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ARTICLE

KRAS mutations drive adenomatoid odontogenic tumor and are independent of clinicopathological features

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Abstract

Adenomatoid odontogenic tumor is a benign encapsulated epithelial odontogenic tumor that shows an indolent clinical behavior. We have reported in a few adenomatoid odontogenic tumors mutations in *KRAS*, which is a proto-oncogene frequently mutated in cancer such as lung, pancreas, and colorectal adenocarcinomas. We aimed to assess *KRAS* mutations in the hotspot codons 12, 13, and 61 in a large cohort of adenomatoid odontogenic tumors and to test the association of these mutations with clinical (age, site, tumor size, follicular/extrafollicular subtypes) and histopathological parameters. Thirty eight central cases were studied. *KRAS* codon 12 mutations were assessed by TaqMan allele-specific qPCR (p.G12V/R) and/or Sanger sequencing, and codon 13 and 61 mutations were screened by Sanger. Histological tumor capsule thickness was evaluated by morphometric analysis. Additionally, the phosphorylated form of the MAPK downstream effector ERK1/2 was investigated. Statistical analysis was carried out to test the association of *KRAS* mutations with clinicopathological parameters. *KRAS* c.35 G >T mutation, leading to p.G12V, was detected in 15 cases. A novel mutation in adenomatoid odontogenic tumor, c.34 G >C, leading to p.G12R, was detected in 12 cases and the other 11 were wild-type. Codon 12 mutations were not associated with the clinicopathological parameters tested. *RAS* mutations are known to activate the MAPK pathway, and we show that adenomatoid odontogenic tumors express phosphorylated ERK1/2. In conclusion, a high proportion of adenomatoid odontogenic tumors (27/38, 71%) have *KRAS* codon 12 mutations, which occur independently of the clinicopathological features evaluated. Collectively, these findings indicate that *KRAS* mutations and MAPK pathway activation are the common features of this tumor and some cancer types. Although it is unclear why different codon 12 alleles occur in different disease contexts and the complex interactions between tumor genotype and phenotype need clarification, on the basis of our results the presence of *KRAS* p.G12V/R favors the adenomatoid odontogenic tumor diagnosis in challenging oral neoplasm cases.

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Introduction

Adenomatoid odontogenic tumor is a benign epithelial odontogenic tumor with a strong predilection for individuals in their second and third decades of life [1, 2]. The adenomatoid odontogenic tumor corresponds to 2.2–7.1% of odontogenic tumors [3]. Few cases of extraosseous (peripheral) adenomatoid odontogenic tumor have been reported, with the vast majority being intraosseous (central), with a strong predilection for the anterior jaws. The central variant can be subclassified into follicular type, in which the tumor is associated with the crown of an unerupted tooth, and into extrafollicular type, in which there is no association of the tumor with the crown of an embedded tooth [1].

Cortical bone expansion and teeth displacement are the common findings in the central variant, with cortical perforation being unusual [1]. Adenomatoid odontogenic tumor shows a slow but progressive growth, with its true neoplastic nature being questioned by some in the past. This tumor exhibits spindle-shaped or cuboidal epithelial cells forming rosette-like and duct-like structures, surrounded by a well-defined fibrous capsule [1].

Few studies have explored the genetic basis of the adenomatoid odontogenic tumor. Samples of this tumor evaluated by HUMARA gene polymorphism assay showed a monoclonal inactivation pattern, suggesting these are monoclonal tumors in origin [4]. At the genomic structural level, a few rare and common copy number variations (CNVs), including gains and losses, have been detected in adenomatoid odontogenic tumor [5]. Specifically, two new CNVs were detected, represented by losses at chromosomes 6q15 and 7p15.3, the latter covering the *IGF2BP3* gene. However, no CNV was detected in regions covering *KRAS* gene, 12p12.1. At the gene level, *AMBN* somatic mutation has been described in one adenomatoid odontogenic tumor sample [6]. In 2016, our research group studied a small adenomatoid odontogenic tumor cohort using a next-generation sequencing approach to sequence a panel of 50 oncogenes and tumor suppressor genes commonly mutated in human cancers. We detected and reported for the first time the presence of *KRAS* p.G12V mutations in adenomatoid odontogenic tumors [5].

