 2015).

The lack of understanding of the core molecular changes involved in tumour initiation and progression represents a critical barrier to developing new strategies for cancer detection and prevention (Campbell et al., 2016). On this basis, it has recently been proposed the development of a "Pre-Cancer Genome Atlas" in a similar fashion to the well-known The Cancer Genome Atlas (TCGA)[3] (Campbell et al., 2016). Considering the efforts in characterizing the molecular basis of early tumourigenesis (Ryan & Falpel-Badger, 2016) and the fact that ameloblastoma and adenomatoid odontogenic tumours can originate from dental follicles, embryonic tissues remnants of teeth formation, we hypothesized that the *BRAF* and *KRAS* mutations might be early events in odontogenic tumours tumourigenesis.

2. Material and methods

2.1. Sample selection and clinical data

This study was approved by The Research Ethics Committee of *Universidade Federal de Minas Gerais* and a written consent was obtained from the patients. Haematoxylin & eosin stained slides of all dental follicle cases were examined to confirm diagnosis. We selected seventy-seven samples of dental follicles, but twenty-nine cases were excluded due to either insufficient odontogenic epithelium amount or in a few cases due to low DNA quality. Therefore, forty-eight dental follicle samples were included in the study, comprising sixteen dental follicles from anterior teeth and thirty-two from around impacted third molar teeth. The formalin-fixed, paraffin embedded (FFPE)[4] samples were obtained from the Oral Pathology Service and the fresh tissue samples were collected in the Oral Surgery clinics at the authors' institution. These dental follicles were surgically removed in association with impacted teeth from healthy individuals. Clinical information including patients' age, sex and teeth location were obtained.

2.2. DNA isolation and mutation detection

FFPE tissue genomic DNA (gDNA)[5] was isolated using a commercially available kit (QIAamp DNA FFPE Tissue Kit, Qiagen, Hilden, Germany) and fresh tissue gDNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen), according to the manufacturer's recommendations. gDNA quantification and purity analysis were performed by spectrophotometry (NanoDrop instrument 2000, Thermo Fisher Scientific, Wilmington, USA), with a 1.8 ratio of absorbance at 260 nm and 280 nm being accepted as optimal. All samples were amplified by conventional PCR prior to qPCR to assess DNA quality, to ensure sufficient amount of amplifiable gDNA.

Samples from dental follicles associated with impacted teeth in the anterior region of the mandible and maxilla were tested for *KRAS* p.G12 V and *KRAS* p.G12R mutations and samples from dental follicles associated with impacted teeth from the posterior region of the jaws were tested for *BRAF* p.V600E. Mutations were assessed by TaqMan allele-specific qPCR, which is a highly specific and sensitive assay that can detect rare amounts of mutated DNA in a background of wild-type DNA. The reactions were performed using: *KRAS* p.G12 V mutation, KRAS_520_mu and KRAS_rf assays; *KRAS* p.G12R mutation, KRAS_518_mu and KRAS_rf assays; *BRAF* p.V600E mutation, BRAF_476_mu and BRAF_rf assays, (Applied Biosystems, Foster City, USA), as reported previously (Coura et al., 2019; Diniz et al., 2015). Briefly, reactions were run on a StepOne Plus instrument (Applied Biosystems) using the universal mutation detection thermal cycling protocol (95 °C for 10 min; 5 cycles: 92 °C for 15 s and 58 °C for 1 min; 40 cycles: 92 °C for 15 s and 60 °C for 1 min). The mutation status was determined using *Taqman Mutation Detector*™ Software (Life Technologies Corporation, Carlsbad, USA). Ameloblastoma and adenomatoid odontogenic tumour samples positive for *BRAF* p.V600E or *KRAS* p.G12 V or p.G12R mutations were used as positive controls for each mutant allele. Six samples were submitted to direct sequencing to confirm the qPCR results, including cases in which there was unspecific amplification of the mutant allele in the qPCR. Briefly, PCR products were sequenced using the BigDye Terminator reagent (Life Technologies, Connecticut, USA) on the ABI3730 (Life Technologies). The chromatograms were interpreted using the SnapGene® software (from GSL Biotech; available at snapgene.com). To assess *KRAS* mutations at codon 12 the following primer pair was used to amplify the DNA of three samples: Forward 5′-AAAAGGTACTGGTGGAGTATTTGA-3′, Reverse 5′-TCATGAAAATGGTCAGAGAAACC-3′. The chromatograms were manually analyzed using *KRAS* reference sequence NG_007524.1. Three samples were sequenced to assess *BRAF* p.V600E mutation and the following primers were used in the PCR: Forward 5′-TCATAATGC TTGCTCTGATAGGA-3′, Reverse 5′−CCAAAAATTTAATCAGTGGA-3′. The chromatograms were manually analyzed using *BRAF* reference sequence NG_007873.3.

