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ORIGINAL ARTICLE

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The relationship between ras protein and odontogenic lesions

Relação entre a proteína ras e as lesões odontogênicas

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ABSTRACT

Odontogenic lesions comprise a diverse group of lesions which usually affect the oral cavity and are derived from embryonic dental tissues. Their mechanisms of development and progression are still not fully known, but there are some studies investigating the participation of specific proteins in these events. **Objective:** The objective of this study was to investigate the expression of KRAS protein in these pathologies and also to associate the protein expression with the behaviour of the lesions studied. **Material and Methods:** Immunohistochemical analysis of 20 cases was performed for each one of the following lesions: dentigerous cyst (DC), odontogenic keratocyst (OKC) and ameloblastoma (AB), totalising 60 cases to be analysed. **Results:** By considering the KRAS immunoexpression, there were 11 cases (55%) presenting overexpression (score 3) in the basal layer of DC and 13 cases (65%) in the suprabasal layer. A similar finding was observed in the analysis of OKC, with eight cases (40%) in the suprabasal layer (score 3) and only five cases (25%) in the basal layer. As for AB, the overexpression was observed in six cases (30%). **Conclusion:** Therefore, it one suggests that the KRAS expression in odontogenic lesions is inversely proportional to their aggressive behaviour.

KEYWORDS

KRAS; Odontogenic lesions; Immunohistochemistry.

RESUMO

As lesões odontogênicas compreendem um grupo diversificado de lesões que comumente acometem a cavidade oral e são derivadas dos tecidos que originam o dente. Seus mecanismos de desenvolvimento e progressão ainda não são completamente conhecidos, porém alguns estudos investigam a participação de algumas proteínas específicas nesses eventos. **Objetivo:** O objetivo desse estudo foi investigar a expressão da proteína KRAS nestas patologias e associar a expressão proteica com o comportamento das lesões estudadas. **Material e Métodos:** A análise imuno-histoquímica foi realizada em 20 casos cada uma das lesões: cisto dentígero (CD), ceratocisto odontogênico (CO) e ameloblastoma (Am), totalizando 60 casos analisados. **Resultados:** Considerando-se a imunoexpressão de KRAS na camada basal do CD, 11 (55%) casos apresentaram hiperexpressão (score 3), já na camada suprabasal em 13 (65%) casos. Resultado semelhante foi observado na análise do CO, onde a camada suprabasal obteve 8 (40%) casos com score 3, enquanto a camada basal atingiu esse nível em apenas 5 (25%) casos. Em relação ao Am a hiperexpressão foi observada em 6 (30%) dos casos. **Conclusão:** Portanto, sugere-se que a expressão de KRAS nas lesões odontogênicas seja inversamente proporcional ao comportamento agressivo dessas lesões.

PALAVRAS-CHAVE

KRAS; Lesões odontogênicas; Imuno-histoquímica.

INTRODUCTION

Odontogenic lesions arise from tissues which give origin to the tooth, constituting a diverse group of oral pathologies [1]. They can originate from epithelium, ectomesenchyme and/or mesenchyme of the region [2]. Their development and progression are associated with several events involving enzymes which degrade the extracellular material, adhesion between molecules, and factors of proliferation, angiogenesis, and osteolysis [3].

The KRAS protein is a member of the family of small G proteins which bind to the nucleotides guanosine triphosphate (GTP) and guanosine diphosphate (GDP), all being associated with regulation of cell response to extracellular stimuli [4]. In its normal state, this protein can be either inactivated when bound to GDP or activated when bound to GTP. Once activated, the protein stimulates regulators of cell proliferation sequentially, such as MAPK, which sends signals to the nucleus for cell proliferation [5]. Activation of this protein was observed as an early event during tumorigenesis as well as a late event [6,7]. In an immunohistochemical reaction, the KRAS protein was detected in both cases, that is, in normal epithelium and neoplastic odontogenic epithelium [8]. Sandros et al. [9] suggested that the expression of KRAS gene can be used as a marker for detection of biological behaviour and prognosis of AM.