Herein, we aimed to investigate *KRAS* codon 12 mutations in a large cohort of adenomatoid odontogenic tumor and to test their association with clinicopathological parameters. Considering some cases were wild-type for codon 12 mutations, we additionally searched for hotspot mutations at codons 13 and 61. As *RAS* mutations are known to activate the MAPK/ERK cell signaling pathway, we also investigated if the MAPK pathway is activated in these lesions, by using phospho-ERK1/2 (pERK1/2) immunohistochemistry.

Materials and methods

Sample selection and clinical data

This study was approved by the Research Ethics Committee of Universidade Federal de Minas Gerais (protocol number 30405514.5.0000.5149). Fifty formalin-fixed paraffin-embedded (FFPE) tissue samples of adenomatoid odontogenic tumor obtained from eight different oral pathology services in Brazil were included in this study. The diagnosis of all the cases was confirmed by three oral pathologists (V. F.B., C.C.G., and R.S.G.). Samples with insufficient tissue availability or low-quality DNA were excluded ($n = 12$) and 38 samples were included in the final analyses. Clinical information was collected for all the cases, including patients' age, sex, tumor site, association of the lesion with impacted teeth, and tumor size.

DNA extraction and mutation detection

FFPE tissue genomic DNA was isolated using a commercially available kit (QIAamp DNA FFPE Tissue Kit; Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Genomic DNA quantification was performed by spectrophotometry (NanoDrop instrument 2000; Thermo Fisher Scientific, Wilmington, USA). *KRAS* p.G12V mutation was assessed by TaqMan allele-specific qPCR using KRAS_520_mu and KRAS_rf assays (Applied Biosystems, Foster City, USA), following the manufacturer's instructions. Reactions were run on a StepOne Plus instrument (Applied Biosystems), and the mutation status was determined using Taqman Mutation Detector™ Software (Life Technologies Corporation, Carlsbad, USA). The *KRAS* p.G12V mutation was confirmed by direct sequencing. Wild-type cases for *KRAS* p.G12V were bidirectionally sequenced to assess the other *KRAS* mutations at codon 12 with the primer pair For: 5′-AAAAGG TACTGGTGGAGTATTTGA-3′ and Rev: 5′-TCATGAAA ATGGTCAGAGAAACC-3′. Considering *KRAS* p.G12R mutation was detected by Sanger sequencing, we further assessed this mutation by TaqMan allele-specific qPCR using KRAS_518_mu and KRAS_rf assays (Applied Biosystems, Foster City, USA). Codon 61 was sequenced with primer pair For: 5′-TGTGTTTCTCCCTTCTCAGGA-3′ and Rev: 5′-AAAGAAAGCCCTCCCCAGT-3′.

The chromatograms were manually analyzed using *KRAS* reference sequence NG_007524.1.

Histological and morphometric analysis and immunohistochemistry

The histological thickness of the fibrous capsule of all tumors was measured using the MMI Cell Tools software

(MMI Molecular Machines & Industries, Tokyo, Japan). The haematoxylin–eosin slides were placed on the platform of the microscope and scanned in 40 x original magnification. Measurements were taken by creating lines from the innermost to the outermost part of the connective tissue surrounding the tumor nests. The mean micrometer values were converted to millimeters.

RAS mutations are known to activate the MAPK pathway [7]. To assess MAPK activation in adenomatoid odontogenic tumor samples, we examined pERK1/2 immunoreactivity in all cases with available material ($n = 35$). Immunohistochemistry was carried out following standard procedures. The 4-μm paraffin-embedded sections were placed on glass slides (Star Frost, Knittel Glass, Germany), epitope retrieval was performed using TRIS-EDTA buffer solution (pH 8.0), and endogenous peroxidase was blockaded with methanol and hydrogen peroxide. The incubation of primary antibody was performed with rabbit monoclonal anti-pERK1/2 (Thr202/Tyr204, CST #4376), diluted at 1:100 in antibody diluent, for 18 h at 4 °C. Then, the reaction was visualized using a polymer-based system (EnVision, Dako Corporation, Carpinteria, USA), and the chromogen used was diaminobenzidine (Dako North America, Carpinteia, USA). Slides were counterstained with Harris haematoxylin for 3 min. Positive controls (pyogenic granuloma) and negative controls (pyogenic granuloma without primary antibody) were included in all the reactions. Results were evaluated by four observers (B.P.C., R.S.G., V.F.B., and C. C.G.) on a light microscope, using blind analysis. Sections were scanned at 20 × using the Pannoramic MIDI Digital Slide Scanner (3DHistech, Hungary). Nuclear and cytoplasmic staining was considered a positive reaction.

