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EGFR is not amplified in ameloblastoma

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Objective. The aim of this study was to investigate alterations in the *EGFR* gene and its protein expression for a better understanding of the biologic behavior of ameloblastoma.

Study Design. Twenty-five samples of ameloblastoma were selected, and dual-color fluorescence in situ hybridization assay was performed. The results of the assay and immunohistochemistry reaction for EGFR and Ki67 were associated with clinicopathologic features and recurrence.

Results. All analyzed cases presented disomy without any gene polysomy or amplification. With regard to EGFR immunoexpression, 3 cases (12%) were considered negative, and 22 (88%) were positive, of which 13 (52%) were weak and 9 (36%) were strong. All samples presented low positivity for Ki67. There was no association between EGFR expression and clinicopathologic features or recurrence ($P > .05$). In some cases, EGFR immunoexpression was observed without gene amplification.

Conclusions. Ameloblastoma development, progression, or recurrence does not appear to be related to *EGFR* amplification or polysomy. (Oral Surg Oral Med Oral Pathol Oral Radiol 2018;125:454–458)

Ameloblastoma is a benign epithelial odontogenic neoplasm that has a tendency to cause local and progressive destruction of bone and soft tissue. This tumor is exclusively found in maxillary bones and occurs mainly between the fourth and fifth decades of life, with no gender predilection. In about 80% of the cases, the lesions are located in the mandible, with a higher tendency toward the posterior region.[1]

In the 2017 World Health Organization's *Classification of Head and Neck Tumors*, the classification of ameloblastoma was simplified and narrowed to "ameloblastoma, unicystic and peripheral."[2] Although the adjective "solid/multicystic" has been discarded, its subtype demonstrates aggressive behavior, infiltrating into cancellous bone trabeculae and recurrence rates of 22%; the unicystic subtype is less aggressive and is usually treated as a cystic lesion, with 29% of recurrence.[3,4]

The epidermal growth factor receptor (EGFR; or ErbB-1 or HER1) is a tyrosine kinase receptor and a member of the ErbB receptor family. Its phosphorylation can activate multiple epithelial mitogenic signaling pathways, such as those of mitogen-activated protein kinases/extracellular signal-regulated kinases (MAPK/ERK) and phosphoinositide 3-kinase/phosphatase and tensin homolog deleted on chromosome 10 (PI3 K/PTEN).[5-10] The activation of these pathways induces several cellular processes, including proliferation, migration, invasion, transformation, differentiation, angiogenesis, and apoptosis inhibition.[8] Mutations in genes that belong to the MAPK pathway are reported in about 90% of ameloblastoma cases,[11] but little is known about the role of *EGFR* mutations/alterations in the pathogenesis of this neoplasm.

For a better understanding of the pathogenesis and biologic behavior of ameloblastoma, some biologic pathways and their associated genes have been studied. In the literature, the role of *EGFR* polysomy and amplification is not clear. The aims of this study were therefore to investigate *EGFR* alteration in ameloblastoma samples, using fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC), and associate the results with the clinicopathologic features and recurrence.

MATERIALS AND METHODS

Ameloblastoma samples

The Research ethics committee of the A.C. Camargo Cancer Center approved this study (Protocol No. 669/05). A retrospective study was performed analyzing cases of ameloblastoma admitted for treatment at the Department of Otolaryngology and Head and Neck Surgery during the period 1953 to 2003, with available paraffin blocks and clinical and follow-up information. Twenty-five

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Statement of Clinical Relevance

Ameloblastoma development, progression, or aggressiveness is not related to *EGFR* alterations. This is the first study using fluorescence in situ hybridization to investigate *EGFR* gene alterations in a series of ameloblastoma cases.

cases of ameloblastoma were found to be suitable for analysis. By following the 2017 WHO guidelines,[1] 2 pathologists (ERF and FAA) reviewed the histopathologic results to confirm the diagnoses.