Studies have shown a relationship between this protein and growth of AB, suggesting that the role of KRAS protein is to regulate cell proliferation and differentiation in both normal and neoplastic odontogenic epithelia. Nevertheless, the literature is scarce regarding the relationship between KRAS protein and odontogenic lesions. In view of this, the objective of this work was to investigate the expression of the KRAS protein in these pathologies and to associate with the behaviour of the lesions studied.

MATERIAL & METHODS

Clinicopathological Analysis

The clinicopathological records of the patients with AB, OKC and DC, who were diagnosed in the histopathological laboratory of the Department of Biosciences and Oral Diagnosis at the Institute of Sciences and Technology of São José dos Campos (UNESP) between 1980 and 2015, were revised retrospectively. The research project was submitted to the local research ethics committee and approved under protocol number CAAE 55685816.3.0000.0077.

The collected data included gender, age, lesion localisation and other clinical-imaging features whenever available. Those cases presenting neither H&E slides nor paraffin blocks were excluded.

Immunohistochemical Reaction

Sections of 3 μm thickness were pre-heated at 56ºC for 12 hours and the slides were submitted to deparaffinization, rehydration, and unmasking using Trilogy™ (Cell Marque Rocklin, CA, USA) at 1:100 dilution in a pressure pot for 15 minutes. Endogen peroxidase was blocked with 6% hydrogen peroxide (Merck) and TBS buffer for 15 minutes at room temperature. Next, the slides were covered with KRAS primary antibody (clone ab55391 Abcam, Cambridge, MA, USA), which was previously diluted at 1:100 (Dako; DakoCytomation, Germany), and then incubated at 4ºC overnight. After, incubated with biotin-free horseradish peroxidase specifications (EnVision, Dako; DakoCytomation), according to the manufacture's, and detected by using DAB chromogenic substrate reagent (diaminobenzidine, DakoCytomation). Finally, the sections were counter-stained with Harris' haematoxylin.

Cytoplasmic labelling was classified into scores according to a modified classification established by Vered et al. [10], namely: 1 (0-10%), 2 (11-50%) and 3 (51-100%). The score

was performed by using a light microscope (Zeiss Axioplan2) at 400x magnification, with each section being divided into five areas containing at least 100 cells and randomly selected before being classified as positive and negative. In the cases of DC and OKC, the score was also subdivided into basal and suprabasal layers before being performed separately. Moreover, the labelling intensity was subjectively analysed.

Statistical Analysis

Associations between the variables in 2x2 contingency tables were determined by using Fisher's exact test or chi-square test, with all tests being performed at significance level of 5%.

RESULTS

Clinicopathological and immunohistochemical results are summarised in Table 1 (A, B and C). Male patients were the most affected, with DC, OKC and AB representing 13 (65%), 11 (55%) and 13 (65%) cases, respectively. As for the age group, 10 (50%) cases of DC affected children and youngsters aged up to 20 years, with a mean age of 25.85 years old and ranging from 5 to 64 years. As for OKC, the mean age was 34.4 years old, ranging from 18 to 63 years. In the cases of AB, the minimum and maximum ages were 18 and 82 years old, respectively, with a mean age being 40.5 years.

Radiographically, all the 20 cases of DC (100%) presented a well-delimited unilocular radiolucent area, 14 cases of AB (70%) and 10 of OKC (50%) showed a multilocular radiolucent area, whereas the other cases had no radiographic examination. The three types of lesions were predominant in Caucasian patients, affecting the posterior region of the mandible.

Microscopically, in the cases of DC, one can observe the presence of a thin stratified pavementous non-keratinised epithelium and, sometimes, regions of epithelial thickening in six cases (30%) (Figure 1A). Twenty-five per cent of the cases presented areas of hydropic degeneration in the suprabasal layer. Two cases (10%) presented capsules of fibrous connective tissue with haemorrhage and islands of odontogenic epithelium. Inflammation was absent in 13 of the slides analysed (65%), and when found, consisted of a discrete, chronic, inflammatory infiltrate.