Statistical analysis

Statistical analysis was performed using the software IBM SPSS Statistics for Windows, Version 21.0 (Armonk, NY: IBM Corp). Data normality was evaluated using Shapiro–Wilk test. The comparison between the "mutated" group and the "wild-type" group for each variable (tumor size, minimum, mean and maximum capsule thickness and age) was performed by Independent Samples *t* Test or Mann–Whitney U test according to data distribution. Fisher's exact Test was used to compare the number of mutated cases between two groups, which are as follows: maxilla versus mandible, anterior versus posterior region, and follicular versus extrafollicular subtypes. Significance level was set at 0.05.

Results

Clinicopathological and molecular results of the 38 adenomatoid odontogenic tumors are shown in Table 1. Patient's mean age was 17.9 years (ranging from 6 to 57) and female-to-male proportion was 2:1. Most cases occurred in the anterior region ($n = 26$) of the maxilla ($n = 24$). Follicular cases were two times more frequent than extrafollicular ones.

KRAS mutation detection

Table 1 shows the mutation status for each individual sample. We detected *KRAS* codon 12 mutations in 27 cases, specifically c.35G > T, leading to p.G12V, in 15 cases and c.34G > C, leading to p.G12R, in 12 cases (Fig. 1a-c). The other 11 samples were wild-type for *KRAS* G12 mutations. Eleven of the wild-type samples were tested by TaqMan allele-specific qPCR and Sanger sequencing, and two of these wild-type cases were tested by TaqMan allele-specific qPCR only, due to insufficient availability or low DNA quality.

Histological and morphometric analysis and immunohistochemistry

The mean number of histological measurements of fibrous capsule per case was 13.8 (ranging from 6 to 41). The mean histological capsule thickness measurement among all cases was 1.7 mm, and the mean value of the fibrous capsule thickness measurements by case is shown in Table 1.

The adenomatoid odontogenic tumor cases included in the cohort exhibited the diverse histopathological features described in the literature [8, 9]. The tumors were composed of a multinodular proliferation of cuboidal or columnar cells, scattered duct-like structures, anastomosing strands of basaloid epithelial cells in a plexiform or lattice-work pattern, calcifications, eosinophilic areas, and a fibrous capsule of variable thickness (Fig. 2a, b). The juxtanodular spindle cells that were adjacent to the clusters of cell-rich nodules were arranged in concentric layers (Fig. 2c). Cystic areas resembling dentigerous cysts and calcifying epithelial odontogenic tumor-like areas were noted in nine cases each (Fig. 2d).

pERK1/2 immunopositivity was observed in all the cases tested (Table 1). Positivity for pERK1/2 was found in the ducts or microcysts, and in the cell-rich nodules and rosettes of columnar cells (Fig. 2e, f). Interestingly, the first layers of the juxtanodular spindle cells were also positive for the protein, but the outer layers were mostly negative (Fig. 2e, f). This pattern was observed in most of the tumors. In one case (no. 22), an inverse pattern was noted, with a more intense staining in the juxtanodular layers of spindle cells. Calcifying epithelial odontogenic tumor-like areas were also positive for pERK1/2 (Fig. 2g). While most of the anastomosing strands of basaloid cells were negative for the protein, the epithelial cells in the dentigerous-like cystic areas were positive (Fig. 2h).

