

# E-cadherin regulators are differentially expressed in the epithelium and stroma of keratocystic odontogenic tumors

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**BACKGROUND:** The epithelial–mesenchymal transition (EMT) is the process where cells lose their epithelial features and acquire properties of typical mesenchymal cells. The dissociation of tumor cells due to changes in cell–cell adhesion is one of the key principles of tumor invasion and EMT. Thus, the knowledge of the molecular features of EMT in keratocyst odontogenic tumor (KOT) can provide useful markers to aid in the diagnosis and prognosis and perhaps contribute to an alternative therapeutic approach as it shows an aggressive clinical behavior and high recurrence rates. This study aimed to evaluate the EMT in KOT by the immunoexpression of E-cadherin, N-cadherin, Snail, and Slug and comparing to radicular cysts and dental follicles.

**METHODS:** Thirty-two KOTs, 15 radicular cysts, and 08 dental follicles were used for immunohistochemistry, evaluating the extent, intensity, labeling pattern, cellular compartment in the epithelium and stroma, and the presence of inflammation.

**RESULTS:** E-cadherin was preserved in most cases of keratocystic odontogenic tumor. N-cadherin was increased in the tumor epithelium, a result that was positively correlated with the heterogeneous and nuclear immunoexpression of Slug in the epithelium; Slug also correlated with high Snail immunoexpression. N-cadherin was positively correlated with Slug in the stroma of keratocystic odontogenic tumors.

**CONCLUSIONS:** The high immunoexpression of Snail and nuclear Slug in keratocystic odontogenic tumors suggests these proteins as transcription factors without necessarily participating in ‘cadherin switching’. However,

the knowledge of their induction of the epithelial–mesenchymal transition in odontogenic tumors is still limited.

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**Keywords:** cell adhesion; E-cadherin; epithelial–mesenchymal transition; immunohistochemistry; odontogenic tumors

## Introduction

Keratocystic odontogenic tumor (KOT), previously known as odontogenic keratocyst, is a benign cystic neoplasia resulting from odontogenic epithelial remnants (1, 2). Its current nomenclature was suggested in 2005, when the World Health Organization (WHO) recognized its distinct clinical behavior, given its aggressive infiltrative pattern and high rate of local recurrence, and reclassified it as benign odontogenic neoplasia (3).

The epithelial–mesenchymal transition (EMT) is a complex process through which epithelial cells lose their polarity and reorganize their cytoskeleton, acquiring a mesenchymal phenotype and increased migration (4). The EMT has key roles during embryogenesis and in pathological conditions, including fibrosis and carcinogenesis (5, 6).

Epithelial and mesenchymal components are also known to participate in the growth regulation of odontogenic cystic lesions and tumors (7). Evidence of the involvement of the EMT in the progression of odontogenic tumors is limited, especially in KOTs. Therefore, it is essential to examine the expression of EMT-regulating proteins in those tumors, including molecules associated with the E-cadherin epithelial adhesion pathway, namely Snail and Slug, and with the expression of mesenchymal markers related to this process, including N-cadherin, as already studied by some authors (8–12). A significant downregulation in the expression of

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cell adhesion proteins as E-cadherin in the epithelium of KOT may explain a molecular mechanism to understand initiating and development of KOTs and an alternative therapeutic approach (9). In ameloblastomas, a high rate was positive for N-cadherin, but no cases of dentigerous cyst showed positivity (10). Transcription factors as Snail and Slug probably play differential roles in mediating local invasiveness in odontogenic tumors. Specifically, it is possible that overexpression of Snail represents the prototype transcription factor involved in inducing EMT in the ameloblastoma (12).

Thus, furthering the understanding of tumor biology should unveil the complex aspects of the interaction between tumor components that may affect the tumor's biological behavior. However, thus far, few studies examining tumor progression through the EMT that focused on cell adhesion in KOTs have been published. This promising approach might assist in defining more refined answers to the biological behavior of these tumors.

This study aimed to evaluate the EMT through the differential immunoexpression of E-cadherin and N-cadherin associated with regulatory molecules (Snail and Slug) in the epithelial and stromal compartments of KOTs, as changes related to some proteins are apparently involved in the growth mechanism, invasion potential, and aggressiveness of KOT. For comparative purposes, radicular cysts (RCs) and dental follicles (DF) as morphologically healthy odontogenic tissue were included.

## Materials and methods

### Biological samples

Thirty-two samples of KOT and 15 samples of RC, embedded in paraffin, were retrospectively retrieved from the reports of the Pathological Anatomy Laboratory records of the School of Dentistry of the Federal University of Bahia (UFBA). They were diagnosed between 2003 and 2013 and obtained from either incisional or excisional biopsies from 2003 to 2013. Two pathologists (J.N.S. and F.C.A.X.) previously reviewed all KOT and RC histological slides stained with hematoxylin–eosin (HE) under an optical microscope to confirm the diagnosis. Those cases with unrepresentative tissue sample were excluded. The main clinical data as age, gender, tumor site and region, and recurrence history were also collected for KOT samples. Eight cases of DF resulting from impacted tooth extraction surgery, representing morphologically healthy tissue with odontogenic epithelial remnants, were also included. This study was approved by the Research Ethics Committee of the School of Dentistry, UFBA, under number 217.452.