3. Results

Clinical and molecular results are shown in Table 1. Patients' mean ages with dental follicles collected from the anterior and posterior jaws were 17 years. Most samples were collected from female individuals (n = 31), the mandible (n = 39) and posterior region (n = 32). All dental follicle specimens obtained from around impacted third molar teeth were from the mandible. When tested by allele-specific qPCR, 48/48 samples were wild-type for *KRAS* p.G12 V and p.G12R (samples from dental follicles associated with impacted teeth in the anterior region of the jaws) or *BRAF* p.V600E mutations (samples from dental follicles associated with impacted third molars). Sanger sequencing of all sequenced cases confirmed the wild-type status for the mutations.

4. Discussion

Normal tissues can already show the presence of somatic mutations that drive benign tumours´ tumourigenesis. Confirming this, a recent study has shown that normal tissues from sun-exposed eyelid can harbour somatic mutations (Martincorena et al., 2015). *FGFR3* mutant clones were among the largest observed in normal skin (Martincorena et al., 2015) and notably, *FGFR3* mutations "drive" the benign skin tumour, seborrheic keratosis (Hafner et al., 2006).

In the present study, we attempted to identify the earliest genetic changes possibly involved in the initiation process of two epithelial odontogenic tumours, ameloblastomas and adenomatoid odontogenic tumours. Between 60–80% (Diniz et al., 2015; Kurppa et al., 2014) of ameloblastomas harbour the *BRAF* p.V600E mutation (Kurppa et al., 2014) and 80% of cases are diagnosed in the mandible, most often in the posterior region (Vered et al., 2017). Therefore, we assessed this mutation in the dental follicle specimens obtained from impacted third molar teeth. Considering that more than 2/3 of adenomatoid odontogenic tumours have *KRAS* codon 12 mutations (Coura et al., 2019), and that there is a strong predilection for the anterior jaws, most cases occurring in association with an unerupted canine, we assessed the presence of *KRAS* p.G12R and p.G12 V mutations in the dental follicle specimens obtained from around impacted anterior teeth, mostly from canines.

Ameloblastomas show a peak incidence of diagnosis in the 4th and 5th decades of life (Vered et al., 2017). Interestingly, for the *BRAF* p.V600E mutant ameloblastoma cases, the mean patient age at diagnosis is lower than in the wild-type cases (34 vs 54 y.o., respectively) (Brown et al., 2014). Almost 90% of adenomatoid odontogenic tumours occur in the 2nd and 3rd decades of life (Philipsen et al., 2007), but there is no difference in the mean age at diagnosis in *KRAS* mutant versus wild-type cases (Coura et al., 2019). One has to consider, though, that

Table 1
Clinicopathological and molecular data of the 48 cases of the dental follicles.