Table 1 Clinicopathological and molecular data of the 38 cases of central adenomatoid odontogenic tumors included in the study

Sample no.	Clinical information						Histopathology		Molecular results
	Age (years)	Sex	Jaw bone	Region	Subclassification	Tumor size (highest dimension in cm)	Capsule thickness (mean) (mm)	pERK1/2 immunohistochemistry	*KRAS* codon 12, 13, and 61 mutations
1	7	M	Mandible	Anterior	Extrafollicular	NA	2.6	Positive	p.G12V
2	22	F	Maxilla	Posterior	Follicular	NA	1.4	Positive	p.G12V
3	24	M	Maxilla	Posterior	Follicular	3	2.6	Positive	p.G12V
4	19	F	Maxilla	Anterior	Follicular	1.8	1.2	Positive	p.G12V
5	12	F	Mandible	Anterior	Extrafollicular	NA	0.4	Positive	p.G12V
6	18	F	Mandible	Posterior	Follicular	3	0.7	Positive	p.G12V
7	15	F	Mandible	Anterior	Extrafollicular	1.2	0.4	Positive	p.G12V
8	6	F	Mandible	Anterior	Follicular	1.5	1.3	Positive	p.G12V
9	17	F	Maxilla	Anterior	Extrafollicular	2	1.3	NA	p.G12V
10	11	M	Mandible	Anterior	Extrafollicular	1	0.7	Positive	p.G12V
11	12	F	Maxilla	Anterior	Follicular	2	1.2	Positive	p.G12V
12	12	M	Maxilla	Anterior	Follicular	2	1.8	Positive	p.G12V
13	14	M	Maxilla	Posterior	Follicular	3.5	2	Positive	p.G12V
14	28	F	Maxilla	Posterior	Extrafollicular	4	0.4	Positive	p.G12V
15	25	M	Maxilla	Anterior	Follicular	5.5	1.1	Positive	p.G12V
16	17	M	Mandible	Posterior	Extrafollicular	1	0.4	NA	p.G12R
17	57	F	Mandible	Posterior	Extrafollicular	4.5	1.8	Positive	p.G12R
18	15	M	Maxilla	Anterior	Follicular	4	2.3	Positive	p.G12R
19	13	F	Maxilla	Posterior	Follicular	NA	0.7	Positive	p.G12R
20	12	F	Maxilla	Anterior	Extrafollicular	4	2.3	Positive	p.G12R
21	29	F	Mandible	Anterior	Extrafollicular	1.5	0.2	Positive	p.G12R
22	17	M	Maxilla	Anterior	Follicular	NA	0.2	Positive	p.G12R
23	13	F	Maxilla	Anterior	Follicular	4	2.1	Positive	p.G12R
24	NA	F	Mandible	Posterior	Extrafollicular	NA	0.3	Positive	p.G12R
25	25	F	Mandible	Anterior	Extrafollicular	2	0.6	Positive	p.G12R
26	13	F	Maxilla	Anterior	Follicular	1	0.4	Positive	p.G12R
27	23	F	Maxilla	Anterior	Extrafollicular	2.9	0.6	Positive	p.G12R
28	22	F	Maxilla	Posterior	Follicular	5	0.9	Positive	Wild-type
29	14	M	Maxilla	Posterior	Follicular	3	0.4	Positive	Wild-type
30	14	F	Maxilla	Anterior	Follicular	3	2.2	Positive	Wild-type
31	17	F	Maxilla	Anterior	Follicular	3	1.3	Positive	Wild-type
32	19	F	Maxilla	Anterior	Follicular	1	1.1	Positive	Wild-type
33	26	M	Mandible	Anterior	NA	3	0.3	Positive	Wild-type
34	15	M	Maxilla	Anterior	Follicular	2	2.1	Positive	Wild-type
35	12	F	Mandible	Anterior	Extrafollicular	2.5	NA	NA	Wild-type
36	17	F	Mandible	Posterior	Follicular	3	1.6	Positive	Wild-type
37	13	M	Maxilla	Anterior	Follicular	4	1.4	Positive	Wld-type*
38	19	F	Maxilla	Anterior	Extrafollicular	0.4	0.7	Positive	Wild-type*

NA not available; *these two cases are wild-type for *KRAS* G12V/R, as only the *KRAS* p.G12V and p.G12R. TaqMan allele-specific qPCR was used to assess mutations in these cases.