In the cases of OKC, 13 cases (65%) showed a stratified pavementous epithelium with a few layers, whereas seven cases (35%) presented a thicker epithelium. All the sections presented a parakeratin layer with a corrugated epithelial surface. The cells of the basal epithelial layer varied from cubic to columnar and were arranged in palisade with a hyperchromatic nuclei (Figure 1B). The suprabasal layer showed areas of hydropic degeneration and acantholysis in nine (45%) and five (25%) cases, respectively. In the capsule of fibrous connective tissue, foci of haemorrhage were observed in four (20%) sections and discrete, chronic, inflammatory infiltrate in nine (45%).

As for AB, a follicular pattern was predominantly observed in 12 cases (60%), followed by a plexiform pattern in eight (40%). In the former, there was a formation of columnar epithelial cells with hyperchromatic nucleus in inverted polarisation, similar to ameloblasts, surrounding loosely arranged fusiform cells resembling a stellate reticulum. In the latter, there was also a formation of epithelial islets developing anastomoses to form long epithelial cords (Figure 1C).

With regard to KRAS expression, of the 60 cases analysed, 48 (80%) showed positive labelling regardless of intensity. DC was the odontogenic lesion with the strongest expression, on average, presenting 11.6 (58%) and 12 (60%) cases with strong labelling in basal and suprabasal layers, respectively (Figure 1D). OKC had strong labelling in the suprabasal layer, with eight cases (40%) on average, whereas the basal layer had only five cases (25%) (Figure 1E). As for AB, a strong expression was observed in 8.6 cases (43%), on average Figure 1F).