### Immunohistochemistry reactions

The paraffin blocks were sectioned (3  $\mu$ m) in a microtome, and the sections were extended on previously silanized 2% in acetone glass slides. The laboratory procedures were conducted in the Immunohistochemistry Laboratory of the School of Dentistry, UFBA, using the immunoperoxidase method. Immunohistochemistry reactions were standardized in paraffin samples of human (human skin for E-cadherin) or animal (mouse embryo for N-cadherin and mouse kidney for Snail and Slug) control tissues with known immunoreactivities to the specific immunostaining of E-cadherin, N-cadherin, and Snail and Slug proteins. The same tissues, wherein the primary antibody was replaced by non-immune serum, were used as negative external controls. The descriptions of each primary antibody, dilution, and detection system used for each protein are outlined in Table 1.

Initially, the sections were deparaffinized through immersion in xylol and were rehydrated in decreasing percentages of ethanol, followed by incubation in PBS-diluted 3% hydrogen peroxide for 40 min. Following antigenic exposure through immersion in 10 mM citrate (pH 6.0) buffer solution in a steamer at 95°C for 20 min, the sections were incubated for 10 min in Protein Block Serum-Free (K0909; Dako, Carpinteria, CA, USA) to block non-specific sites, followed by incubation with a primary antibody in a wet chamber at 4°C for 18 h. Incubation with the EnVision™ Dual Link (K4061; Dako) detection system occurred for 30 min, and incubation with LSAB™ (K0690; Dako) and ADVANCE™ (K4068; Dako) occurred in two phases lasting 30 min each. The reaction development was performed using 3,3'-diaminobenzidine (K3468; Dako Liquid DAB Plus, Dako), and the slides were counterstained with Mayer's hematoxylin. The sections were washed twice with PBS (pH 7.4) buffer solution supplemented with 0.1% Triton between those passages. The sections were then dehydrated, diaphanized, and mounted in Permount resin (Fisher Scientific, Fair Lawn, NJ, USA) for observation under a light microscope.

### Immunostaining analysis

Protein immunoexpression was analyzed semiquantitatively and qualitatively for immunostaining extent and intensity (weak, moderate, and strong), staining pattern (homogeneous and heterogeneous), cellular compartment (nucleus, cytoplasm, and/or cell membrane), and effect of the presence of inflammation in immunostained areas (absent, non-significant, and significant) in KOTs, RCs, and DFs.

The variable immunoexpression extent was specifically categorized for each antibody analyzed: E-cadherin (11), N-cadherin (10), and Snail and Slug (12). Only the

**Table 1** Types of primary antibodies, host animals, brands, catalog numbers, clones, blocking of non-specific interactions, and detection systems used in the immunohistochemistry reactions

Antibody	Type of antibody	Host animal	Brand	Catalog number	Clone	Blocking of non-specific interactions	Primary antibody dilution	Detection system
E-cadherin	Monoclonal	Mouse	Dako	M3612	NCH38	No blocking	1:50	EnVision
N-cadherin	Polyclonal	Rabbit	Abcam	ab12221	–	Blocking solution	1:250	EnVision
Snail	Polyclonal	Goat	Abcam	ab53519	–	Blocking solution	1:50	LSAB
Slug	Polyclonal	Rabbit	Abcam	ab27568	–	No blocking	1:100	Advance

immunostaining of dental follicles was qualitatively defined as positive or negative given the sample representativeness. The variables extent, intensity, and immunoreactivity pattern were analyzed in the epithelium and/or stroma depending on the antibody.

#### Statistical analysis

All data recorded were tabulated and submitted to statistical analysis, with a 95% confidence interval, using Statistical Package for the Social Sciences (SPSS, Inc., Chicago, IL, USA) version 13.0. A  $P$  value  $< 0.05$  was considered significant. The association of antibody expression between groups (KOT, RC, and DF), clinical data, and comparisons of the extent of N-cadherin and Snail and Slug antibody staining in the epithelium and stroma were assessed using Fisher's exact test. Spearman's rank correlation coefficient was used for the correlation between the immunostaining extent of antibodies with each other in KOTs.

## Results

Clinical data of 24 patients with KOT demonstrated ages ranging from eight to 73 years (mean age 32.33, SD = 17.58). The gender distribution was higher in males than females (16:8). The mandible, predominantly the posterior region, was in fact the most affected site. Twenty cases had the first occurrence of the disease, and three cases were tumoral recurrence (Table 2). There was no statistically significant association between age, gender, recurrence, and the extent of immunostaining of proteins ( $P > 0.05$ ). However, it was noted that the following: (i) cases exclusively in the posterior region had preserved E-cadherin ( $> 50\%$ ) compared to cases with anterior mandible involvement ( $P = 0.046$ ); (ii) mandibular KOT expressed significantly N-cadherin and Slug in epithelium in a high extent (scores 3 and 4) immunostaining (Fisher's exact test  $P = 0.000$  and  $P = 0.005$ , respectively); and (iii) when lesion was located in the mandible and maxilla simultaneously had no expression of N-cadherin in stroma ( $P = 0.006$ ).