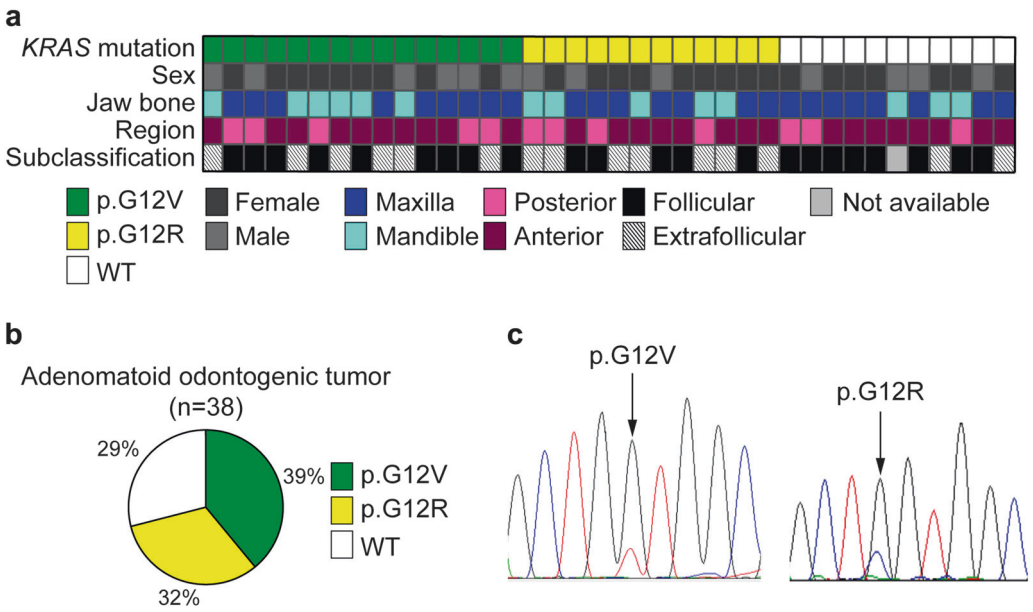

Fig. 1 Spectrum of *KRAS* mutations in adenomatoid odontogenic tumor. *KRAS* mutations and clinical information of the 38 cases (**a**). A high proportion (71%) of the samples showed *KRAS* codon 12 mutations (**b**), specifically *KRAS* c.35 G > T mutation, leading to p.G12V (left) or c.34 G > C, leading to p.G12R (right) (**c**)

Association between KRAS mutations and clinicopathological parameters

There was no statistically significant association between the presence of *KRAS* codon 12 mutations and clinicopathological parameters, including patient's age, tumor size and location, follicular or extrafollicular variants, and fibrous capsule thickness ($p > 0.05$).

Discussion

Adenomatoid odontogenic tumors have been reported as a component of Schimmelpenning syndrome (OMIM #163200) [10] caused by autosomal dominant *RAS* lethal mutations that survive by somatic mosaicism [11]. On this basis, in 2016, using next-generation sequencing in an adenomatoid odontogenic tumor sample from a Schimmelpenning syndrome patient as an index sample we reported for the first time *KRAS* p.G12V mutation in this syndromic-patient tumor, as well as in a few cases of sporadic adenomatoid odontogenic tumors [5]. Now, we have identified *KRAS* codon 12 mutations leading either to p.G12V or to G12R substitutions in 71% (27/38) of adenomatoid odontogenic tumor samples. *KRAS* p.G12R is reported in adenomatoid odontogenic tumors for the first time. In two out of the 11 wild-type cases we only interrogated p.G12V and p.G12R by TaqMan allele-specific qPCR, due to an insufficient quantity/quality of DNA, and there is a remote possibility that these two cases are false negative for other codon 12 (or 13 and 61) mutations. It is noteworthy that the proportion of adenomatoid odontogenic tumors with these *KRAS* driver mutations is similar to the proportion of other benign neoplasms previously shown to have a driver mutation [12].

KRAS is the most frequently mutated oncogene in human cancers, occurring in approximately one-third of them. Interestingly, transgenic Hras mice develop jaw tumors compatible with odontogenic tumors [13–15]. In addition, Hras-G12V mutant mice show compromised ameloblasts [16, 17].