Sample number	Clinical information					Molecular results*
	Age (years)	Sex	Jaw bone	Region	Associated Tooth	*KRAS* and *BRAF* mutations
1	25	M	Mandible	Anterior	Canine	Wild-type
2	33	M	Mandible	Anterior	Canine	Wild-type
3	13	M	Maxilla	Anterior	Canine	Wild-type
4	20	M	Maxilla	Anterior	Canine	Wild-type
5	14	F	Mandible	Anterior	Canine	Wild-type
6	15	F	Maxilla	Anterior	Canine	Wild-type
7	12	F	Mandible	Anterior	Canine	Wild-type
8	08	M	Maxilla	Anterior	NA	Wild-type
9	13	F	Maxilla	Anterior	Canine	Wild-type
10	12	F	Maxilla	Anterior	Canine	Wild-type
11	12	F	Maxilla	Anterior	Incisive	Wild-type
12	14	F	Mandible	Anterior	Canine	Wild-type
13	16	F	Maxilla	Anterior	Canine	Wild-type
14	51	M	Mandible	Anterior	NA	Wild-type
15	13	F	Mandible	Anterior	Canine	Wild-type
16	12	F	Maxilla	Anterior	Canine	Wild-type
17	15	F	Mandible	Posterior	3rd Molar	Wild-type
18	16	M	Mandible	Posterior	3rd Molar	Wild-type
19	16	M	Mandible	Posterior	3rd Molar	Wild-type
20	17	F	Mandible	Posterior	3rd Molar	Wild-type
21	17	F	Mandible	Posterior	3rd Molar	Wild-type
22	18	M	Mandible	Posterior	3rd Molar	Wild-type
23	20	F	Mandible	Posterior	3rd Molar	Wild-type
24	25	F	Mandible	Posterior	3rd Molar	Wild-type
25	30	F	Mandible	Posterior	3rd Molar	Wild-type
26	17	F	Mandible	Posterior	3rd Molar	Wild-type
27	13	F	Mandible	Posterior	3rd Molar	Wild-type
28	15	M	Mandible	Posterior	3rd Molar	Wild-type
29	16	M	Mandible	Posterior	3rd Molar	Wild-type
30	16	F	Mandible	Posterior	3rd Molar	Wild-type
31	17	M	Mandible	Posterior	3rd Molar	Wild-type
32	17	F	Mandible	Posterior	3rd Molar	Wild-type
33	17	F	Mandible	Posterior	3rd Molar	Wild-type
34	17	M	Mandible	Posterior	3rd Molar	Wild-type
35	17	M	Mandible	Posterior	3rd Molar	Wild-type
36	17	F	Mandible	Posterior	3rd Molar	Wild-type
37	17	F	Mandible	Posterior	3rd Molar	Wild-type
38	17	M	Mandible	Posterior	3rd Molar	Wild-type
39	17	M	Mandible	Posterior	3rd Molar	Wild-type
40	17	F	Mandible	Posterior	3rd Molar	Wild-type
41	17	F	Mandible	Posterior	3rd Molar	Wild-type
42	18	F	Mandible	Posterior	3rd Molar	Wild-type
43	18	F	Mandible	Posterior	3rd Molar	Wild-type
44	18	F	Mandible	Posterior	3rd Molar	Wild-type
45	19	F	Mandible	Posterior	3rd Molar	Wild-type
46	19	F	Mandible	Posterior	3rd Molar	Wild-type
47	19	F	Mandible	Posterior	3rd Molar	Wild-type
48	17	M	Mandible	Posterior	3rd Molar	Wild-type

F = female, M = male, NA = detailed information not available.

* all samples from dental follicles associated with impacted teeth in the anterior region of the mandible and maxilla were tested for *KRAS* p.G12 V and *KRAS* p.G12R mutations and all samples from dental follicles specimens obtained from around impacted third molar teeth were tested for *BRAF* p.V600E.

both, ameloblastoma and adenomatoid odontogenic tumour derive from residual epithelial components of tooth development, and therefore, their tumourigenesis start during the odontogenesis process. Seminal work developed by Stanley et al. (1965) shows that the epithelial component of dental follicles undergoes changes and that while epithelial rests are numerous in the young individuals' dental follicles, they gradually disappear with age (Stanley et al., 1965). Interestingly, nor the cord-shaped odontogenic epithelium rests nor enamel organ epithelium were observed in dental follicles from individuals after 26 years (Stanley et al., 1965). To overcome this natural epithelial age change limitation, we studied dental follicles from young individuals and selected only cases in which odontogenic epithelium was present, and therefore several samples had to be excluded from the research. The epithelium lining or the remnants of the dental lamina in dental follicles are the only epithelium sources for odontogenic tumours progression in these tissues.

For all tested samples, the result (i.e. the deltaCT value) was greater than the detection cutoff value, meaning that the sample is either mutation negative (wild-type) or below the limit of detection for the TaqMan allele-specific mutation detection assay. As this assay has a sensitivity limit of 0.1%, it can detect somatic mutations down to 1 mutant cell in a 1000 wild-type background. Therefore, our results point to a wild-type genetic background for the *BRAF* and *KRAS* mutations tested in the dental follicles. As these mutations are highly prevalent in ameloblastomas and adenomatoid odontogenic tumour, we would expect to detect them in at least a few dental follicle samples. There is also the remote possibility that they could not be detected due to the sensitivity limit of the assay. Ultimately, the absence of this odontogenic tumours' driver mutations in the dental follicles also raises the possibility that these mutations occur in odontogenic epithelial remnants outside the dental follicle, such as from the odontogenic epithelial rests inside the bone or in the gubernacular cord.

We used odontogenic tissue remnants in order to try to identify the presence of epithelial odontogenic tumours mutations at early stages, during initiation or promotion steps. *BRAF* p.V600E, highly prevalent in ameloblastomas, was not detected in the dental follicles associated with impacted 3rd molars, nor *KRAS* p.G12 V/R, highly frequent in adenomatoid odontogenic tumours, were detected in the samples of dental follicles associated with anterior impacted teeth.

5. Conclusions

In conclusion, we were unable to showcase that *BRAF* p.V600E and *KRAS* p.G12R or p.G12 V mutations are the early genetic events associated with odontogenic tumours development in dental follicles.

Declaration of Competing Interest

None.

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