#### Differential protein immunoreactivity in KOT, RC, and DF

##### E-cadherin

The analysis of E-cadherin immunoreactivity in the three study groups is outlined in Table 2. Most KOTs (62.5%)

**Table 2** Main clinical data of keratocystic odontogenic tumor cases in the studied sample

Clinical data	Features	KOT	
		N	%
Age	≤ 28 years	13	5.1
	> 28 years	11	45.8
Gender	Male	8	33.3
	Female	16	66.6
Tumor site	Maxilla	0	0
	Mandible	22	91.6
	Maxilla and mandible	2	8.3
Tumor region	Anterior	2	12.5
	Posterior	11	68.7
	Anterior and posterior	3	18.7
Recurrence	No	12	80
	Yes	3	20

showed preserved immunoreactivity. All of the eight cases of DF showed positive immunoreactivity. Immunostaining was detected in the plasma membrane of the epithelial lining, odontogenic epithelial remains, and a focal area of the reduced epithelium of the enamel organ (Fig. 1A–I). A comparison between the three study groups revealed a significant difference in the marked effect of inflammation on E-cadherin immunoreactivity in RCs ( $P = 0.000$ , Table 3).

##### N-cadherin

N-cadherin immunoreactivity was detected in both the plasma membrane and the cytoplasm and/or nucleus in the epithelium and the stroma/capsule (Fig. 1J–O), ranging considerably from case to case and showing a rather granular appearance (Fig. 1M). The eight cases of DF showed positive N-cadherin immunoreactivity in the epithelium. Stronger combined immunostaining in the membrane and cytoplasm was observed in lesions (KOTs and RCs), with predominant cytoplasmic immunostaining alone in DF, which was statistically significant ( $P = 0.001$ , Table 4). A significant difference was also observed regarding the marked effect of inflammation on the N-cadherin immunoreactivity of RCs ( $P = 0.000$ , Table 4) when analyzing inflammation in the three groups.

A statistically significant difference ( $P = 0.004$ ) was observed when comparing the N-cadherin immunoreactivity extent in the epithelium with that in the stroma of KOTs, with the epithelium showing stronger N-cadherin immunoreactivity than the stroma.

##### Snail

The KOT, RC, and DF cases showed high nuclear and/or cytoplasmic Snail immunoreactivity in the epithelium and/or stroma (Fig. 2A–F, Table 5). Endothelial cells and fibroblasts were immunostained in the stroma (Fig. 2A–F).

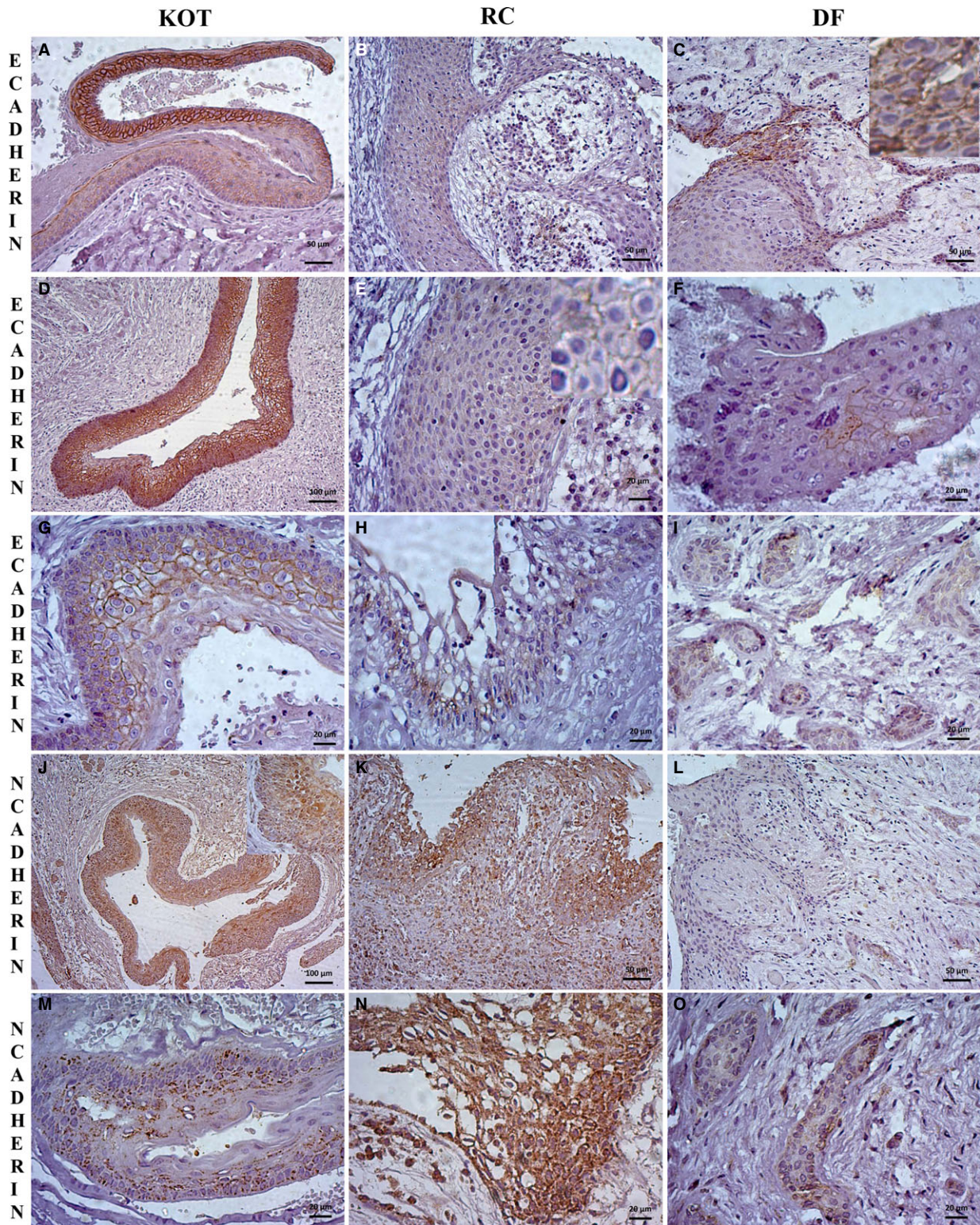
No variable analyzed in the epithelium exhibited statistically significant results when the three study groups were compared ( $P > 0.05$ ; Table 5). Statistically significant differences were only assessed in the stroma when analyzing the extent ( $P = 0.031$ ) and the presence of inflammation changing the immunoreactivity of Snail ( $P = 0.000$ ) (Table 4). No statistically significant difference ( $P = 0.543$ ) occurred when comparing the Snail immunostaining extent in the epithelium with that in the stroma of KOTs.