There is an overall preponderance of *KRAS* codon 12 mutations in different cancer types, such as non-small cell lung cancer, and colorectal and pancreatic ductal adenocarcinoma [18–21]. In these three above-mentioned cancer types, p.G12V, the most frequent *KRAS* mutant allele in our cohort, is the second most common mutant allele. Conversely, *KRAS* p.G12R, the second most common mutant allele in adenomatoid odontogenic tumor, is the third most common allele in pancreatic ductal adenocarcinomas, but it is not as frequent in non-small cell lung cancer and colorectal adenocarcinoma (reviewed by Haigis [18]). Future studies may shed light on the many complexity layers that drive the tumorigenic process, specially on the genotype–phenotype correlation. The prognostic value of codon 12 mutations is context-dependent, and it is not clear why different codon 12 alleles occur in different disease contexts [18]. For example, in colorectal carcinomas, p.G12V mutation is associated with a more aggressive behavior [22], and non-small cell lung carcinoma patients

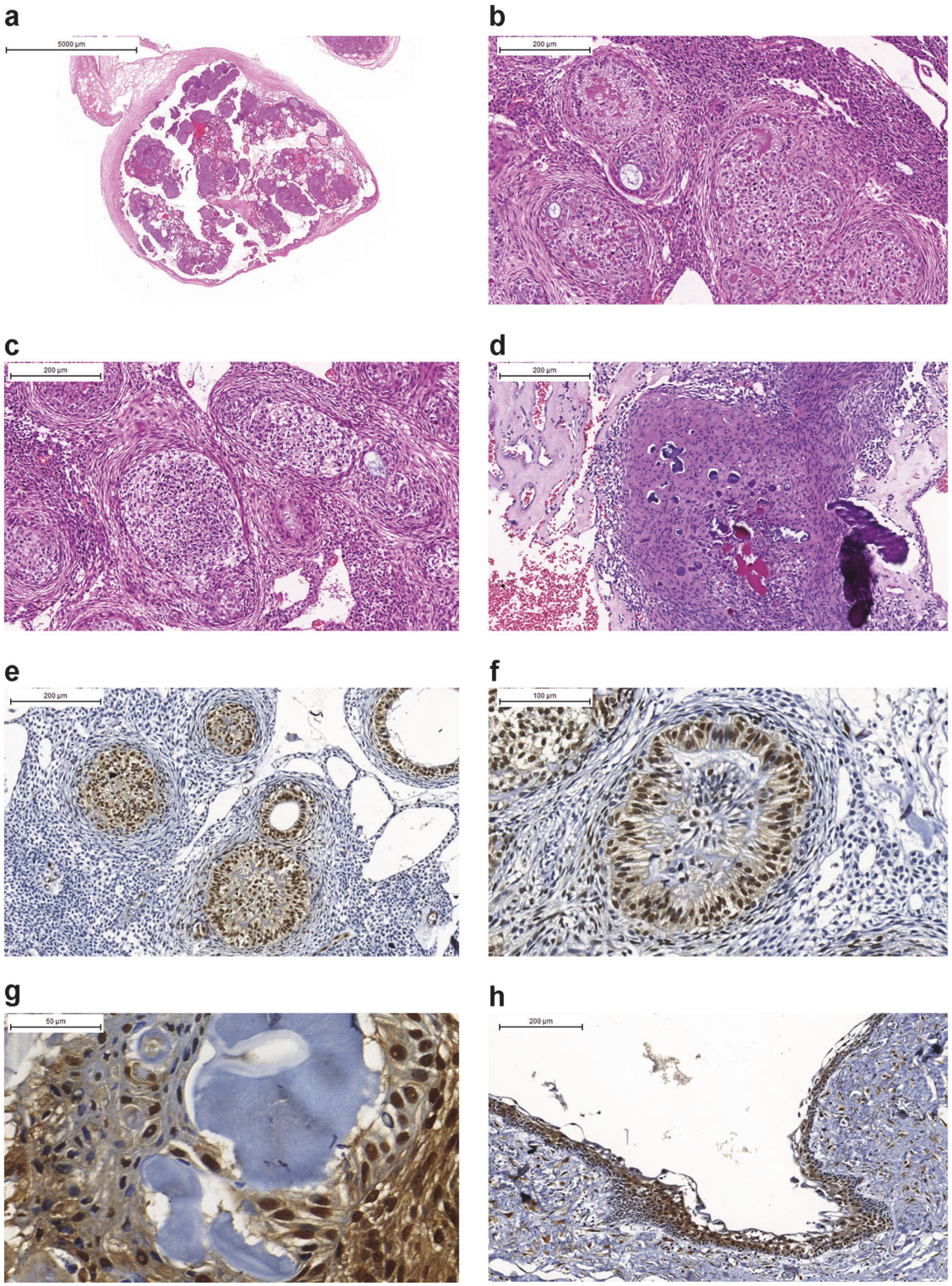

Fig. 2 Histopathological and immunohistochemical features of adenomatoid odontogenic tumor. Microscopic features of adenomatoid odontogenic tumor showing a well-defined capsule of varying thickness and multinodular proliferation of cuboidal or columnar cells with some scattered duct-like structures (**a**–**c**). Calcifying epithelial odontogenic tumor-like areas were observed in some cases (**d**). Immunohistochemical reactions showing pERK1/2 expression in nodules (**e**) and rosettes (**f**). Epithelial cells in the calcifying epithelial odontogenic tumor-like areas (**g**) and in the lining of dentigerous cyst-like areas were also positive (**h**)

with p.G12V and p.G12C, the most common codon 12 mutations, do better than those with rare codon 12 mutations [23]. We tested the associations between the presence of *KRAS* codon 12 mutations and clinicopathological parameters, but no clear association was observed.

Recently, driver oncogenic mutations have been reported in several benign neoplasms, including the odontogenic ones [24–26]. In the last World Health Organization Classification of the Head and Neck Tumors published in 2017, there were no important modifications in the adenomatoid odontogenic tumor [9]. In the past, some advocated that adenomatoid odontogenic tumors were hamartomas rather than true neoplastic lesions. On the basis of our findings, the high proportion of adenomatoid odontogenic tumor cases with recurrent *KRAS* pathogenic mutations provides evidence of its neoplastic nature.