Tissue microarray (TMA) blocks were constructed, as described by Fregnani et al.[12]

Dual-color fluorescence in situ hybridization

One slide of the TMA block was subjected to FISH analysis, using the ZytoLight Spec *EGFR*/CEN7 Dual Color Probe (ZytoVision, Bremerhaven, Germany) that contains both the fluorescently labeled *EGFR* gene and chromosome 7 centromere (CEN7) probes. In brief, the sections were incubated overnight at 56°C and then deparaffinized by being washed in xylene, ethanol, and distilled water. After incubation in 0.2 M hydrochloric acid for 20 minutes at room temperature, the sections were heat pretreated in a citrate buffer (2 × saline-sodium citrate [SSC], pH 6.0) for 1 hour at 80°C. Next, the sections were digested with pepsin for 8 minutes at room temperature, rinsed in 2 × SSC for 2 minutes at room temperature, and dehydrated in an increasing ethanol series (75%, 80%, and 100%) for 2 minutes each. The *EGFR*/CEN7 probe mix was applied to the dried slides, and the tissue area was coverslipped and sealed with rubber cement. The slides were then incubated in a hybridizer (Hybridizer for in situ hybridization; S2450, Dako, Glostrup, Denmark) for denaturation at 75°C for 10 minutes and hybridization at 37°C for approximately 18 hours. Posthybridization washes were performed in urea/ 0.1 × SSC for 30 minutes at 45°C and in 2 × SSC for 2 minutes at room temperature. The slides were dehydrated in serial ethanol solutions, after which 15 µL of mounting medium containing 4',6'-diamidino-2-phenylindole (DAPI) was applied, and the tissue area was then coverslipped.

The criteria proposed by Jiang et al.[13] were used to establish whether the FISH results were evaluable. In each case, 30 non-overlapping, intact interphasic tumor nuclei identified by DAPI staining were evaluated, and the copy numbers of the *EGFR* gene (green signal) and CEN7 (red signal) in each nucleus were determined. Amplification was defined as an average copy number ratio (*EGFR*/CEN7) of 2.0 or greater in all nuclei evaluated or when the *EGFR* signals formed a tight gene cluster.

TMA was constructed by using 73 cases of ameloblastoma; however, in this study, only 25 cases were suitable for FISH analyses. In addition, the EGFR and Ki67 immunoexpressions from another study of our group[12] have been associated with FISH results.

Statistical analysis

All statistical analyses were performed by using the SPSS software program, version 23.0 (SPSS Inc., Chicago, IL). Categorical variables were compared by using Pearson's χ^2 test or Fisher's exact test, depending on the expected values found in the contingency table. In all statistical tests, the alpha error was set at 5%.

RESULTS

The clinicopathologic data of the participants are summarized in Table I.

All analyzed cases presented disomy (Figure 1A), and no gene polysomy or amplification was found. In EGFR IHC analysis, 3 cases (12%) were considered negative