##### Slug

The KOT, RC, and DF cases showed nuclear and/or cytoplasmic Slug immunostaining in the epithelium and/or stroma (Fig. 2G–L, Table 5). Immunostaining was detected in the keratin layer in two cases of KOT (Fig. 2G).

Statistically significant differences were identified in intensity ( $P = 0.046$ ), with a predominance of moderate and strong intensity in KOTs and intense expression in DFs, with a heterogeneous pattern in KOTs ( $P = 0.011$ ) characterized by staining in the nuclear/cytoplasmic cellular compartment and exclusively cytoplasmic staining in DFs ( $P = 0.001$ ; Table 5) when the three groups were compared in terms of epithelial staining. A statistically significant difference was only assessed in the stroma when analyzing





**Figure 1** Immunorexpression of E-cadherin and N-cadherin in keratocystic odontogenic tumors (KOTs), radicular cysts (RCs), and dental follicles (DFs). E-cadherin shows moderate (A) and intense (A and D) immunorexpression in KOTs, with a membranous pattern (G) primarily distributed in the suprabasal stratum (A and G) or in all stratum (D). E-cadherin shows weak (B and E) immunostaining in RCs, with a membranous pattern (E, insert) in all stratum (E) or in the suprabasal stratum (H). E-cadherin shows focal immunostaining in the lining epithelium (C, insert) and in the reduced epithelium of the enamel organ (F) in DFs. N-cadherin shows intense (J) and moderate (M) immunostaining with membranous, cytoplasmic, or nuclear patterns (J, insert) distributed in all stratum (J) and sometimes with a granular appearance (M) in the KOT epithelium. The stroma shows immunostaining (J). N-cadherin shows moderate (K) and intense (N) immunostaining with a membranous/cytoplasmic pattern (N) in the RC epithelium and in inflammatory cells (K). N-cadherin is noticeably absent from the lining epithelium (L), with weak membranous/cytoplasmic immunostaining in the odontogenic epithelium (O), in DFs. The scales indicate 20  $\mu$ m (E, F, G, H, I, M, N and O), 50  $\mu$ m (A, B, C, K, and L) and 100  $\mu$ m (D and J).



**Table 3** E-cadherin immunoeexpression characterizations in the KOT, RC, and DF lesions

E-cadherin	KOT		RC		DF		P
	n	%	n	%	n	%	
Extent							
Absent	2	6.3	2	13.3	–	–	0.291
<20%	4	12.5	4	26.7	–	–	
20–50%	6	18.8	4	26.7	–	–	
>50%	20	62.5	5	33.3	–	–	
Intensity							
Absent	2	6.3	2	13.3	0	0	0.144
Weak	6	18.8	4	26.7	0	0	
Moderate	12	37.5	7	46.7	2	25	
Strong	12	37.5	2	13.3	6	75	
Pattern							
Homogeneous	25	78.1	9	60	–	–	0.295
Heterogeneous	7	21.9	6	40	–	–	
Inflammation							
Absent	17	53.1	0	0	6	75	0.000*
Non-significant	8	25	4	26.7	2	25	
Significant	7	21.9	11	73.3	0	0	

\*Fisher's exact test,  $P < 0.05$ .

intensity ( $P = 0.023$ ), with all cases of KOTs with moderate or strong intensity (Table 5). The presence of inflammation markedly changed Slug immunostaining in RCs ( $P = 0.000$ ; Table 5). No statistically significant difference was assessed ( $P = 0.153$ ) when comparing the extent of Slug immunostaining in the epithelium with that in the stroma of KOTs.

#### Correlations between protein immunoeexpression levels in KOTs

The following statistically significant and positive correlations were observed when assessing the correlations existing between the study proteins in KOTs, either through their epithelial or stromal expression: N-cad (epithelium) X Slug (epithelium;  $P = 0.024$ , 0.328 Spearman's rank correlation coefficient); N-cad (stroma) X Slug (stroma;  $P = 0.019$ , 0.346 Spearman's rank correlation coefficient); and Snail (epithelium) X Slug (epithelium;  $P = 0.021$ , 0.335 Spearman's rank correlation coefficient).

## Discussion

Our results showed epithelial E-cadherin immunostaining and epithelial/stromal N-cadherin, Snail, and Slug immunostaining in KOTs, without an apparent loss of E-cadherin immunoeexpression in most cases. The results were not different from those detected in RCs. However, the expression levels of all proteins were markedly affected by inflammation in the RCs and that characteristic enables one to confidently differentiate KOTs and DFs from RCs. N-cadherin expression was increased in the tumor epithelium compared to the stroma, specially in mandible tumors, a result that was correlated with the positive expression of Slug in the epithelium; in turn, Slug expression was correlated with positive Snail expression. N-cadherin was positively correlated with Slug in the stroma of KOTs. To our knowledge, this study is the first to address the simultaneous involvement of these markers in KOTs.