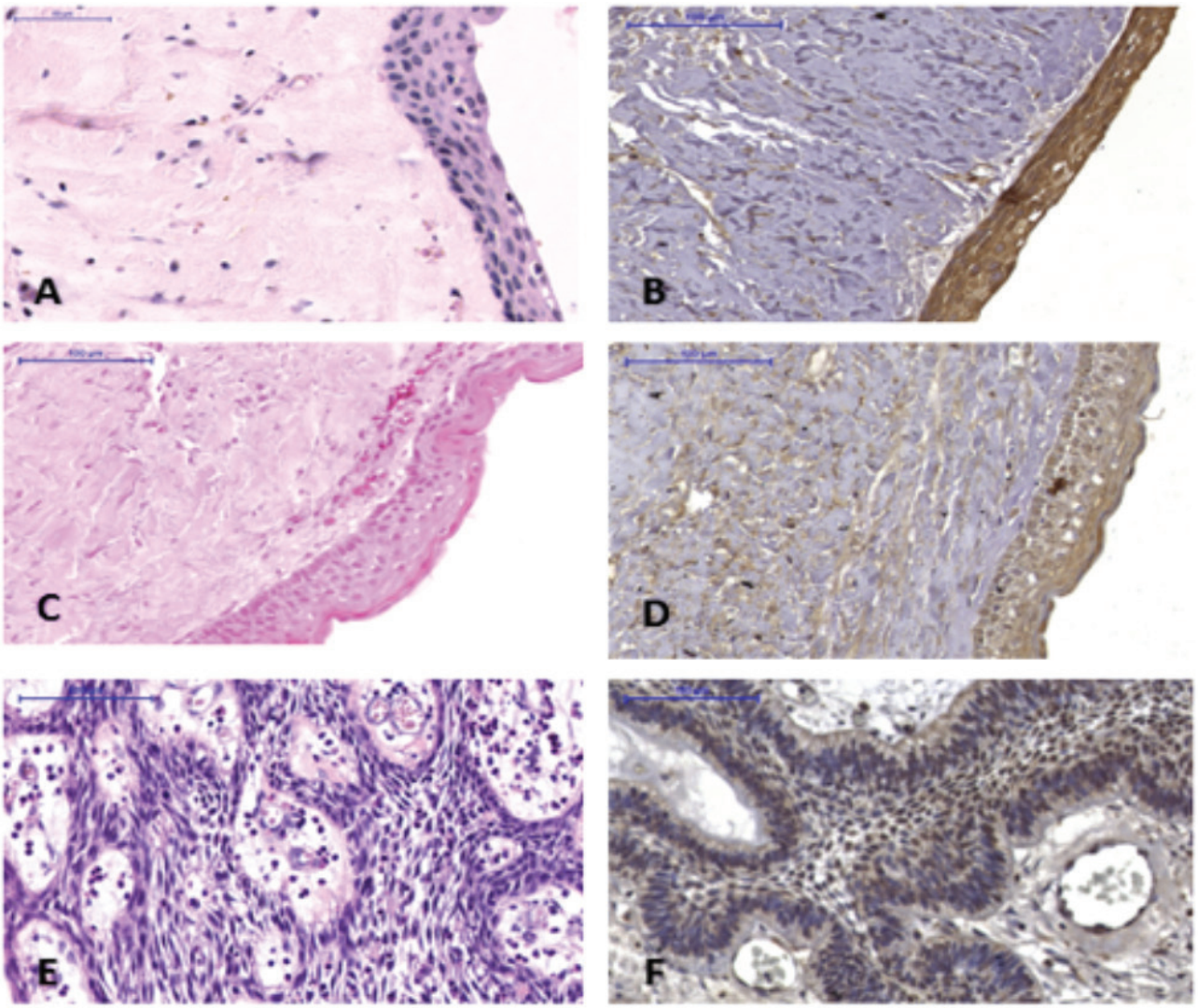

Figure 1 - Microphotograph showing histopathological characteristics of the cases of DC (A): thin, stratified, pavementous, nonkeratinised epithelium; of OKC (B): thin, stratified, pavementous, parakeratinised epithelium with corrugated surface and cells, with hyperchromatic nuclei, arranged in palisade manner in the basal layer; of AB (C): plexiform pattern with formation of cords of peripheral, columnar, epithelial cells with hyperchromatic nuclei in inverted polarisation. Immunohistochemical expression of KRAS in the cases of DC (D): strong labelling in basal and suprabasal layers; of OKC (E): more evident labelling in the suprabasal layer; and of AB (F): stronger labelling in peripheral cells.

With regard to DC, 11 (55%) and 13 (65%) of the cases were classified with score 3 in the basal and suprabasal layers, respectively. As for OKC, eight cases (40%) with more than 50% of cells in the suprabasal layer were positive, whereas only five cases (25%) were in the basal layer. With regard to AB, over-expression was observed in six cases (30%).

There was no association between KRAS expression, age ($p = 0.339$) and gender ($p = 0.668$), but a greater expression was observed in the basal layer for DC compared to OKC despite the lack of statistically significant difference ($p = 0.057$). With regard to the suprabasal layer, there was no statistically significant difference either ($p = 0.357$). By comparing the non-stratified layers of the three lesions between the groups, no statistically significant difference was found ($p = 0.623$).

Table 1 - A - General data on the cases of DC studied.

Cases	Group	Age	Gender	Race	Site	RX	KRAS	Score B	Score SB
1	1	40	1	1	1	1	1	3	3
2	1	15	1	2	1	1	1	3	3
3	1	6	1	1	4	1	1	3	3
4	1	27	2	1	3	1	4	1	1
5	1	64	1	2	1	1	3	2	3
6	1	53	2	1	1	1	3	2	3
7	1	50	1	1	1	1	3	1	2
8	1	31	1	1	1	1	3	2	2
9	1	13	2	1	4	1	2	3	3
10	1	36	1	1	1	1	4	1	1
11	1	13	2	1	1	1	3	3	3
12	1	21	2	1	1	1	1	3	3
13	1	18	1	1	3	1	4	1	1
14	1	11	2	1	2	1	4	1	1
15	1	11	2	1	2	1	1	3	3
16	1	17	1	1	1	1	4	1	1
17	1	14	1	1	3	1	2	3	3
18	1	5	1	2	1	1	1	3	3
19	1	49	1	1	1	1	1	3	3
20	1	25	1	1	1	1	3	3	3

Groups: 1=DC; 2=OKC; 3=AB / Gender: Male=1; Female=2 / Race: Caucasian=1 Others=2 / Site: Posterior Mandible=1;

Anterior Mandible=2; Posterior Maxilla=3; Anterior Maxilla =4 / RX: Unilocular=1; Multilocular=2 / RAS (Labelling intensity): Strong=1; Intermediate=2; Weak=3; Absent=4

Table 1 - B - General data on the cases of OKC studied.