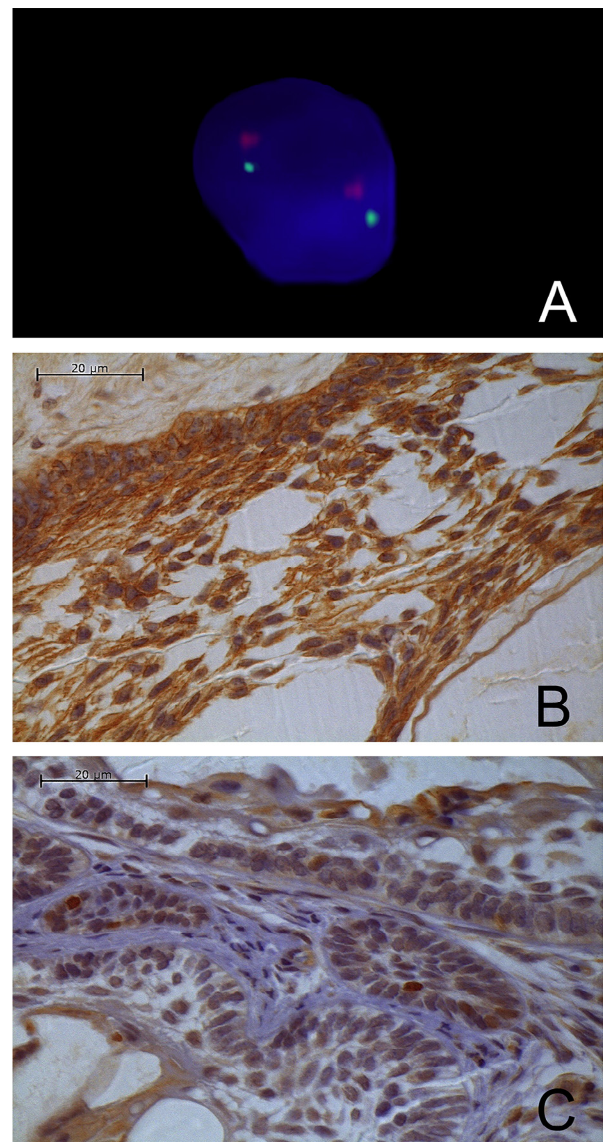

Fig. 1. **A,** *EGFR* disomy in nuclei of ameloblastoma cell (blue stained with DAPI), 2 signals of *EGFR* (green probe) and 2 signals of *CEN7* (red probe). **B,** Ameloblastoma cells showing strong epidermal growth factor receptor (EGFR) membrane expression. **C,** Ameloblastoma cells showing expression of Ki-67 less than 10% of nuclear cells stained (Mayer's hematoxylin counterstain).

Table I. Clinicopathologic features, EGFR status, and Ki-67 label of patients with ameloblastoma

	Age (y)	*Gender*	*Race*	*Location*	*Radiographic features*	*Histology*	*Treatment*	*EGFR FISH*	*EGFR expression*	*Ki-67*	*Relapse*
1	21	F	White	Mandible	Unilocular	Follicular	Conservative	D	2	Low	Yes
2	40	M	White	Mandible	Unilocular	Follicular	Aggressive	D	2	Low	No
3	35	F	White	Mandible	Multilocular	Follicular	Conservative	D	1	Low	Yes
4	22	M	White	Mandible	Multilocular	Plexiform	Conservative	D	1	Low	Yes
5	36	F	Other	Mandible	Multilocular	Plexiform	Aggressive	D	2	Low	No
6	52	M	White	Mandible	Multilocular	Plexiform	Conservative	D	2	Low	No
7	17	M	Other	Mandible	Unilocular	Plexiform	Conservative	D	2	Low	No
8	49	M	Other	Mandible	Unilocular	Plexiform	Aggressive	D	1	Low	No
9	32	F	Other	Mandible	Multilocular	Follicular	Conservative	D	1	Low	No
10	19	F	White	Mandible	Unilocular	Plexiform	Conservative	D	1	Low	No
11	12	M	White	Mandible	Unilocular	Plexiform	Conservative	D	1	Low	No
12	35	F	Other	Mandible	Multilocular	Follicular	Conservative	D	1	Low	Yes
13	25	M	Other	Mandible	Unilocular	Plexiform	Conservative	D	1	Low	No
14	17	F	White	Mandible	Multilocular	Plexiform	Conservative	D	0	Low	Yes
15	64	M	White	Mandible	Multilocular	Follicular	Aggressive	D	1	Low	No
16	28	F	White	Mandible	Unilocular	Follicular	Aggressive	D	1	Low	Yes
17	46	M	White	Maxilla	Multilocular	Plexiform	Conservative	D	0	Low	No
18	59	M	White	Mandible	Unilocular	Plexiform	Conservative	D	0	Low	No
19	12	F	White	Mandible	Unilocular	Plexiform	Conservative	D	2	Low	No
20	27	F	White	Mandible	Unilocular	Plexiform	Conservative	D	2	Low	No
21	78	F	White	Maxilla	Unilocular	Plexiform	Conservative	D	1	Low	No
22	18	M	White	Mandible	Unilocular	Plexiform	Conservative	D	2	Low	No
23	23	F	White	Mandible	Unilocular	Plexiform	Conservative	D	1	Low	No
24	24	F	Other	Mandible	Multilocular	Plexiform	Conservative	D	2	Low	No
25	30	F	Other	Mandible	Multilocular	Plexiform	Aggressive	D	1	Low	Yes

0, negative; *1*, underexpression; *2*, overexpression; *D*, disomy; *EGFR*, epidermal growth factor receptor; *F*, female; *FISH*, fluorescence in situ hybridization; *low*, <10%; *M*, male.

and 22 (88%) were positive, of which 13 (52%) were weak, and 9 (36%) were strong (Figure 1B). In Ki67 IHC analysis, all samples presented less than 10% of positivity (Figure 1C). No statistically significant association was observed between Ki67 or EGFR expressions and the clinicopathologic features and recurrence ($P > .05$).