Adhesion molecules, including E-cadherin, have been studied in odontogenic tumors with the goal of understanding their roles in neoplastic progression (13–17), cell differentiation, and the development and capacity of tumor invasion (9–11, 18). Although decreased E-cadherin immunoeexpression might explain the invasive growth of KOTs (9), that evidence was not confirmed in the present study because 62.5% of KOT cases showed preserved E-cadherin immunoeexpression. Similar results were reported by Kusafuka et al. (10) and Mello et al. (11). The latter authors suggested that a high level of cell adhesion exists between the parenchymal cells of this tumor.

Some E-cadherin immunoeexpression difference presumably exists between the study lesions, based on the hypothesis that the EMT is involved in the progression of KOTs, as shown by Hakim et al. (9), who observed significant decreases in the membrane expression levels of  $\beta$ -catenin and E-cadherin in all 18 samples of sporadic and syndromic KOTs, especially in the suprabasal layer, while the eight dentigerous cysts conversely showed preserved immunostaining from the basal membrane of the epithelial layer to the luminal surface. However, this study detected no statistically significant difference when comparing the extent of E-cadherin immunoeexpression between KOT, RC, and FOL, although 37.6% of KOTs showed reduced E-cadherin immunoeexpression. Alves Pereira et al. (19) also failed to observe differences in E-cadherin immunoeexpression in ameloblastomas and tooth germs because all cases showed positive E-cadherin immunoeexpression. Conversely, Mello et al. (11) noted that all 18 cases of KOT and four cases of calcifying cystic odontogenic tumor showed significantly stronger E-cadherin immunoeexpression than that observed in 20 ameloblastomas, suggesting its key role in cell adhesion mediation in odontogenic tumors.

Cadherin switching, represented by increased N-cadherin expression and concomitant low E-cadherin expression, is reportedly a crucial characteristic of the EMT (20). In the present study, high N-cadherin expression was shown in the epithelium of KOTs and in RCs. N-cadherin was expressed at higher levels in the tumor epithelium than in the stroma in KOTs. Only one study was identified in which N-cadherin expression in KOTs was assessed: Kusafuka et al. (10) identified four cases of KOT (40.0%) positive for N-cadherin expression but with negative immunoreactivity in all seven dentigerous cysts that were evaluated. Therefore, according to the results from the present study, N-cadherin is apparently involved in KOT progression and may operate in EMT signaling through interactions with other regulators. Furthermore, the presence of inflammation contributed to increased N-cadherin expression in RCs.

No studies on odontogenic tumors evaluating the differential expression patterns of N-cadherin in cellular compartments have been reported. The N-cadherin staining pattern in this study was similar to that observed in head and neck carcinomas (17, 21): most KOTs and RCs showed membranous and cytoplasmic staining, and most DFs showed only cytoplasmic staining. However, it is noteworthy that no RC or DF showed nuclear immunostaining, which was significantly detected in 33.4% of KOTs. Based on their study results, DI Domenico et al. (22) hypothesized that the N-cadherin in cellular compartments may be a

**Table 4** N-cadherin immunoeexpression characterizations in KOT, RC, and DF lesions

N-cadherin	KOT		RC		DF		P
	n	%	n	%	n	%	
Epithelial extent							
Absent	2	6.3	0	0	–	–	0.073
<10%	1	3.1	2	13.3	–	–	
10–20%	0	0	0	0	–	–	
>20%	8	25	0	0	–	–	
>50%	21	65.6	13	86.7	–	–	
Stromal extent							
Absent	4	12.9	1	6.7	–	–	0.803
<10%	10	32.3	6	40	–	–	
10–20%	3	9.7	2	13.3	–	–	
>20%	8	25.8	2	13.3	–	–	
>50%	6	19.4	4	26.7	–	–	
Epithelial intensity							
Absent	2	6.3	0	0	0	0	0.710
Weak	5	15.6	2	13.3	1	12.5	
Moderate	12	37.5	8	53.3	2	25	
Strong	13	40.6	5	33.3	5	62.5	
Stromal intensity							
Absent	4	12.9	1	6.7	0	0	0.542
Weak	8	25.8	3	20	3	50	
Moderate	10	32.3	3	20	1	16.7	
Strong	9	29	8	53.3	2	33.3	
Epithelial pattern							
Homogeneous	16	50	7	46.7	–	–	1.000
Heterogeneous	16	50	8	53.3	–	–	
Stromal pattern							
Homogeneous	10	32.3	6	40	–	–	0.744
Heterogeneous	21	67.7	9	60	–	–	
Inflammation							
Absent	8	26.7	0	0	7	87.5	0.000*
Non-significant	16	53.3	4	26.7	1	12.5	
Significant	6	20	11	73.3	0	0	
Cellular compartment							
Membrane	3	10	0	0	0	0	0.001*
Cytoplasm	2	6.7	4	26.7	6	75	
Membrane + Cytoplasm	15	50	11	73.3	2	25	
Cytoplasm + nucleus	5	16.7	0	0	0	0	
Membrane + Cytoplasm + nucleus	5	16.7	0	0	0	0	

\*Fisher's exact test,  $P < 0.05$ .

useful diagnostic and prognostic tool in tumor assessment, as the N-cadherin nuclear staining pattern was particularly observed in undifferentiated oral carcinomas.