Cases	Group	Age	Gender	Race	Site	RX	KRAS	Score B	Score SB
21	2	19	2	1	2	1	3	2	3
22	2	28	2	1	4	1	3	1	3
23	2	56	2	1	1	1	1	3	3
24	2	62	1	2	2	2	3	1	2
25	2	20	1	1	3	2	3	1	2
26	2	14	1	2	1	1	4	1	1
27	2	13	2	2	3	1	3	1	2
28	2	54	1	1	3	1	3	1	2
29	2	52	1	2	2	2	2	3	3
30	2	26	1	1	1	1	2	2	3
31	2	18	1	1	1	2	1	3	3
32	2	31	2	1	4	1	3	2	2
33	2	24	2	1	1	2	3	1	2
34	2	63	1	1	1	1	2	3	3
35	2	35	1	1	1	1	4	1	1
36	2	52	2	1	1	2	3	1	1
37	2	18	1	1	1	2	3	2	2
38	2	22	2	2	4	2	3	1	1
39	2	56	1	2	1	2	2	3	3
40	2	25	2	1	1	2	4	1	1

Groups: 1=DC; 2=OKC; 3=AB / Gender: Male=1; Female=2 / Race: Caucasian=1 Others=2 / Site: Posterior Mandible=1;

Anterior Mandible=2; Posterior Maxilla=3; Anterior Maxilla =4 / RX: Unilocular=1; Multilocular=2 / RAS (Labelling intensity): Strong=1; Intermediate=2; Weak=3; Absent=4

Table 1 - C - General data on the cases of AB studied.

Cases	Group	Age	Gender	Race	Site	RX	KRAS	Score B	Score SB
41	3	48	2	1	1	2	2	2	3
42	3	23	1	2	1	1	2	2	3
43	3	57	1	2	1	2	3	2	3
44	3	20	1	1	1	2	2	3	2
45	3	22	2	2	1	2	3	2	2
46	3	43	1	2	1	2	4	1	1
47	3	74	2	1	1	1	3	3	2
48	3	82	2	1	1	2	2	3	2
49	3	15	1	1	4	1	3	1	3
50	3	60	1	2	1	2	2	2	3
51	3	34	2	2	1	2	3	2	3
52	3	34	2	2	1	2	4	1	2
53	3	18	1	1	1	1	4	1	2
54	3	64	1	1	1	2	3	2	3
55	3	18	1	2	1	2	4	1	1
56	3	39	2	2	1	2	2	3	1
57	3	31	1	1	1	2	3	3	2
58	3	40	1	1	1	1	3	2	1
59	3	40	1	1	1	1	2	3	3
60	3	48	1	1	1	2	2	3	1

Groups: 1=DC; 2=OKC; 3=AB / Gender: Male=1; Female=2 / Race: Caucasian=1 Others=2 / Site: Posterior Mandible=1;

Anterior Mandible=2; Posterior Maxilla=3; Anterior Maxilla =4 / RX: Unilocular=1; Multilocular=2 / RAS (Labelling intensity): Strong=1; Intermediate=2; Weak=3; Absent=4

DISCUSSION

Odontogenic lesions are among the most common lesions found in the oral cavity and they have been studied in various parts of the world [11]. DC is the most common of the developmental odontogenic cysts, representing 20-24% of the cases. This lesion develops around the crown of a non-erupted tooth, that is, from the fluid accumulated between the reduced epithelium of the enamel organ and the impacted tooth [12,13]. Demirkol et al. [14] found a higher prevalence of DC in male patients, reporting an approximate ratio of 1.5:1 in relation to females, a finding also observed in our study. The other clinical and histological characteristics obtained were also supported by the literature. DC was more commonly found in young patients aged up to 30 years old, affecting the posterior mandible next to the third molar region, being radiographically represented by a well-defined, unilocular, radiolucent image associated with an impacted tooth [12,13,14].