DISCUSSION

Ameloblastoma, the most common odontogenic neoplasm (excluding odontomas), is characterized by a tendency toward local infiltration of bone and soft tissue and recurrence.[1] In the present study, FISH analysis revealed none of our samples to have gene amplification or polysomy, a result that is supported by the molecular study by Rotellini et al.,[14] who did not find *EGFR* gene alterations through FISH or gene sequencing in metastasizing ameloblastoma. Cells of pericoronal follicles that express EGFR respond to proliferative stimuli, exercising an important role in the pathogenesis of odontogenic lesions.[15,16]

In ameloblastoma, EGFR immunopositivity is reported in about 30% to 100% of cases.[3,13,17-20] However, EGFR overexpression is reported in only about 9% to 16% of cases.[3,18,19] A previous study by our group showed an EGFR immunopositivity of 77.1%, with a strong positivity in 22 (30.1%) cases.[12] The discrepancy in IHC results may have been caused by the use of different types of antibody clones and the criteria adopted for the IHC analysis, such as EGFR staining at the membrane only, cytoplasm only, or membrane and cytoplasm together. In addition, intratumor molecular heterogeneity[21] can influence EGFR expression mainly in paraffin-embedded blocks. IHC and FISH can be used on TMA samples, with minimal changes to standard protocols. However, antigen retrieval techniques or enzymatic digestion, such as deproteination for FISH, may cause tissue detachment from the TMA slide and loss of the sample.[22] Another difficulty was in standardization of the FISH protocol for ameloblastoma samples because a large number of samples did not present positivity even when standard protocols were used.

EGFR overexpression in ameloblastoma is related to the reactive increase of matrix metalloproteinase (MMP) secretion, and the signals generated by this molecular network are transduced by the ERK1/2 pathway, contributing to the tumor's aggressive behavior.[23] da Rosa et al.[24] used a primary cell line from human ameloblastoma samples transduced with human papillomavirus type 16 and suggested an interplay between the MMPs and EGFR in regulating ameloblastoma biology.[23]

In this study, no association was found between *EGFR* amplification and protein overexpression. Some events can contribute to EGFR expression, regardless of *EGFR* amplification. EGFR can be overproduced at the level

of transcription, which could be activated through tumor-derived p53 mutants (p53-143 A, p53-175 H, p53-248 W, p53-273 H, and p53-281 G), even in the absence of gene amplification.[25,26] Additionally, the inhibition of protein tyrosine phosphatases spontaneously activates EGFR in the absence of the ligand by EGF binding, and when EGFR is overexpressed, the phosphorylation levels of EGFR may exceed the capacity of the phosphatases, resulting in the enhanced activation of EGFR.[26]

Previous studies have recommended the use of anti-EGFR agents to treat ameloblastoma.[3,19,27] However, anti-EGFR therapy may not be effective in view of the low rate of EGFR membrane immunostaining and in cases of nuclear EGFR expression.[28] The most successful treatment for ameloblastoma is wide-excision surgery, including 1-cm margins in bone and a margin of the soft tissue plane; this approach greatly reduces the recurrence rate. Simple enucleation has a recurrence rate of 60% to 80%.[1,29] In this study, recurrence was not associated with the type of treatment.

Despite EGFR expression, the Ki-67 proliferation index was found to be always low in this study and was not associated with recurrence or the histologic type of tumor. However, some authors have suggested the use of Ki-67 as a marker to predict the prognosis and recurrence of ameloblastoma.[13,19,30]

CONCLUSIONS

The development and progression of ameloblastoma does not seem to occur as a result of *EGFR* amplification or polysomy. However, a high membrane expression of EGFR may play an important role in the pathogenesis of this disease, activating signaling pathways that may influence the proliferation, migration, and invasion of ameloblastoma cells.

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