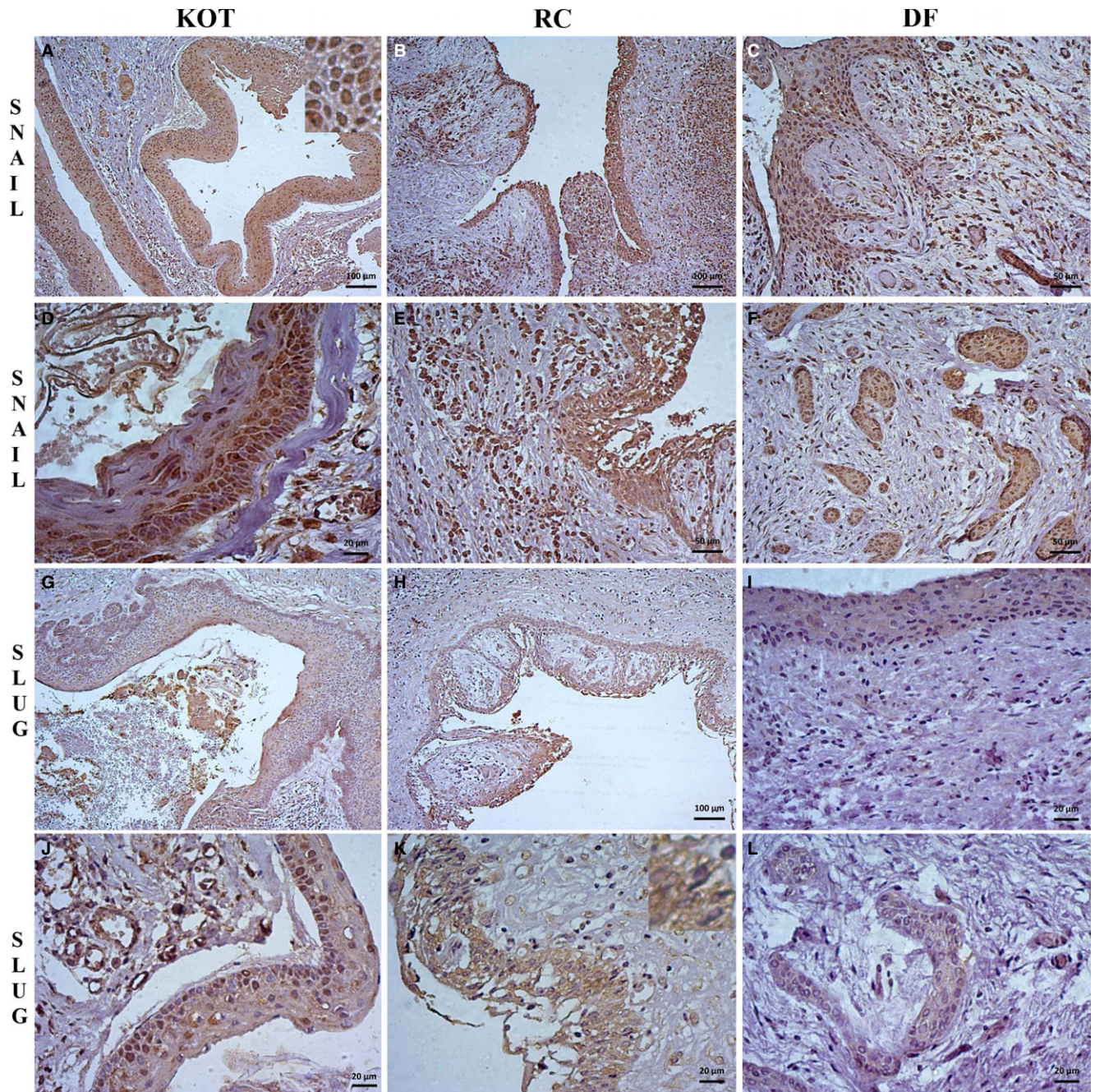
In KOTs, N-cadherin immunoeexpression was significantly stronger in the epithelium than in the stroma. Although no apparent reduction of E-cadherin expression correlated with that finding toward characterizing the 'cadherin switch' phenomenon was observed, 37.6% of KOTs exhibited reduced E-cadherin immunoeexpression. Thus, an analysis of a larger group of tumors with follow-up data becomes necessary to establish whether that protein profile is related to a tumor group with different biological behavior.

Snail is a key regulator that promotes the EMT (17, 23, 24). Studies have shown that increased Snail expression induces morphological changes and decreases E-cadherin expression (17, 23, 25). No significant difference in Snail in epithelium immunoreactivity was found in the KOT and RC groups in this study, and both showed high immunoreactivity. All DFs also showed Snail immunoeexpression. Conversely, Siar and Ng (12) observed high Snail immunoeexpression in ameloblastomas. Although proteins from

the Snail family are necessary for EMT processes, that requirement does not necessarily mean that EMT induction is an exclusive role of genes of the Snail family. The genes also have different cellular functions independent from EMT induction, including protecting cells from induced death, either through the loss of survival factors or through direct apoptotic stimuli. The mechanism through which EMT induction is avoided in these cases is unknown (26). Therefore, Snail positivity expressed in follicles is apparently unrelated to the EMT. The preserved expression of E-cadherin in this tissue also supports this hypothesis.