We observed strong immunohistochemical expression of pERK1/2 in adenomatoid odontogenic tumor cell-rich nodules and rosettes of columnar cells, consistent with MAPK/ERK pathway activation. MAPK activation occurred even in wild-type cases for *KRAS* codon 12, 13, and 61 mutations, indicating that in these wild-type cases either we missed the *KRAS* mutation probably due to a low variant allele frequency or a low proportion of mutant cells in the samples, or there are other mechanisms that lead to MAPK activation in this subset of wild-type tumors. Whole-exome sequencing of *KRAS* wild-type cases can help in the future to elucidate if there is another mutation signature in these cases.

While an adenomatoid odontogenic tumor shows an indolent clinical behavior, being encapsulated and growing slowly, ameloblastomas are odontogenic tumors of aggressive clinical course. Ameloblastomas are known to have *BRAF* p.V600E mutations [27]. *BRAF* is downstream of *KRAS* in the MAPK signaling pathway, and both adenomatoid odontogenic tumors and ameloblastomas show MAPK-activating mutations despite the different clinical behaviors. Notably, *KRAS* p.G12R mutations have also been reported in *BRAF* wild-type ameloblastomas in 4/50 (8%) [28] and 4/28 (15%) [29] samples. Since *KRAS* p.G12V has not been reported in ameloblastoma, it can be used in the differential diagnosis between this tumor and adenomatoid odontogenic tumor. It remains to be established what modulates this complex genotype–phenotype relation in these odontogenic tumors.

In conclusion, we show that a high proportion of adenomatoid odontogenic tumors have *KRAS* codon 12 mutations, showing MAPK pathway activation. In our cohort, there was no clear association between the presence of these mutations and clinicopathological parameters. *KRAS* mutations are yet not directly actionable [18], but considering adenomatoid odontogenic tumors are successfully treated by conventional enucleation, the presence of *KRAS* mutations is not important in their clinical management. Notably, *KRAS* p.G12V or p.G12R can favor the adenomatoid odontogenic tumor diagnosis in the diagnostic process of challenging cases.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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