The keratocystic odontogenic tumour was re-classified by the World Health Organisation (WHO) in 2017, returning to the category of odontogenic cyst [15,16,17]. Among the justifications for this change, there is the fact that marsupialisation is an effective treatment method for OKC, in which the lining epithelium resembles more the oral mucosa than the characteristic epithelium of this pathology after decompensation – a feature not associated with neoplasias [16, 17]. Vázquez-Romero et al. [18] described the lesion as being predominantly found in Caucasian males, which was also corroborated in the present study.

OKC occurs predominantly in the mandible, particularly in the third molar region involving the mandibular angle and ramus, with a mandible-maxilla ratio of 2:1. The lesion can appear at any age, but it is more frequently seen in young adults aged 20-30 years old [18]. Characteristics similar to those described in the literature were also observed: 13 (65%) cases of OKC occurred in patients within the age group of 21-40 years, with a mean age of 34.4 years old, commonly affecting the posterior mandible region.

AB represents approximately 1% of all head and neck tumours as well as 9-11% of the odontogenic tumours. Its growth is usually slow, but locally invasive [19]. With a higher incidence in the third and fourth decades of life, AB frequently affects males and females at a ratio of 1:1, but which varies depending on the region where the study was performed. For example, there is a slightly higher incidence of AB among males in the Indian population [19.20]. As for

the cases of AB analysed in the present study, the mean age of the affected patients was 40.5 years old, being more prevalent in the 21-40-year age group (40%). Of the 20 cases analysed, 13 were of male patients, thus demonstrating the slight predilection for this gender. AB is more common in the posterior mandible region and can be radiographically seen as a unilocular or multilocular radiolucent image presenting a soap-bubble aspect, with the latter being more frequently observed [20,21,22]. Milman et al. [21] conducted a microscopical analysis and found a prevalence of the follicular histological pattern, followed by plexiform and more rarely by desmoplastic forms. Our cases of AB showed the characteristics described in the literature, with 95% occurring in the posterior mandible. Of the 20 cases, 14 (70%) presented multilocular (radiographically) and 12 (60%) follicular (histologically) patterns, with the remaining cases presenting a plexiform pattern (40%).

KRAS is one of the proteins participating in the regulation of the cell cycle and proliferation [23]. Mutations in this oncogene, observed in 30% of all human cancers, stimulate GTPases and sometimes are associated with resistance to chemotherapy and targeted-therapy [24]. Cox et al. [25] suggest that mutations in the KRAS gene are initial genetic events in the progression of tumours, with its continuous expression being necessary for their maintenance. In the literature, a number of studies demonstrated associations between the mutated gene and malignant lesions, but there is no evidence showing the protein labelling in benign lesions, such as cysts and odontogenic tumours. The basal layer had the highest expression of KRAS in the cases of DC compared to the cases of OKC, with the difference being statistically borderline significant ($p = 0.057$) probably due to the small sample size ($n = 20$). This difference was not statistically significant for the suprabasal layer because of the microscopical aspects in the cases of DC, in which the epithelium was sometimes very thin, making stratification of the layers difficult. In the cases of OKC, over-expression was observed in the suprabasal layer. In general, KRAS expression is limited to cells of the basal layer or follicular ones in the skin of mice, a fact suggesting that these cells lack a renewal capacity, whereas the expression in other epithelial compartments can suggest the beginning and progression of some neoplasms [26]. On the other hand, in the basal layer of the epithelium, over-expression of KRAS can be enough for malignant transformation due to the presence of stem cells in this layer [27].

Alteration in cell signalling pathways (e.g. MAPK) is reported in cysts and odontogenic tumours [28]. The greatest association of KRAS and Raf kinase (BRAF) in ameloblastoma result in higher activity of ERK-1 and -2 in both cytoplasm and nucleus [28]. In the present study, the expression of KRAS was similar between odontogenic cysts when the layers were compared as groups.

CONCLUSION

The expression of KRAS was predominant in cells of the basal layer for DC and in cells of the suprabasal layer for OKC, but this expression was similar between OKC and AB when the layers were not stratified. This suggests that the lower the expression, the greater the aggressiveness of the lesion, since DC was the odontogenic lesion which most strongly expressed KRAS among all the cases studied.

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