A significant association of Snail immunoeexpression was observed when comparing KOTs and RCs, with 73.3% of RCs showing increased Snail expression (> 50%), when Snail was analyzed in the stroma. Siar and Ng (12) noted that fibroblasts adjacent to the tumor epithelium of ameloblastomas often expressed Snail. However, no studies assessing the expression of that marker in KOTs have been reported. Franz et al. (23) and Schwock et al. (27) assessed that Snail positivity mainly occurs in the stroma invasive front, in the vicinity of tumor islands and inflammation, when evaluating the immunoreactivity of Snail in oral





**Figure 2** Immunoeexpression of Snail and Slug in keratocystic odontogenic tumors (KOTs), radicular cysts (RCs), and dental follicles (DFs). Snail shows intense immunostaining in nuclear and cytoplasmic (A, insert, and D) patterns distributed in all epithelial stratum (A and D) in KOTs. The stroma shows immunostained fibroblasts and endothelial cells. Snail also shows intense immunostaining (B and E) in nuclear and cytoplasmic patterns (E) distributed in all epithelial stratum (B and E) and in inflammatory cells (B and E) in RCs. Snail shows immunostaining in the lining epithelium (C) and in the reduced epithelium of the enamel organ (F) in DFs. Slug shows moderate immunostaining (G and J) in a heterogeneous cytoplasmic pattern distributed in all stratum (J) in the KOT epithelium. Immunostaining of fibroblasts and endothelial cells is observed (G and J). Slug shows moderate (H and K) immunostaining in a homogeneous cytoplasmic pattern distributed in all stratum (K, insert) in the RC epithelium. DFs show weak cytoplasmic immunostaining of Slug in the lining epithelium (I) and odontogenic epithelium (L). The scales indicate 20  $\mu$ m (D, I, J, K, and L), 50  $\mu$ m (C, E, and F), and 100  $\mu$ m (A, B, G, and H).

squamous cell carcinoma, which may explain the increased expression in RCs in the present study.

Snail and Slug are not equivalent despite their similar structural conformations, as specific combinations of zinc fingers (ZF1/ZF2 in Snail1 and ZF3/ZF4 in Snail2) are necessary for complete repression activity. Therefore, the proteins may play non-equivalent roles in gene repression,

DNA binding conformation, and the capacity to induce the EMT (28). Depending on the tissue analyzed, Slug has indeed shown a different behavior from Snail. In the present study, no differences in Slug immunoeexpression extent were assessed in KOTs, RCs, and DFs. However, a statistically significant result was detected when analyzing the expression intensities in the epithelium and stroma. DFs intensely



**Table 5** Snail and Slug immunoeexpression characterizations in KOT, RC, and DF lesions

	<i>Snail</i>						P	<i>Slug</i>						P
	<i>KOT</i>		<i>RC</i>		<i>DF</i>			<i>KOT</i>		<i>RC</i>		<i>DF</i>		
	n	%	n	%	n	%		n	%	n	%	n	%	
<b>Epithelial extent</b>														
Absent	1	3.1	0	0	–	–	0.681	1	3.1	0	0	–	–	0.690
<25%	1	3.1	1	6.7	–	–		8	25	2	13.3	–	–	
25–50%	5	15.6	1	6.7	–	–		8	25	5	33.3	–	–	
>50%	25	78.1	13	86.7	–	–		15	46.9	8	53.3	–	–	
<b>Stromal extent</b>														
Absent	0	0	0	0	–	–	0.031*	0	0	0	0	–	–	0.515
<25%	10	33.3	1	6.7	–	–		3	9.7	3	20	–	–	
25–50%	10	33.3	3	20	–	–		11	35.5	6	40	–	–	
>50%	10	33.3	11	73.3	–	–		17	54.8	6	40	–	–	
<b>Epithelial intensity</b>														
Absent	1	3.1	0	0	0	0	0.839	1	3.1	0	0	0	0	0.046*
Weak	1	3.1	1	6.7	0	0		4	12.5	6	40	1	12.5	
Moderate	8	25	6	40	2	25		14	43.8	3	20	0	0	
Strong	22	68.8	8	53.3	6	75		13	40.6	6	40	7	87.5	
<b>Stromal intensity</b>														
Absent	0	0	0	0	0	0	0.629	0	0	0	0	0	0	0.023*
Weak	0	0	0	0	0	0		0	0	3	20	3	37.5	
Moderate	3	10	1	6.7	0	0		13	41.9	5	33.3	1	12.5	
Strong	27	90	14	93.3	8	100		18	58.1	7	46.7	4	50	
<b>Epithelial pattern</b>														
Homogeneous	24	77.4	13	86.7	–	–	0.696	8	25.8	10	66.7	–	–	0.011*
Heterogeneous	7	22.6	2	13.3	–	–		23	74.2	5	33.3	–	–	
<b>Inflammation</b>														
Absent	4	13.3	0	0	7	87.5	0.000*	6	19.4	0	0	5	62.5	0.000*
Non-significant	13	43.3	0	0	0	0		18	58.1	2	13.3	2	25	
Significant	13	43.3	15	100	1	12.5		7	22.6	13	86.7	1	12.5	
<b>Cellular compartment</b>														
Nucleus	15	48.4	7	46.7	5	62.5	0.441	4	12.5	0	0	0	0	0.001*
Cytoplasm	3	9.7	1	6.7	2	25		7	21.9	9	60	8	100	
Nucleus + cytoplasm	13	41.9	7	46.7	1	12.5		21	65.6	6	40	0	0	

\*Fisher's exact test,  $P < 0.05$ .

expressed Slug in the epithelium, while all KOTs showed moderate or intense expression in the stroma, in contrast to RCs and DFs, which were well distributed among the categories.

In the present study, epithelial Slug immunostaining showed a rather heterogeneous distribution pattern in KOTs (74.2%), unlike the homogeneity observed in RCs (66.7%). A similar Slug expression pattern was observed in 64 ameloblastomas evaluated by Siar and Ng (12). However, no studies assessing Slug expression in KOTs have been reported. Thus, the heterogeneity detected might be specific to tumor tissues, which was not observed in the inflammatory cystic lesion in this study.

Regarding the protein's role as a transcription factor, several lines of evidence indicate that the nuclear forms are functionally active molecules, whereas the forms expressed in the cytoplasm are phosphorylated and functionally inactive (24, 29). Most KOTs showed notable nuclear staining in the present study, unlike the mainly cytoplasmic staining in RCs and DFs, which would suggest greater Slug activity as a transcription factor in KOTs. Different results were reported by Siar and Ng (12), who observed a predominantly cytoplasmic rather than nuclear Slug staining pattern in ameloblastomas and a predominantly nuclear Snail staining pattern, concluding that the transcription factors play different roles in EMT induction in ameloblas-

tomas and that Snail may be the key repressor involved in this process. The results of the present study suggest that the process is apparently reverse in the case of KOTs, with the Slug protein playing the predominant role.

The EMT is characterized by the downregulation of epithelial markers and the upregulation of mesenchymal markers (30–32). In the present study, we observed a correlation between Slug and N-cadherin expression. Indeed, Snail and Slug have either direct or indirect effects on genes other than E-cadherin (28). Thus, the involvement of Slug in the EMT in KOTs may regulate N-cadherin expression because we detected positive and statistically significant correlations between Slug and N-cadherin expression in the epithelium and stroma. The same correlation was reported by Zhang et al. (33) in samples from 119 primary head and neck carcinomas, with Slug expression levels negatively correlated with E-cadherin and positively correlated with N-cadherin expression. However, no evidence has shown that Slug signaling is involved in the direct regulation of N-cadherin. Therefore, further research studies involving functional assays in odontogenic tumors are required to clarify that correlation.

The increased expression of the transcription factors Snail and Slug mostly repressed E-cadherin function; they are considered key molecules in the EMT, with significant relevance in the progression of different tumors (23, 25, 34).



Although an inverse correlation between the expression of E-cadherin and Snail/Slug was not detected in the KOTs of the present study, a positive correlation between Snail and Slug expression was observed in the epithelium of KOTs. That result indicates how much these factors are interconnected, despite exhibiting non-equivalent roles in epithelial promoter repression, DNA binding, and the capacity to induce EMT (28). Siar and Ng (12) assessed inverse relationships between Snail and Slug in the epithelium of ameloblastomas with low Slug expression, suggesting a limited role of Slug in EMT induction in this neoplasia. The reasons for these different patterns of expression between Snail and Slug are not clear, although new evidence suggests that these transcription repressors may operate either alone or together (35, 36).

Inflammation causes structural tissue changes (37) and functional alterations in KOTs, mainly resulting from the effect of inflammatory cells on epithelial proliferative activity (38–40) and cytokeratin expression (41) and with a smaller impact on stromal vascularization (37). Inflammation significantly affected the immunoreactivity of the study proteins in most RCs.

Protein interactions in different tissue compartments are crucial for the EMT process. In this study, in KOTs, epithelial N-cadherin expression was correlated with positive epithelial Slug expression, which, in turn, was correlated with positive Snail expression. N-cadherin expression was positively correlated with Slug in the stroma of KOTs. In another study, E-cadherin expression was decreased in Snail-induced cell cultures, while the expression levels of the mesenchymal markers N-cadherin and Vimentin were increased (42). Conversely, Gasparotto et al. (43) noted that the expression levels of Twist and Slug were positively correlated with the increased expression of mesenchymal markers, including N-cadherin, and that the upregulation of these mesenchymal markers was not necessarily associated with the loss of E-cadherin expression, suggesting that the activation of a mesenchymal factor may be at least partly unrelated to the transcriptional downregulation of E-cadherin.

In conclusion, we have shown that the E-cadherin, N-cadherin, Snail, and Slug proteins are differentially expressed in the epithelium and stroma of KOTs and that the immunoreactivity profile of E-cadherin was preserved in KOTs. No significant differences in protein expression occurred between KOTs, RCs, and DFs. However, protein immunoreactivity was noticeably markedly affected by inflammation in RCs. An N-cadherin expression gain occurred in the epithelium of KOTs compared to the stroma, which was correlated with Slug upregulation in the epithelium. This upregulation was likewise correlated with Snail upregulation. Furthermore, N-cadherin was positively correlated with Slug in the stroma of KOTs. The increased expression of Snail and the heterogeneous and nuclear expression pattern of Slug in KOTs suggest that these proteins are putative transcription factors in that type of odontogenic tumor, without necessarily being involved in cadherin switching. However, knowledge of the actual involvement of E-cadherin, N-cadherin, Snail, and Slug in EMT signaling in KOTs is still limited and requires further studies to define the role of each marker and their combined actions in odontogenic tumors.

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## Conflict of interest

All authors have declared no conflict of interests.